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<210> SEQ ID NO 174
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 174

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 175
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 175

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met
1 5 10 15

Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 176
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 176

Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser
1 5 10 15

Gly Leu Gly Cys
20

<210> SEQ ID NO 177
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 177

Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly
1 5 10 15

Leu Gly Cys

<210> SEQ ID NO 178
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 178

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
1 5 10 15

Gly Cys

<210> SEQ ID NO 179
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 179

Gly Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 180
<211> LENGTH: 53
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 180

Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu Leu
1 5 10 15

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
20 25 30

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
35 40 45

Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 181
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 181

Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 182
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 182

Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly
1 5 10 15

Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Asn
20 25 30

Ser Gly Leu Gly Cys

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<210> SEQ ID NO 183
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic Polypeptide

<400> SEQUENCE: 183

Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Asn Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 184
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 184

Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys Leu
1 5 10 15

Asp Arg Ile Gly Ser Asn Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 185
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 185

Pro Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 186
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 186

Pro Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 187

<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 187

Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys
1 5 10 15

Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> SEQ ID NO 188
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 188

Pro Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 189
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 189

Pro Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 190
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 190

Met Asp Leu Arg Val Asp Thr Lys Ser Arg Ala Ala Trp Ala Arg Leu
1 5 10 15

Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
20 25 30

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
35 40 45

Met Ser Gly Leu Gly Cys
50

<210> SEQ ID NO 191
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 191

Met Gly Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 192
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 192

Met Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys Lys
1 5 10 15

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 193
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 193

Met Gly His Lys Ser Glu Val Ala His Arg Phe Lys Gly Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 194
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 194

Met Gly Ala Asn Arg Arg Gly Leu Ser Arg Gly Cys Phe Gly Leu Lys
1 5 10 15

Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 195
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 195

Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro
1 5 10 15

Arg Gly Ser His Thr Gly Asp Asp Asp Asp Lys His Met Asp

```
            20                  25                  30

<210> SEQ ID NO 196
<211> LENGTH: 142
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 196

Met Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys
1               5                   10                  15

Pro Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile
            20                  25                  30

Ala Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala
        35                  40                  45

Arg His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His
    50                  55                  60

Leu Glu Arg Gln Trp Asn Met Trp Ile Met Gly Ser Ser His His His
65                  70                  75                  80

His His His Ser Ser Gly Leu Val Pro Arg Gly Ser His Thr Gly Asp
                85                  90                  95

Asp Asp Asp Lys His Met Asp Pro Gly Gln Glu His Pro Asn Ala Arg
            100                 105                 110

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
        115                 120                 125

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
    130                 135                 140


<210> SEQ ID NO 197
<211> LENGTH: 72
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 197

Val Leu Thr Lys Lys Lys Leu Gln Asp Leu Val Arg Glu Val Cys Pro
1               5                   10                  15

Asn Glu Gln Leu Asp Glu Asp Val Glu Glu Met Leu Leu Gln Ile Ala
            20                  25                  30

Asp Asp Phe Ile Glu Ser Val Val Thr Ala Ala Cys Gln Leu Ala Arg
        35                  40                  45

His Arg Lys Ser Ser Thr Leu Glu Val Lys Asp Val Gln Leu His Leu
    50                  55                  60

Glu Arg Gln Trp Asn Met Trp Ile
65                  70


<210> SEQ ID NO 198
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 198

Pro Gly Gln Glu His Pro Asn Ala Arg
1               5


<210> SEQ ID NO 199
<211> LENGTH: 30
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 199

Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly
1 5 10 15

Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
20 25 30

<210> SEQ ID NO 200
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 200

Gly Ala Asn Lys Pro
1 5

<210> SEQ ID NO 201
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 201

Gly Ala Asn Pro Lys
1 5

<210> SEQ ID NO 202
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 202

Gly Ala Asn Pro Gln
1 5

<210> SEQ ID NO 203
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 203

Gly Ala Asn Gln Lys
1 5

<210> SEQ ID NO 204
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 204

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys

```
        35                  40


<210> SEQ ID NO 205
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 205

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys
1               5                   10                  15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
            20                  25                  30

Ala Met Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 206
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 206

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Arg Gly Arg Ser Lys Lys
1               5                   10                  15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
            20                  25                  30

Met Ser Gly Leu Gly Cys
        35


<210> SEQ ID NO 207
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Triakis scyllium

<400> SEQUENCE: 207

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1               5                   10                  15

Met Ser Gly Leu Gly Cys
            20


<210> SEQ ID NO 208
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 208

Arg Leu Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1               5                   10                  15

Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg
            20                  25                  30

Ile Gly Ala Met Ser Gly Leu Gly Cys
        35                  40


<210> SEQ ID NO 209
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 209

Leu Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys
1               5                   10                  15

Lys Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly
            20                  25                  30
```

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Ala Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 210
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 210

Lys Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg Ser Lys Lys
1 5 10 15

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
20 25 30

Met Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 211
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 211

Phe Lys Gly Arg Ser Lys Lys Gly Pro Ser Arg Gly Cys Phe Gly Val
1 5 10 15

Lys Leu Asp Arg Ile Gly Ala Met Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 212
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Lamna ditropis

<400> SEQUENCE: 212

Gly Pro Ser Arg Gly Cys Phe Gly Val Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 213
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 213

Arg Leu Leu Gln Asp Leu Ser Asn Asn Pro Leu Arg Phe Lys Gly Arg
1 5 10 15

Ser Lys Lys Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg
20 25 30

Ile Gly Ala Met Ser Gly Leu Gly Cys
35 40

<210> SEQ ID NO 214
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Squalus acanthias

<400> SEQUENCE: 214

Gly Pro Ser Arg Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 215
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 215

Gly Trp Asn Arg Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 216
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 216

Pro Met Val Ala Gly Gly Gly Cys Phe Gly Met Lys Met Asp Arg Ile
1 5 10 15

Gly Ser Ile Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 217
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 217

Gly Arg Ser Ser Met Val Gly Gly Arg Gly Cys Phe Gly Met Lys Ile
1 5 10 15

Asp Arg Ile Gly Ser Ile Ser Gly Leu Gly Cys
20 25

<210> SEQ ID NO 218
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 218

Gly Gly Met Arg Ser Cys Phe Gly Val Arg Leu Glu Arg Ile Gly Ser
1 5 10 15

Phe Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 219
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 219

Gly Gly Leu Arg Ser Cys Phe Gly Val Arg Leu Ala Arg Ile Gly Ser
1 5 10 15

Phe Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 220
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Oryzias latipes

<400> SEQUENCE: 220

Gly Gly Ser Thr Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg
1 5 10 15

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Ile Gly Thr Ile Ser Gly Met Gly Cys
20 25

<210> SEQ ID NO 221
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Arothron maculatus

<400> SEQUENCE: 221

Gly Gly Ser Ser Arg Ser Gly Cys Phe Gly His Lys Met Asp Arg Ile
1 5 10 15

Gly Thr Ile Ser Gly Met Gly Cys
20

<210> SEQ ID NO 222
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 222

Leu Leu His Asp His Pro Asn Pro Arg Lys Tyr Lys Pro Ala Asn Lys
1 5 10 15

Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
20 25 30

Ser Thr Ser Gly Leu Gly Cys
35

<210> SEQ ID NO 223
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Ornithorhynchus anatinus

<400> SEQUENCE: 223

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Thr Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 224
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 224

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ala
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 225
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 225

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Gln Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 226
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 226

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Ala Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 227
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 227

Ser Leu Arg Arg Ser Ser Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly
1 5 10 15

Ser Met Ser Gly Leu Gly Cys
20

<210> SEQ ID NO 228
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 228

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

<210> SEQ ID NO 229
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 229

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Gln Ser Gly Leu Gly
1 5 10 15

Cys Asn Ser Phe Arg Tyr
20

<210> SEQ ID NO 230
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetnic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 230

Ile Glu Gly Arg Xaa

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1 5

<210> SEQ ID NO 231
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(6)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 231

Asp Asp Asp Asp Lys Xaa
1 5

<210> SEQ ID NO 232
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)..(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 232

Glu Xaa Xaa Tyr Xaa Gln Gly
1 5

<210> SEQ ID NO 233
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 233

Val Asp Asp Arg
1

<210> SEQ ID NO 234
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 234

Gly Ser Asp Arg
1

<210> SEQ ID NO 235
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 235

Ile Thr Asp Arg
1

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<210> SEQ ID NO 236
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 236

Pro Gly Asp Arg
1

<210> SEQ ID NO 237
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 237

Ala Phe Leu Gly Pro Gly Asp Arg
1 5

What is claimed is:

**1**. A variant of C-type natriuretic peptide (CNP) selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Met-CNP53)

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS GLGC [CNP-53(M48N)].

**2**. A pharmaceutical composition comprising a CNP variant of claim **1**, and a pharmaceutically acceptable excipient, carrier or diluent.

**3**. The composition of claim **2**, which is a lyophilized formulation prepared from a formulation that comprises a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6.

**4**. The composition of claim **3**, wherein the lyophilized formulation is prepared from a formulation that further comprises (a) an isotonicity-adjusting agent or a bulking agent or (b) an antioxidant.

**5**. A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a CNP variant to a subject in need thereof, wherein the CNP variant is a CNP variant according to claim **1**, and wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

**6**. A method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Gly-CNP53);

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Pro-CNP53);

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS GLGC (Met-CNP53);

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNS GLGC [CNP-53(M48N)].

**7**. The method of claim **6**, wherein the cleavable peptide or protein is selected from the group consisting of histidine tags, human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E), ketosteroid isomerase, maltose-binding protein, β-galactosidase, glutathione-S-transferase, thioredoxin, chitin-binding domain, BMP-2, BMP-2 mutants, BMP-2(C/A), and mutants and fragments thereof.

**8**. The method of claim **6**, wherein the host cell is bacterial.

**9**. The method of claim **6**, wherein the fusion polypeptide is expressed as a soluble protein or as an inclusion body.

**10**. The method of claim **6**, further comprising isolating the expressed fusion polypeptide from the host cell or culture medium.

**11**. The method of claim **10**, further comprising contacting the isolated fusion polypeptide with a cleaving agent selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.

**12**. A CNP variant produced according to the method of claim **6**, wherein the CNP variant is selected from the group consisting of:

(SEQ ID NO: 179)
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Gly-CNP53));

(SEQ ID NO: 185)
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Pro-CNP53));

(SEQ ID NO: 190)
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMS
GLGC (Met-CNP53));

(SEQ ID NO: 180)
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSG
LGC [CNP-53(M48N)]).

**13**. A method of increasing long bone growth, comprising administering a CNP variant according to claim **1** to a subject in need thereof, where the administration increases long bone growth.

**14**. A CNP variant according to claim **1** useful for increasing long bone growth or treating achondroplasia, hypochondroplasia, short stature, dwarfism, or homozygous achondroplasia in a subject in need thereof.

* * * * *

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| | 2 | 2009015011 | WO | A1 | 2009-01-29 | MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH | | |
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| | 1 | Anonymous: "UniProtKB - Q9QZ96 (Q9QZ96_CAVPO)", 1 May 2000 (2000-05-01), pages 1-4, SP055463352, Retrieved from the internet: URL:http/www.uniprot.org/uniparc/UPI00000E7708 (retrieved on 2018-03-28) | |
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(71) Applicant: **Daiichi Sankyo Company, Limited**
**Chuo-ku, Tokyo (JP)**

(72) Inventors:
- **WAKABAYASHI, Naomi**
  **Ohra-gun**
  **Gunma 370-0503 (JP)**
- **SATO Seiji**
  **Ohra-gun**
  **Gunma 370-0503 (JP)**

(74) Representative: **Vossius & Partner**
**Siebertstrasse 4**
**81675 München (DE)**

# (54) PEPTIDE CAPABLE OF EXTENDING HALF-LIFE OF PEPTIDE OF INTEREST IN PLASMA

(57) A peptide of the following (I) or (II).
(I) a peptide represented by the formula B, A-B, B-C or A-B-C in which A, B and C each is represented by the following (1), (2) and (3) and, when it is bonded to other object peptide, it is able to extent the half-life in plasma as compared with the object peptide where the physiological activity of the object peptide is still retained.
(II) a peptide comprising a reversed sequence of the peptide of (I); a sequence which is represented by A-B in (I) and A or B is reversed; a sequence which is represented by B-C in (I) and B or C is reversed; or a sequence which is represented by A-B-C in (I) and A, B, C, A and B, B and C or A and C is reserved.
(1) A is a peptide comprising 1 to 14 of any amino acid(s)
(2) B is a peptide represented by the formula 1:
(Wk-X1-Y-Zm-Wn)-(Wo-Xp-Y-Zq-Wr)s
(In the formula 1, W is a basic amino acid; X and Z are any amino acids; Y is an acidic amino acid; k is 1 or 2; 1 is an integer of $4 \geq 1 \geq 0$; m is an integer of $2 \geq m \geq 0$; $4 \geq 1 + m \geq 0$; n is 1 or 2 ; O is 1 or 2; p is an integer of $4 \geq p \geq 0$; q is an integer of $2 \geq q \geq 0$; $4 \geq p + q \geq 0$; r is 1 or 2; and s is 0 or 1.)
(3) C is a peptide comprising 2 to 14 of any amino acids.

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## Description

TECHNICAL FIELD

**[0001]** The present invention relates to a peptide which is able to give therapeutic usefulness by improving the pharmacokinetics *in vivo* of the object peptide and giving the pharmacokinetics *in vivo* meeting the treating object, to a chimeric peptide where the pharmacokinetics *in vivo* is improved and the physiological activity of the object peptide is available, to a pharmaceutical composition containing the chimeric peptide and to a process for producing the chimeric peptide.

BACKGROUND ART

**[0002]** Even when a physiologically peptide which is able to be applied as a drug is present, some of the peptide has a short half-life in plasma and, in its application for therapeutic purpose, it is necessary to attempt the achievement by means of the continuous administration by intravenous or subcutaneous means or by administration of a DDS preparation of a sustained release type. Therefore, much time and cost for a long period of time are necessary for actual application.

**[0003]** For example, as an example where the half-life in plasma of the physiologically active peptide is extended and development as a drug is conducted, there is a case of glucagons-like peptide-1 (Non-Patent Document 1). In clinical studies for clarifying the action of the physiologically active peptide, a sustained release intravenous administration has been much used (Non-Patent Document 2) and, for the treatment of type 2 diabetes mellitus, a peptide where half-like is extended whereby a bolus subcutaneous administration is possible has been created. As such, in order to develop as an actual treating agent, there is a case where development of derivatives where a half-life is extended is necessary and, in such a case, physiologically active peptide derivatives are chemically synthesized or, even in the case of different type of natural peptide, that having a long half-life has been developed (Non-Patent Documents 3 to 5).

**[0004]** When a physiologically active peptide having a short half-life is to be applied as a drug, there is an attempt where it is bonded to a protein having a long half-life so that the half-life of the physiologically active peptide is made near the half-life of the protein. For example, an attempt such as that the object physiologically active protein and the protein are bonded via a spacer having a bonding group to a serum albumin has been in a testing stage (Non-Patent Document 6).

**[0005]** Accordingly, for subjecting the physiologically active peptide having a short half-life to a practical use, it has been expected that the pharmacokinetics *in vivo* is improved by adding a substance which is able to extent the half-life whereby the physiologically active peptide which was unable to exhibit a therapeutic effect by the prior art is applied to a medical purpose.

**[0006]** Attempts where the physiologically active peptide having a short half-like is modified so as to apply it as a drug have been widely carried out already. It has been attempted to stabilize the peptide, to give a sustaining property by an improvement in the preparation, etc. and examples where the practical implementation was successful have been known as well.

**[0007]** On the other hand, ghrelin is a hormone which was found in the stomach in 1999, has an amino acid sequence comprising 28 residues and is a peptide having a very unusual chemical structure where the third amino acid from the amino terminal of the sequence is acylated with fatty acid (Non-Patent Document 6 and Patent Document 1). Ghrelin is an endogenous brain- and digestive tract-hormone (Non-Patent Document 7) which acts on a growth hormone secretagogue-receptor la (GHS-R1a) (Non-Patent Document 7) to promote the secretion of growth hormone (GH) from pituitary gland.

**[0008]** Further, ghrelin was firstly isolated and purified from rats as an endogenous GHS for GHS-R1a. Moreover, ghrelin having the similar primary structure has been also isolated from vertebrate animals other than rat such as human, mouse, swine, domestic fowl, eel, cattle, horse, sheep, frog, rainbow trout or dog and the amino acid sequence thereof has been known already (Patent Document 1 and Non-Patent Document 8). All of the above ghrelin is a peptide where a side-chain hydroxyl group of the serine residue or threonine residue at 3-position is acylated by fatty acid such as octanoic acid or decanoic acid and, with regard to the physiologically active peptide having such a hydrophobically modified structure, there has been no example of being isolated from living body except ghrelin.

**[0009]** In the recent studies, it has been clarified that ghrelin promotes the appetite, that a subcutaneous administration of ghrelin increases body weight and body fat (Non-Patent Documents 9 to 11) and that an action such as improving the cardiac function is available (Non-Patent Documents 12 to 14). Further, ghrelin has a promoting action for GH secretion and a promoting action for appetite and it has been expected that an action where fat is burned via the action of GH so as to convert into energy or an effect where anabolic action of GH is expressed so as to potentiate the muscle is able to be more effectively brought out by promotion of appetite (Non-Patent

**[0010]** Document 15).

**[0011]** However, the current status is that, although the active center of ghrelin is shown to be an N-terminal moiety

having an acyl group (Patent Document 1), there are still ambiguous points for the physiological significance of the C terminal moiety.

CITATION LIST

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**[0012]** [PTL 1] WO 01/07475

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SUMMARY OF INVENTION

TECHNICAL PROBLEM

**[0014]** An object of the present invention is to provide a peptide having an action of extending the half-life of a physiologically active peptide which is not suitable as a drug due to its short half-life in plasma; a chimeric peptide having a physiological activity of the object peptide and being able to be practically used as a drug due to its extension of half-life in plasma; and a process for producing the chimeric peptide, etc.

SOLUTION TO PROBLEM

**[0015]** The present inventors have attempted to develop a peptide which is able to extend its half-life in plasma by addition of a peptide having an aimed physiological activity (hereinafter, it will be called the object peptide) which had to be intravenously or subcutaneously injected continuously for applying as a drug due to its short half-like in plasma and have found an amino acid sequence which is to become a half-life-extension motive in the C terminal moiety of the amino acid sequence of ghrelin. The peptide having an amino acid sequence which is able to extent the half-life is called a half-life-extension peptide in the present invention.

**[0016]** Thus, it has been found that, when the half-life-extension peptide is bonded to an N terminal side, C terminal side or both terminals of the object peptide (such as atrial natriuretic peptide (ANP), C type natriuretic peptide (CNP) or motilin), the half-life in plasma is able to be extended while the physiological activity of the object peptide is still retained. In the present invention, a peptide which is produced by bonding the half-life-extension peptide with the object peptide and has an extended half-life in plasma as compared with the object peptide where the physiological activity of the object

peptide is still able to be retained is called a chimeric peptide.

**[0017]** It has been also found that the amino acid sequence concerning the half-life-extension peptide is able to be used not only in the case based on the natural amino acid sequence but also in the case based on the reversed sequence where the N terminal and C terminal thereof are reversed or on the case based on the sequence in which the N terminal and the C terminal are partly reversed.

**[0018]** It has been further found that the chimeric peptide has resistance to an enzyme which decomposes the object peptide (such as neutral endopeptidase) and, the same as in the case of an object peptide of a native, it does not show an antigenic property even upon repeated administrations but is able to be safely used.

**[0019]** Furthermore, when the object peptide of a native and the chimeric peptide are administered to individuals in the same dose and the same method of use, a sufficient aimed physiological action is able to be achieved in living body if a half-life-extension peptide is bonded to the peptide so as to extent the half-life even when the dose is unable to give a sufficient aimed physiological action by the use of the object peptide only. As a result, it has been shown that the method of use which is not a continuous injection is able to give the adaptability as a drug. Thus, it has been found that, when the half-life-extension peptide according to the present invention is added to the object peptide where a continuous intravenous or subcutaneous administration is necessary due to its short half-life, a bolus administration is now possible and, moreover, the aimed activity is able to be easily achieved in the chimeric peptide as compared with the object peptide of a native.

**[0020]** The present invention has been achieved based on the above finding and it relates to the followings.

Item 1:

**[0021]** A peptide of the following (I) or (II).

(I) a peptide represented by the formula B, A-B, B-C or A-B-C in which A, B and C each is represented by the following (1), (2) and (3) and, when it is bonded to other object peptide, it is able to extent the half-life in plasma as compared with the object peptide where the physiological activity of the object peptide is still retained.
(II) a peptide comprising a reversed sequence of the peptide of (I); a sequence which is represented by A-B in (I) and A or B is reversed; a sequence which is represented by B-C in (I) and B or C is reversed; or a sequence which is represented by A-B-C in (I) and A, B, C, A and B, B and C or A and C is reserved.

(1) A is a peptide comprising 1 to 14 of any amino acid(s)
(2) B is a peptide represented by the formula 1: (Wk-X1-Y-Zm-Wn)-(Wo-Xp-Y-Zq-Wr)s
(In the formula 1, W is a basic amino acid; X and Z are any amino acids; Y is an acidic amino acid; k is 1 or 2; 1 is an integer of $4 \geq 1 \geq 0$; m is an integer of $2 \geq m \geq 0$; $4 \geq 1 + m \geq 0$; n is 1 or 2; o is 1 or 2; p is an integer of $4 \geq p \geq 0$; q is an integer of $2 \geq q \geq 0$; $4 \geq p + q \geq 0$; r is 1 or 2; and s is 0 or 1.)
(3) C is a peptide comprising 2 to 14 of any amino acids.

**[0022]** Item 2:

The peptide according to item 1, wherein B is a peptide where s is 1 in the formula 1.

**[0023]** Item 3:

The peptide according to item 2, wherein o is 0, p is 0, q is 0 and r is 2 in the formula 1.

**[0024]** Item 4:

The peptide according to item 1, wherein C contains 4 to 9 amino acid sequences being able to take $\alpha$ helix structure.

**[0025]** Item 5:

The peptide according to item 4, wherein C has a Pro sequence between its terminal and the amino acid sequence being able to take $\alpha$ helix structure.

**[0026]** Item 6:

A peptide represented by the formula B, A-B, B-C or A-B-C and, in the formula, A, B and C each is the following (1) or (2) and, when it is bonded to other peptide, its half-life is able to be extended where the physiological activity is

still retained.

(1) A is an amino acid sequence of one or more amino acid numbers 1 to 4 continuing to B in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;
B is an amino acid sequence of amino acid numbers 5 to 9 in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and
C is an amino acid sequence of one or more amino acid numbers 10 to 17 continuing to B in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added.
(2) A is an amino acid sequence of one or more amino acid numbers 1 to 8 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;
B is an amino acid sequence of amino acid numbers 9 to 13 in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and
C is an amino acid sequence of one or more amino acid numbers 14 to 17 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added.

**[0027]** Item 7:

The peptide according to item 6, wherein it is represented by the formula: B in which
B is a sequence selected from the group consisting of the amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 57; the amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; the amino acid numbers 2 to 6 in SEQ ID Nos: 58 to 60; the amino acid numbers 3 to 6 in SEQ ID Nos: 61 and 62: the amino acid numbers 3 to 7 in SEQ ID Nos: 63 to 65; and the amino acid numbers 1 to 3 in SEQ ID No: 66.

**[0028]** Item 8:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C in which
B is a sequence selected from the group consisting of the amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 57; the amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; the amino acid numbers 2 to 6 in SEQ ID Nos: 58 to 60; the amino acid numbers 3 to 6 in SEQ ID Nos: 61 and 62: the amino acid numbers 3 to 7 in SEQ ID Nos: 63 to 65; and the amino acid numbers 1 to 3 in SEQ ID No: 66,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 4 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 52 to 57; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 3 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; a sequence of amino acid number 1 in SEQ ID Nos: 58 to 60; and a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 2 in SEQ ID Nos: 61 to 65,
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from N-terminal side of amino acid numbers 10 to 17 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 54; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 9 to 16 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 10 to 16 in SEQ ID No: 57; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 7 to 20 in SEQ ID No: 58; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 7 to 13 in SEQ ID No: 61; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 8 to 14 in SEQ ID No: 63; and a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 4 to 18 in SEQ ID No: 66.

**[0029]** Item 9:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 8.

**[0030]** Item 10:

The peptide according to item 6, wherein it is represented by the formula: B and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 87; amino acid numbers 15 to 19 in SEQ ID No: 91; amino acid numbers 8 to 11 in SEQ ID No: 94; amino acid numbers 8 to 12 in SEQ ID No: 96; and amino acid numbers 16 to 18 in SEQ ID No: 99.

**[0031]** Item 11:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 87; amino acid numbers 8 to 12 in SEQ ID No: 90; amino acid numbers 15 to 19 in SEQ ID No: 91; amino acid numbers 8 to 11 in SEQ ID No: 94; amino acid numbers 8 to 12 in SEQ ID No: 96; and amino acid numbers 16 to 18 in SEQ ID No: 99,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 8 in SEQ ID Nos: 67 to 87; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 7 in SEQ ID No: 90; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 14 in SEQ ID No: 91; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 7 in SEQ ID Nos: 94 and 96; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 15 in SEQ ID No: 99 and
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from the N terminal side of amino acid numbers 14 to 17 in SEQ ID Nos: 67, 69, 72 to 74, 80, 81, 83 and 86 to 87; a sequence comprising at least one amino acid continuing from the N terminal side of amino acid numbers 14 to 16 in SEQ ID Nos: 68, 70, 71, 75 to 79, 82 and 85; a sequence comprising at least one amino acid continuing from the N terminal side of amino acid numbers 13 to 16 in SEQ ID No: 90; an amino acid number 20 in SEQ ID No: 91, a sequence comprising at least one amino acid continuing from the N terminal side of amino acid numbers 12 to 13 in SEQ ID No: 94; and a sequence comprising at least one amino acid continuing from the N terminal side of amino acid numbers 13 to 14 in SEQ ID No: 96.

**[0032]** Item 12:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 11.

**[0033]** Item 13:

The peptide according to item 6, wherein it is represented by the formula: B and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 57; amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; and amino acid numbers 2 to 6 in SEQ ID Nos: 58 to 60.

**[0034]** Item 14:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 57; amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; and amino acid numbers 2 to 6 in SEQ ID Nos: 58 to 60,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 4 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48, 50 and 53 to 57; a sequence comprising at least one amino acid continuing from the C terminal side of amino acid numbers 1 to 3 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; and an amino acid number 1 in SEQ ID Nos: 58 to 60 and
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 10 to 17 in SEQ ID Nos: 34 , 36 , 39 to 41, 47, 48, 50 and 53 to 54; a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 9 to 16 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52; a sequence comprising at least one amino acid continuing from C terminal side in SEQ ID Nos: 57; and a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 7 to 20 in SEQ ID No: 58.

**[0035]** Item 15:

The peptide according to item 14, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 14.

**[0036]** Item 16:

The peptide according to item 6, wherein it is represented by the formula: B and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 87; amino acid numbers 8 to 12 in SEQ ID No: 90; and amino acid numbers 15 to 19 in SEQ ID No: 91.

**[0037]** Item 17:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 87; amino acid numbers 8 to 12 in SEQ ID No: 90; and amino acid numbers 15 to 19 in SEQ ID No: 91,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 8 in SEQ ID Nos: 67 to 87; a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 7 in SEQ ID No: 90; and a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 14 in SEQ ID No: 91 and
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 14 to 17 in SEQ ID Nos: 67, 68, 72 to 74, 80, 81, 83 and 86 to 87; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 14 to 16 in SEQ ID Nos: 68, 70, 71, 75 to 79, 82 and 85; a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 13 to 16 in SEQ ID No: 90; and an amino acid number 20 in SEQ ID No: 91.

**[0038]** Item 18:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 17.

**[0039]** Item 19:

The peptide according to item 6, wherein it is represented by the formula: B and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 4 7, 48 and 50; and amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52.

**[0040]** Item 20:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 5 to 9 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48 and 50; and amino acid numbers 4 to 8 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 4 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48 and 50; and a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 3 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52 and
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 10 to 17 in SEQ ID Nos: 34, 36, 39 to 41, 47, 48 and 50; and a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 9 to 16 in SEQ ID Nos: 35, 37, 38, 42 to 46, 49 and 52.

**[0041]** Item 21:

The peptide according to item 6, where it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 20.

**[0042]** Item 22:

The peptide according to item 6, where it is represented by the formula: B and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 85.

**[0043]** Item 23:

The peptide according to item 6, where it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence selected from the group consisting of amino acid numbers 9 to 13 in SEQ ID Nos: 67 to 85,
A is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 8 in SEQ ID Nos: 67 to 85 and
C is a sequence selected from the group consisting of a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 14 to 17 in SEQ ID Nos: 67, 69, 72 to 74, 80, 81 and 83 and a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 14 to 16 in SEQ ID Nos: 68, 70, 71, 75 to 79, 82 and 85.

**[0044]** Item 24:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 23.

**[0045]** Item 25:

The peptide according to item 6, wherein it is represented by the formula,: B and, in the formula,
B is a sequence of amino acid numbers 5 to 9 in SEQ ID No: 34.

**[0046]** Item 26:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence of amino acid numbers 5 to 9 in SEQ ID No: 34,
A is a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 4 in SEQ ID No: 34 and
C is a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 10 to 17 in SEQ ID No: 34.

**[0047]** Item 27:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 26.

**[0048]** Item 28:

The peptide according to item 6, wherein it is represented by the formula: B and, in the formula,
B is a sequence of amino acid numbers 9 to 13 in SEQ ID No: 67.

**[0049]** Item 29:

The peptide according to item 6, wherein it is represented by the formula: A-B or B-C and, in the formula,
B is a sequence of amino acid numbers 9 to 13 in SEQ ID No: 67,
A is a sequence comprising at least one amino acid continuing from C terminal side of amino acid numbers 1 to 8 in SEQ ID No: 67 and
C is a sequence comprising at least one amino acid continuing from N terminal side of amino acid numbers 14 to 17 in SEQ ID No: 67.

**[0050]** Item 30:

The peptide according to item 6, wherein it is represented by the formula: A-B-C and, in the formula, A, B and C are the same as those in item 29.

**[0051]** Item 31:

A chimeric peptide where a peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to N terminal, C terminal or both of the object peptide and its half-life in plasma is extended as

compared with the object peptide while physiological activity of the object peptide is still retained.

**[0052]** Item 32:

The chimeric peptide according to item 31, wherein the peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to N terminal of the object peptide.

**[0053]** Item 33:

The chimeric peptide according to item 31, wherein the peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to C terminal of the object peptide.

**[0054]** Item 34:

The chimeric peptide according to item 31, wherein the peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to both terminals of the object peptide.

**[0055]** Item 35:

The chimeric peptide according to any of items 31 to 34, wherein the object peptide is an atrial natriuretic peptide, a C type natriuretic peptide or motilin or derivatives thereof.

**[0056]** Item 36:

A pharmaceutical composition in which the chimeric peptide mentioned in any of items 31 to 35 or a pharmaceutically acceptable salt thereof is an effective ingredient.

**[0057]** Item 37:

The pharmaceutical composition according to item 36, wherein the chimeric peptide is the peptide mentioned in item 35.

**[0058]** Item 38:

The pharmaceutical composition according to item 37, wherein the object peptide in the chimeric peptide is an atrial natriuretic peptide or a derivative thereof.

**[0059]** Item 39:

The pharmaceutical composition according to item 38, wherein it is for the treatment of diseases selected from acute cardiac insufficiency, chronic cardiac insufficiency, obliterative arteriosclerosis, ischemic cardiac disease, hypertension, edema disease, myocardial disease, retinitis, diabetic renal disease, nephrosclerosis and myocardial infarction.

**[0060]** Item 40:

The pharmaceutical composition according to item 37, wherein the object peptide in the chimeric peptide is a C type natriuretic peptide or a derivative thereof.

**[0061]** Item 41:

The pharmaceutical composition according to item 40, wherein it is for the treatment of diseases selected from atypical chondrogenesis insufficiency,restenosis after PTCA after coronary artery stenosis, pulmonary hypertension, peripheral artery obliterative disease, osteoarthritis, rheumatoid arthritis, pulmonary fibrosis, hepatic fibrosis, renal fibrosis, myocardial infarction and myocarditis.

**[0062]** Item 42:

The pharmaceutical composition according to item 37, wherein the object peptide in the chimeric peptide is motilin

or a derivative thereof.

**[0063]** Item 43:

The pharmaceutical composition according to item 42, wherein it is for the treatment of diseases selected from functional dyspepsia, reflux esophagitis, diabetic gastric motility paralysis, constipation-type irritable bowel syndrome, chronic peudoileus, postoperative ileus, chronic gastritis and atrophic gastritis.

**[0064]** Item 44:

A process for producing a chimeric peptide where half-life in plasma is extended as compared with the object peptide and a physiological activity of the object peptide is available, characterized in that, a peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to N terminal, C terminal or both of the object peptide.

**[0065]** Item 45:

The process according to item 44, wherein a peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to N terminal of the object peptide.

**[0066]** Item 46:

The process according to item 44, wherein a peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to C terminal of the object peptide.

**[0067]** Item 47:

The process according to item 44, wherein a peptide selected from the group consisting of the peptides mentioned in any of items 1 to 30 is bonded to both terminals of the object peptide.

**[0068]** Item 48:

The process according to any of items 44 to 47, wherein the object peptide is a natriuretic peptide or motilin or derivatives thereof.

**[0069]** Item 49:

The process according to item 48, wherein the object peptide is an atrial natriuretic peptide or a derivative thereof.

**[0070]** Item 50:

The process according to item 48, wherein the object peptide is a C type natriuretic peptide or a derivative thereof.

**[0071]** Item 51:

The process according to item 48, wherein the object peptide is motilin or a derivative thereof.

**[0072]** Item 52:

A method for the treatment of a disease which is able to be treated by the object peptide contained in a pharmaceutical composition, comprising administration of the pharmaceutical composition selected from the pharmaceutical compositions mentioned in any of items 36 to 43 to an individual.

**[0073]** Item 53:

The method for the treatment according to item 52, wherein the pharmaceutical composition is the pharmaceutical composition mentioned in item 37.

**[0074]** Item 54:

The method for the treatment according to item 53, wherein the pharmaceutical composition is the pharmaceutical composition mentioned in item 38.

**[0075]** Item 55:

The method for the treatment according to item 54, wherein the disease is a disease selected from acute cardiac insufficiency, chronic cardiac insufficiency, obliterative arteriosclerosis, ischemic cardiac disease, hypertension, edema disease, myocardial disease, retinitis, diabetic renal disease, nephrosclerosis and myocardial infarction.

**[0076]** Item 56:

The method for the treatment according to item 53, wherein the pharmaceutical composition is the pharmaceutical composition mentioned in item 40.

**[0077]** Item 57:

The method for the treatment according to item 56, wherein the disease is a disease selected from atypical chondrogenesis insufficiency, restenosis after PTCA after coronary artery stenosis, pulmonary hypertension, peripheral artery obliterative disease, osteoarthritis, rheumatoid arthritis, pulmonary fibrosis, hepatic fibrosis, renal fibrosis, myocardial infarction and myocarditis.

**[0078]** Item 58:

The method for the treatment according to item 53, wherein the pharmaceutical composition is the pharmaceutical composition mentioned in item 42.

**[0079]** Item 59:

The method for the treatment according to item 58, wherein the disease is a disease selected from functional dyspepsia, reflux esophagitis, diabetic gastric motility paralysis, constipation-type irritable bowel syndrome, chronic peudoileus, postoperative ileus, chronic gastritis and atrophic gastritis.

**[0080]** Item 60:

A method for extension of half-life in plasma of the object peptide, characterized in that, the peptide selected from the peptides mentioned in any of items 1 to 30 is bonded to N terminal, C terminal or both of the object peptide.

**[0081]** Item 61:

The method according to item 60, wherein the peptide selected from the peptides mentioned in any of items 1 to 30 is bonded to N terminal of the object peptide.

**[0082]** Item 62:

The method according to item 60, wherein the peptide selected from the peptides mentioned in any of items 1, to 30 is bonded to C terminal of the object peptide.

**[0083]** Item 63:

The method according to item 60, wherein the peptide selected from the peptides mentioned in any of items 1 to 30 is bonded to both terminals of the object peptide.

**[0084]** Item 64:

The method according to any of items 60 to 63, wherein the object peptide is a natriuretic peptide or motilin or derivatives thereof.

**[0085]** Item 65:

The method according to item 64, wherein the object peptide is an atrial natriuretic peptide or a derivative thereof.

**[0086]** Item 66:

The method according to items 64, wherein the object peptide is a C type natriuretic peptide or a derivative thereof.

**[0087]** Item 67:

The process according to item 64, wherein the object peptide is motilin or a derivative thereof.

## ADVANTAGEOUS EFFECTS OF INVENTION

**[0088]** When the peptide where half-life is extended according to the present invention is added to an object peptide having a short half-life, the pharmacokinetics *in vivo* are improved and a product having a half-life which is practical as a drug is achieved. Since its antigenic property is almost the same as those of the object peptide of a native, it has an excellent safety and shows resistance to enzymes which decompose a physiologically active peptide *in vivo*. When the peptide where the half-life is extended is used, it is now possible to greatly reduce the cost and time which have been necessary for the development of physiologically active peptides where the pharmacokinetics *in vivo* are improved. In addition, it is possible that an administering method for the physiologically active peptide used for the treatment by a continuous intravenous administration or a continuous subcutaneous administration is conducted in a single-time bolus administration whereby it is now expected to improve the compliance of the individual (patient) and of a medical field.

## BRIEF DESCRIPTION OF DRAWINGS

**[0089]**

[Fig. 1] This is a drawing which shows changes in an. ANP immunoreactivity concentration in plasma when ANP or a chimeric ANP (A to C) thereof (0.1 mg/kg) is intravenously or subcutaneously administered to male rats.
[Fig. 2] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma when native CNPs-22 or a chimeric CNP (A and B) thereof (0.1 mg/kg) is intravenously or subcutaneously administered at one time to male rats.
[Fig. 3] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma and in cGMP concentration in plasma when native CNP-22 or a chimeric CNP (B) thereof (0.1 mg/kg) is subcutaneously administered to male rats.
[Fig. 4] This is a drawing which shows the influence of NEP inhibitor on changes in CNP immunoreactivity concentration in plasma when native CNP-22 and a chimeric CNP (B) thereof (0.02 mg/kg) are intravenously administered to male rats.
[Fig. 5] This is a drawing which shows changes in body length, tail length and body weight when native CNP-22 or a chimeric CNP (B) thereof (0.25 mg/kg) are subcutaneously administered twice daily for 29 days to female mice repeatedly.
[Fig. 6] This is a drawing which shows changes in a motilin immunoreactivity concentration in plasma when motilin or a chimeric motilin thereof (A to M) (10 nmol/kg) is intravenously administered to male rats.
[Fig. 7] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma when native CNP-22 or a chimeric CNP (C to I) (10 nmol/kg) is intravenously administered to male rats.
[Fig. 8] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma when a chimeric motilin (N to V, X to Z and I to XIII) (10 nmol/kg) is intravenously administered.
[Fig. 9] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma when native CNP-22 or a chimeric CNP (D, J and K) (20 nmol/kg) is intravenously administered to male rats.
[Fig. 10] This is a drawing which shows changes in body length, tail length and body weight when a chimeric CNP (D and J) (50 and 200 nmol/kg) is subcutaneously administered once daily for 56 days repeatedly to female mice.
[Fig. 11] This is a drawing which shows a total body image after a chimeric CNP (J) (50 and 200 nmol/kg) is subcutaneously administered once daily for 56 days repeatedly to female mice.
[Fig. 12] This is a drawing which shows changes in body length, tail length and body weight when a chimeric CNP (D) (12.5, 50 and 200 nmol/kg) is subcutaneously administered once daily for 28 days repeatedly to female rats.
[Fig. 13] This is a drawing which shows changes in a CNP immunoreactivity concentration in plasma when native CNP-22 , native CNP-53 or a chimeric CNP (D) (10 nmol/kg) are intravenously administered.

DESCRIPTION OF PREFERRED EMBODIMENTS

**[0090]** The present invention will now be illustrated in detail as follows.

(1) Half-life-extended peptide

**[0091]** The half-life-extended peptide of the present invention is represented by B, A-B, B-C and A-B-C and, in the formulae, A, B and C are the following (1) to (3) and it is a peptide which is able to extent the half-life in plasma, when bonded to other object peptide (physiologically active peptide) , as compared with the object peptide while the physiological activity of the object peptide is still retained.

(1) As shown by the amino acid sequence (14 amino acids: Table 5) in 1 to 14 positions in C terminal fragment (SEQ ID No: 91) derived from frog ghrelin, chimeric motilin (A to F and G to K) (they are in Table 14), MG-d12/14 (Table 16), chimeric CNP (J) and chimeric CNP (K) (Table 17), A may be absent or a sequence of amino acid in any number may be present. As to the length when the sequence is present, a sequence of about 1 to 14 amino acid(s) may be selected and it is preferably 3 to 10 amino acids and, more preferably, 3 amino acids. Specific examples include VQQ and AGSVDHKGKQ.
(2) B is a part which corresponds to the structure (core sequence: RKESKK sequence part) necessary for an action for extending the half-life of the peptide of the present invention derived from human ghrelin which will be mentioned later and examples for B of the peptide of the present invention derived from human ghrelin include the followings. The specific peptides mentioned below are described in Tables 16 and 17.

**[0092]** As chimeric motilin (T) (MG-17E/N), (U) (MG-17E/Q) and (W) (MG-dES) show, the E(Glu) in RKESKK is an essential amino acid. Further, as chimeric motilin (V) (MG-ES/DS), (X) (MG-17E/D), chimeric CNP (J) and chimeric CNP (K) show, it may be D(Asp) as well. Thus, acidic amino acid will do for the amino acid at the position of the above E(Glu) and Glu is preferred.

**[0093]** As chimeric motilin (O) (MG-18S/F), (P) (MG-18S/T), (Q) (MG-18S/P), (R) (MG-18S/L) and (S) (MG-18S/A) show, the S(Ser) in RKESKK is able to be substituted with amino acid having any side chain such as aromatic amino acid, hydrophobic amino acid and polar non-charged amino acid. Thus, the amino acid at the above position of S(Ser) may be an amino acid having any side chain structure. It is preferably Ser, Pro, Leu, Phe or Ala, more preferably Ser, Thr, Pro or Ala and, most preferably, Ser. Further, as shown the peptide MG-dS, the amino acid at the position or the above S (Ser) may be absent.

**[0094]** As shown by chimeric motilin (X) (MG-17E/D), R (Arg) and K (Lys) in RKESKK are able to be substituted provided that it is a basic amino acid.

**[0095]** RKESKK is characterized in containing a basic amino acid cluster (a continuous sequence of two basic amino acids) and an acidic amino acid and, as shown in chimeric motilin (Z) (MG-i17G), (I) (MG-i19G), (II) (MG-i17G2,) and (III) (MG-il17G2-i19G) , the distance between the clusters is able to be expanded by insertion of any amino acid.

**[0096]** As shown in each peptide mentioned in Tables 16 and 17, the distance between the basic amino acid clusters is preferred to be in such an extent that 1 to 5 amino acid(s) is/are present.

**[0097]** Further, any amino acid may be present between the basic amino acid cluster and E (Glu) in RKESKK as shown in chimeric motilin (Z) (MG-i17G), (I) (MG-i19G), (II) (MG-i17G2), (III) (MG-i17G2-19iG), (IV) (MG-dPP), (V) (MG-dPPH1), (VI) (MG-H1), (VII) (MG-H3), (VIII) (MGH4), (XI) (MG-d12/14), (XII) (MGP1), (XIII) $(MGP)_2$ etc. and chimeric CNP (K). For example, G, A, Y, SP and/or V may be made to exit between the basic amino acid cluster (RK-corresponding part) and E and, between E and the basic amino acid cluster (KK- corresponding part), S, H and/or P may be made to exist.

**[0098]** In view of the above, the structure B (core sequence: RKESKK part for human) necessary for an action of half-life-extension concerning the invention of the present application has, firstly, a sequence represented by the following formula 2.

Wk-Xl-Y-Zm-Wn Formula 2:

**[0099]** In the formula, W is a basic amino acid; X and Z each is any amino acid; and Y is an acidic amino acid. Each k, 1, m and n is 0 or a natural number, k is 1 or 2, $4 \geq 1 \geq 0$, $2 \geq m \geq 0$ (where 1 and m are $4 \geq 1 + m \geq 0$) and n is 1 or 2.

**[0100]** As shown in chimeric motilin (Y) (MG-BR), the way of alignment of RKESKK may be reversed (KKSEKR).

**[0101]** Further, as shown in chimeric motilin (XII) (MGP1) and (XIII) (MGP2), since a half-life-extension action is potentiated when the core sequence is aligned in two tandems (such as KKAYSPK + ERK), it is also an effective means in achieving longer half-life extension that plural core sequences are available in a molecule as shown by the following formula.

**[0102]** Accordingly, the structure (core sequence: RKESKK part) necessary for the half-life-extension action concerning

the invention of the present application is expressed by the following formula 1:

(Wk-X1-Y-Zm-Wn) + (Wo-Xp-Y-Zq-Wr)s Formula 1:

**[0103]** In the formula, W, X, Y, Z, K, 1, m and n are the same as those in the above formula 2 and s is 0 or 1. Further, o, p, q and r each is 0 or a natural number, $2 \geq o \geq 0$, $4 \geq p \geq 0$, $2 \geq q \geq 0$ (where p and q are $4 \geq p + q \geq 0$) and r is 1 or 2.

**[0104]** The acidic amino acid is E(Glu) or D(Asp) and, preferably, E(Glu). With regard to X and Z which are any amino acids, X and Z may be same or different and, when there are plural ones, each of them may be same or different. Preferably, X and Z each is Ser, Pro, Leu, Phe or Ala, more preferably Ser, Thr, Pro or Ala and, most preferably, Ser. With regard to the basic amino acid W, both W may be same or different and, when there are plural ones, each of them may be same or different. W is preferably R(Arg) or K(Lys).

**[0105]** Preferably, in the formula 1, s is 1; o, p and q each is 0; and r is 2.

**[0106]** C is able to select any sequence of amino acid numbers such as about 2 to 14 amino acids as shown in the amino acid sequence (two amino acids: Table 14) at 22 to 23 positions in chimeric motilin (J), the amino acid sequence (14 amino acids: Table 5) at 7 to 20 positions in C terminal fragment (SEQ ID No: 58) derived from frog ghrelin and the chimeric motilin (VI) (MG-H1), (VII) (MG-H3) and (VIII) (MG-H4) aligned with a sequence (such as AK (or E) LAALK (or E) A) predicted to form an α-helix structure by a known method for predicting the secondary structure (such as Chou-Fasman method: Biochemistry. 1974 Jan 15;13(2):222-45 Prediction of protein conformation. Chou PY, Fasman GD; or Garnier method: J Mol Biol. 1978 Mar 25;120(1):97-120. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. Garnier J, Osguthorpe DJ, Robseon B. etc.).

**[0107]** It is preferred to be any sequence comprising 3 to 11 amino acids being able to form an α helix structure, more preferably KKPPAKLQPR (a sequence comprising amino acids at 22nd to 29th positions from N terminal in chimeric motilin (C)) or PPAELAALEA (a sequence comprising amino acids at 22nd to 31st positions from N terminal in chimeric motilin (VII) (MG-H3)) and, still more preferably, PPAELAALEA. It is also possible to use any sequence which is able to form a sheet structure by the above prediction method.

**[0108]** Further, when no Pro is present between the amino acid sequence corresponding to the formula C of the half-life-extension peptide according to the invention of the present invention and the amino acid sequence corresponding to the formula B thereof, it is preferred that Pro sequence (Pro numbers within a range by which amino acid numbers of C become 2 to 14 or, preferably, Pro-Pro) is aligned at the terminal site of the amino acid sequence corresponding to the formula C as shown by a comparison of chimeric motilin (IV) (MG-dPP) with chimeric motilin (D), a comparison of chimeric motilin (V) (MG-dPPH1) with chimeric motilin (VI) (MG-H1) and a comparison of chimeric motilin (IX) (MG-dPPS) with chimeric motilin (X) (MG-S).

**[0109]** Further, since a chimeric CNP containing a sequence corresponding to B (core sequence) of the formula of the half-life-extension peptide concerning the invention of the present application also shows a half-life-extension action as shown in the chimeric CNP (J) and (K), it is easily able to be predicted by persons skilled in the art that the same half-life-extension action is able to be achieved when the requirements for A and C of the formula of the half-life-extension peptide concerning the invention of the present application introduced from the comparison in half-lives of chimeric motilin is applied to the chimeric CNP. It is also able to be easily predicted by persons skilled in the art that the same half-life-extension action is able to be achieved when the requirements for B and C of the formula of the half-life-extension peptide concerning the invention of the present application introduced from the comparison in half-lives of chimeric CNP is applied to the chimeric motilin.

**[0110]** Further, it is also able to be easily predicted by persons skilled in the art that the same half-life-extension action is able to be achieved when the requirements for A, B and C of the formula of the half-life-extension peptide concerning the invention of the present application is applied to the chimeric peptide of other object peptides.

**[0111]** A, B and/or C each may be in the normal direction or the reversed direction. Thus, A, B and C each independently may be in a normal direction or a reversed direction or A-B, B-C and A-B-C may be in the normal direction or the reversed direction as a whole. The normal direction and the reversed order used here mean that the sequence direction from N terminal and C terminal is reversed each other.

**[0112]** When the peptide of the present invention is a sole B, it includes the case where a sequence satisfying the requirements of A and/or C is present in the object peptide side.

**[0113]** In the half-life-extension peptide of the present invention, the preferred one is a peptide of the following (1) or (2).

(1) A is an amino acid sequence of one or more amino acid numbers 1 to 4 continuing to B in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;
B is an amino acid sequence of one or more amino acid numbers 5 to 9 in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and
C is an amino acid sequence of one or more amino acid numbers 10 to 17 continuing to B in SEQ ID No: 34 or an

amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added.

(2) A is an amino acid sequence of one or more amino acid numbers 1 to 8 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;

B is an amino acid sequence of amino acid numbers 9 to 13 in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and

C is an amino acid sequence of one or more amino acid numbers 14 to 17 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added. SEQ ID No: 67 is a reversed sequence of SEQ ID No: 34. B is an essential core sequence.

**[0114]** In other words, the half-life-extension peptide according to the present invention is a peptide containing the amino acid sequence of amino acid numbers 5 to 9 in SEQ ID No: 34 or amino acid sequence of amino acid numbers 9 to 13 in SEQ ID No: 67 which is a reversed sequence of the above and comprising 17 or less amino acids or a peptide in which one or some amino acid(s) is/are deleted, substituted and/or added in the amino acid sequence. The term "one or some" used hereinabove means 1 to 35 for example, preferably 1 to 15, more preferably 1 to 10, still more preferably 1 to 8, still more preferably 1 to 6, still more preferably 1 to 5, still more preferably 1 to 4, still more preferably 1 to 3 and, still more preferably, 1 to 2.

**[0115]** The half-life-extension peptide of the present invention includes a half-life-extension peptide comprising a C terminal partial sequence of ghrelin of mammals such as human, monkey, cattle, horse, swine, dog, deer, sheep, goat, cat, rabbit, rat, *Suncus* (musk rat) or whale, birds such as turkey or domestic fowl, reptiles such as turtle, amphibians such as frog and fish such as eel, catfish or shark.

**[0116]** As to deletion, substitution and/or addition of one or some amino acid(s) in the amino acid sequence of A, B and C each of SEQ ID No: 34 or SEQ ID No: 67, persons skilled in the art are able to appropriately select the position and type of the suitable amino acid for conducting the modification as such by comparison among the amino acid sequences derived from various species.

**[0117]** Amino acid sequence of ghrelin derived from various organisms, hale-life-extending peptide of C terminal part thereof, full length of core sequence B, full length of sequence A which may be present in the N terminal side of the core sequence and full length of sequence C which may be present in the C terminal side of the core sequence are shown in the following Tables 1 to 7.

Table 1

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Human | SEQ ID No: 1 | SEQ ID No: 34<br>A: 1 to 4<br>B:5 to 9<br>C:10 to 17 | SEQ ID No:67<br>A: 1 to 8<br>B:9 to13<br>C: 14 to 17 |
| | SEQ ID No: 2 | SEQ ID No: 35<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 68<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| Monkey | SEQ ID No: 3 | SEQ ID No: 36<br>A: 1 to 4<br>B: 5 to 9<br>C:10 to 17 | SEQ ID No: 69<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |

(continued)

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Cattle | SEQ ID No: 4 | SEQ ID No: 37<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 70<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| | SEQ ID No: 5 | SEQ ID No: 38<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 71<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| Horse | SEQ ID No: 6 | SEQ ID No: 39<br>A: 1 to 4<br>B: 5 to 9<br>C: 14 to 17 | SEQ ID No: 72<br>A: 1 to 8<br>B: 9 to 13<br>C:14 to 17 |
| Swine | SEQ ID No: 7 | SEQ ID No: 40<br>A: 1 to 4<br>B:5 to 9<br>C:10 to 17 | SEQ ID No: 73<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |

Table 2

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Dog | SEQ ID No:8 | SEQ ID No: 41<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 17 | SEQ ID No: 74<br>A: 1 to 8<br>B:9 to 13<br>C: 14 to 17 |
| Deer | SEQ ID No: 9 | SEQ ID No:42<br>A: 1 to 3<br>B: 4 to 8<br>C:9 to 16 | SEQ ID No: 75<br>A: 1 to 8<br>B: 9 to 13<br>C:14 to 16 |
| | SEQ ID No: 10 | SEQ ID No:43<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No:76<br>A: 1 to 8<br>B: 9 to13<br>C: 14 to 16 |
| Sheep | SEQ ID No: 11 | SEQ ID No:44<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 77<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| Goat | SEQ ID No: 12 | SEQ ID No: 45<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 78<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| Cat | SEQ ID No: 13 | SEQ ID No: 46<br>A: 1 to 3<br>B: 4 to 8 | SEQ ID No: 79<br>A: 1 to 8<br>B: 9 to 13 |

(continued)

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| | | C: 9 to 16 | C: 14 to 16 |
| Rabbit | SEQ ID No: 14 | SEQ ID No: 47<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 17 | SEQ ID No: 80<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |

Table 3

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Rat | SEQ ID No: 15 | SEQ ID No: 48<br>A: 1 to 4<br>B:5 to 9<br>C: 10 to 17 | SEQ ID No: 81<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |
| | SEQ ID No: 16 | SEQ ID No: 49<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 82<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| | SEQ ID No: 17 | SEQ ID No: 50<br>A: 1 to 4<br>B:5 to 9<br>C: 10 to 17 | SEQ ID No: 83<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |
| *Suncus* | SEQ ID No: 18 | SEQ ID No: 51<br>A: 1 to 2<br>B: 3 to 8<br>C: 9 to 15 | SEQ ID No: 84<br>A: 1 to 7<br>B: 8 to 13<br>C: 14 to 15 |
| Whale | SEQ ID No: 19 | SEQ ID No: 52<br>A: 1 to 3<br>B: 4 to 8<br>C: 9 to 16 | SEQ ID No: 85<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 16 |
| Turkey | SEQ ID No: 20 | SEQ ID No: 53<br>A: 1 to 4<br>8: 5 to 9<br>C: 10 to 17 | SEQ ID No: 86<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |

Table 4

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Domestic Fowl | SEQ ID No: 21 | SEQ ID No: 54<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 17 | SEQ ID No: 87<br>A: 1 to 8<br>B: 9 to 13<br>C: 14 to 17 |
| | SEQ ID No:22 | SEQ ID No:55<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 15 | SEQ ID No: 88<br>A: 1 to 6<br>B:7 to 11<br>C: 12 to 15 |
| | SEQ ID No: 23 | SEQ ID No: 56<br>A: 1 to 4<br>B: 5 to 9<br>C:10 to 13 | SEQ ID No: 89<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 13 |
| Turtle | SEQ ID No: 24 | SEQ ID No: 57<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 16 | SEQ ID No: 90<br>A: 1 to 7<br>B: 8 to 12<br>C: 13 to 16 |

Table 5

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Frog | SEQ ID No: 25 | SEQ ID No: 58<br>A: 1<br>B: 2 to 6<br>C: 7 to 20 | SEQ ID No: 91<br>A: 1 to 14<br>B: 15 to 19<br>C: 20 |
| | SEQ ID No: 26 | SEQ ID No: 59<br>A: 1<br>B: 2 to 6<br>C: 7 to 18 | SEQ ID No: 92<br>A: 1 to 12<br>B: 13 to 17<br>C: 18 |
| | SEQ ID No: 27 | SEQ ID No: 60<br>A: 1<br>B :2 to 6<br>C: 7 to 17 | SEQ ID No: 93<br>A: 1 to 11<br>B: 12 to 16<br>C: 17 |

Table 6

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Eel | SEQ ID No: 28 | SEQ ID No: 61<br>A:1 to 2<br>B: 3 to 6<br>C: 7 to 13 | SEQ ID No: 94<br>A: 1 to 7<br>B: 8 to 11<br>C: 12 to 13 |
| | SEQ ID No: 29 | SEQ ID No: 62<br>A: 1 to 2<br>B: 3 to 6<br>C: 7 to 10 | SEQ ID No: 95<br>A: 1 to 4<br>B: 5 to 8<br>C: 9 to 10 |

Table 7

| Species | Sequence No. of Ghrelin | Amino Acid No. of each Part of Sequence No. (Normal Direction) of Half-life-extension Peptide | Amino Acid No. of each Part of Sequence No. (Reversed Direction) of Half-life-extension Peptide |
|---|---|---|---|
| Catfish | SEQ ID No: 30 | SEQ ID No: 63<br>A: 1 to 2<br>B: 3 to 7<br>C: 8 to 14 | SEQ ID No: 96<br>A: 1 to 7<br>B: 8 to 12<br>C: 13 to 14 |
| | SEQ ID No: 31 | SEQ ID No: 64<br>A: 1 to 2<br>B: 3 to 7<br>C: 8 to 12 | SEQ ID No: 97<br>A: 1 to 5<br>B: 6 to 10<br>C: 11 to 12 |
| | SEQ ID No: 32 | SEQ ID No: 65<br>A: 1 to 2<br>B: 3 to 7<br>C: 8 to 11 | SEQ ID No: 98<br>A: 1 to 4<br>B: 5 to 9<br>C: 10 to 11 |
| Shark | SEQ ID No: 33 | SEQ ID No: 66<br>A: nil<br>B: 1 to 3<br>C: 4 to 18 | SEQ ID No: 99<br>A: 1 to 15<br>B: 16 to 18<br>C: nil |

**[0118]** Amino acid sequences of ghrelin derived from various organisms in Tables 1 to 7 are shown as hereunder. The underlined ones are the parts corresponding to the hale-life-extending peptide.

| | | |
|---|---|---|
| Human | GSSFLSPEHQRVQQRKESKKPPAKLQPR | (SEQ ID No: 1) |
| | GSSFLSPEHQRVQ-RKESKKPPAKLQPR | (SEQ ID No: 2) |
| Monkey | GSSFLSPEHQRAQQRKESKKPPAKLQPR | (SEQ ID No: 3) |
| Cattle | GSSFLSPEHQKLQ-RKEAKKPSGRLKPR | (SEQ ID No: 4) |
| | GSSFLSPEHQKLQ-RKEPKKPSGRLKPR | (SEQ ID No: 5) |
| Horse | GSSFLSPEHHKVQHRKESKKPPAKLKPR | (SEQ ID No: 6) |
| Swine | GSSFLSPEHQKVQQRKESKKPAAKLKPR | (SEQ ID No: 7) |
| Dog | GSSFLSPEHQKLQQRKESKKPPAKLQPR | (SEQ ID No: 8) |
| Deer | GSSFLSPEHQKLQ-RKEPKKPSGRLKPR | (SEQ ID No: 9) |
| | GSSFLSPDHQKLQ-RKEPKKPSGRLKPR | (SEQ ID No: 10) |
| Sheep | GSSFLSPEHQKLQ-RKEPKKPSGRLKPR | (SEQ ID No: 11) |
| Gout | GSSFLSPEHQKLQ-RKEPKKPSGRLKPR | (SEQ ID No: 12) |

(continued)

| | | |
|---|---|---|
| Cat | GSSFLSPEHQKVQ-RKESKKPPAKLQPR | (SEQ ID No: 13) |
| Rabbit | GSSFLSPEHQKVQQRKESKKPAAKLKPR | (SEQ ID No: 14) |
| Rat | GSSFLSPEHQKAQQRKESKKPPAKLQPR | (SEQ ID No: 15) |
| | GSSFLSPEHQKAQ-RKESKKPPAKLQPR | (SEQ ID No: 16) |
| | GSSFLSPEHQKTQQRKESKKPPAKLQPR | (SEQ ID No: 17) |
| Suncus | GSSFLSPEHQKGP-KKDPRKPPKLQPR | (SEQ ID No: 18) |
| Whale | GSSFLSPEHQKLQ-RKEAKKPSGRLKPR | (SEQ ID No: 19) |
| Turkey | GSSFLSPAYKNIQQQKDTRKPTARLHPR | (SEQ ID No: 20) |
| Dom. Fowl | GSSFLSPTYKNIQQQKDTRKPTARLHRR | (SEQ ID No: 21) |
| | GSSFLSPTYKNIQQQKDTRKPTARLH | (SEQ ID No: 22) |
| | GSSFLSPTYKNIQQQKDTRKPTAR | (SEQ ID No: 23) |
| Turtle | GSSFLSPEYQNTQQRKDPKKHTK-LNRR | (SEQ ID No: 24) |
| Frog | GLTFLSPADMQKIAERQSQNKLRHGNMNRR | (SEQ ID No: 25) |
| | GLTFLSPADMQKIAERQSQNKLRHGNMN | (SEQ ID No: 26) |
| | GLTFLSPADMQKIAERQSQNKLRHGNM | (SEQ ID No: 27) |
| Eel | GSSFLSPS-QRPQG-KD-KKP-PRVGRR | (SEQ ID No: 28) |
| | GSSFLSPS-QRPQG-KD-KKP-PRV-$NH_2$ | (SEQ ID No: 29) |
| Catfish | GSSFLSPT-QKPQNR-GDRKPP-RVGRR | (SEQ ID No: 30) |
| | GSSFLSPT-QKPQNR-GDRKPP-RVG | (SEQ ID No: 31) |
| | GSSFLSPT-QKPQNR-GDRKPP-RV-$NH_2$ | (SEQ ID No: 32) |
| Shark | GVSFHPRLKEKDDNSSGNSRKSNPKR | (SEQ ID No: 33) |

**[0119]** Amino acid sequences of the half-life-extension peptide (normal sequences) derived from various organisms in Tables 1 to 7 are shown as hereunder. The underlined ones are the parts corresponding to the core sequence B, the N terminal side of the core sequence B is the part corresponding to the sequence A and the C terminal side of the core sequence B is the part corresponding to the sequence C.

| | | |
|---|---|---|
| Human | VQQRKESKKPPAKLQPR | (SEQ ID No: 34) |
| | VQ-RKESKKPPAKLQPR | (SEQ ID No: 35) |
| Monkey | AQQRKESKKPPAKLQPR | (SEQ ID No: 36) |
| Cattle | LQ-RKEAKKPSGRLKPR | (SEQ ID No: 37) |
| | LQ-RKEPKKPSGRLKPR | (SEQ ID No: 38) |
| Horse | VQHRKESKKPPAKLKPR | (SEQ ID No: 39) |
| Swine | VQQRKESKKPAAKLKPR | (SEQ ID No: 40) |
| Dog | LQQRKESKKPPAKLQPR | (SEQ ID No: 41) |
| Deer | LQ-RKEPKKPSGRLKPR | (SEQ ID No: 42) |
| | LQ-RKEPKKPSGRLKPR | (SEQ ID No: 43) |
| Sheep | LQ-RKEPKKPSGRLKPR | (SEQ ID No: 44) |
| Goat | LQ-RKEPKKPSGRLKPR | (SEQ ID No: 45) |
| Cat | VQ-RKESKKPPAKLQPR | (SEQ ID No: 46) |
| Rabbit | VQQRKESKKPAAKLKPR | (SEQ ID No: 47) |
| Rat | AQQRKESKKPPAKLQPR | (SEQ ID No: 48) |
| | AQ-RKESKKPPAKLQPR | (SEQ ID No: 49) |
| | TQQRKESKKPPAKLQPR | (SEQ ID No: 50) |
| Suncus | GP-KKDPRKPPKLQPR | (SEQ ID No: 51) |
| Whale | LQ-RKEAKKPSGRLKPR | (SEQ ID No: 52) |
| Turkey | IQQQKDTRKPTARLHPR | (SEQ ID No: 53) |
| Dom. Fowl | IQQQKDTRKPTARLHRR | (SEQ ID No: 54) |
| | IQQQKDTRKPTARLH | (SEQ ID No: 55) |
| | IQQQKDTRKPTAR | (SEQ ID No: 56) |
| Turtle | TQQRKDPKKHTK-LNRR | (SEQ ID No: 57) |

(continued)

| | | |
|---|---|---|
| Frog | QKIAERQSQNKLRHGNMNRR | (SEQ ID No: 58) |
| | QKIAERQSQNKLRHGNMN | (SEQ ID No: 59) |
| | QKIAERQSQNKLRHGNM | (SEQ ID No: 60) |
| Eel | QK-KD-KKP-PRVGRR | (SEQ ID No: 61) |
| | QK-KD-KKP-PRV | (SEQ ID No: 62) |
| Catfish | QNR-GDRKPP-RVGRR | (SEQ ID No: 63) |
| | QNR-GDRKPP-RVG | (SEQ ID No: 64) |
| | QNR-GDRKPP-RV | (SEQ ID No: 65) |
| Shark | KEKDDNSSGNSRKSNPKR | (SEQ ID No: 66) |

**[0120]** Amino acid sequences of the half-life-extension peptide (reversed sequences) derived from various organisms in Tables 1 to 7 are shown as hereunder. The underlined ones are the parts corresponding to the core sequence B, the N terminal side of the core sequence B is the part corresponding to the sequence A and the C terminal side of the core sequence B is the part corresponding to the sequence C.

| | | |
|---|---|---|
| Human | RPQLKAPPKKSEKRQQV | (SEQ ID No: 67) |
| | RPQLKAPPKKSEKR-QV | (SEQ ID No: 68) |
| Monkey | RPQLKAPPKKSEKRQQA | (SEQ ID No: 69) |
| Cattle | RPKLRGSPKKAEKR-QL | (SEQ ID No: 70) |
| | RPKLRGSPKKPEKR-QL | (SEQ ID No: 71) |
| Horse | RPKLKAPPKKSEKRHQV | (SEQ ID No: 72) |
| Swine | RPKLKAAPKKSEKRQQV | (SEQ ID No: 73) |
| Dog | RPQLKAPPKKSEKRQQL | (SEQ ID No: 74) |
| Deer | RPKLRGSPKKPEKR-QL | (SEQ ID No: 75) |
| | RPKLRGSPKKPEKR-QL | (SEQ ID No: 76) |
| Sheep | RPKLRGSPKKPEKR-QL | (SEQ ID No: 77) |
| Goat | RPKLRGSPKKPEKR-QL | (SEQ ID No: 78) |
| Cat | RPQLKAPPKKSEKR-QV | (SEQ ID No: 79) |
| Rabbit | RPKLKAAPKKSEKRQQV | (SEQ ID No: 80) |
| Rat | RPQLKAPPKKSEKRQQA | (SEQ ID No: 81) |
| | RPQLKAPPKKSEKR-QA | (SEQ ID No: 82) |
| | RPQLKAPPKKSEKRQQT | (SEQ ID No: 83) |
| Suncus | RPQLKPPKRPDKK-PG | (SEQ ID No: 84) |
| Whale | RPKLRGSPKKAEKR-QL | (SEQ ID No: 85) |
| Turkey | RPHLRATPKRTDKQQQI | (SEQ ID No: 86) |
| Dom. Fowl | RRHLRATPKRTDKQQQI | (SEQ ID No: 87) |
| | HLRATPKRTDKQQQI | (SEQ ID No: 88) |
| | RATPKRTDKQQQI | (SEQ ID No: 89) |
| Turtle | RRNL-KTHKKPDKRQQT | (SEQ ID No: 90) |
| Frog | RRNMNGHRLKNQSQREAIKQ | (SEQ ID No: 91) |
| | NMNGHRLKNQSQREAIKQ | (SEQ ID No: 92) |
| | MNGHRLKNQSQREAIKQ | (SEQ ID No: 93) |
| Eel | RRGVRP-PKK-DK-GQ | (SEQ ID No: 94) |
| | VRP-PKK-DK-GQ | (SEQ ID No: 95) |
| Catfish | RRGVR-PPKRDG-RNQ | (SEQ ID No: 96) |
| | GVR-PPKRDG-RNQ | (SEQ ID No: 97) |
| | VR-PPKRDG-RNQ | (SEQ ID No: 98) |
| Shark | RKPNSKRSNGSSNDDKEK | (SEQ ID No: 99) |

The half-life-extension peptide as such is preferred to be used for the individual of the species wherefrom the peptide is derived. For human for example, it is preferred to use the C terminal sequence of ghrelin derived from human. When

the half-life-extension peptide comprises the sequence of AB, BC or ABC, it is preferred to use the sequence A, B and C derived from the same organism species although it is also possible to use a chimera peptide in which the sequences A, B and C are derived from different species. In the chimera peptide, combination of sequences derived from mammals, birds, reptiles and amphibians is preferred, combination of sequences derived from mammals is more preferred and combination of sequences derived from human is still more preferred.

**[0121]** In the present invention, the expression reading that, when bonded to an object peptide, its physiological activity is retained means that, when bonded to any of N terminal, C terminal or both terminals of the object peptide, not less than 1% of the activity of the object peptide is still retained. The expression reading that the half-life of the object peptide is extended means that, when bonded to any of N terminal, C terminal or both terminals of the object peptide, the resulting half-life is extended as compared with the object peptide even a little.

(2) Chimeric peptide

**[0122]** When the above-illustrated half-life-extension peptide of the present invention is bonded to N terminal side, C terminal side or both terminal sides of the peptide having a physiological activity, the chimeric peptide of the present invention having an improved pharmacokinetics *in vivo* is prepared. When the half-life-extension peptide is bonded to both terminals, the both peptides may be same or different.

**[0123]** A and/or C may be bonded to N terminal and/or C terminal of the object peptide. Alternatively, addition of A and/or C to side chain of the amino acid contained in B such as amino group of lysine resin, carboxyl group of glutamic acid or aspartic acid, etc. is also an embodiment of the present invention. As to the embodiment for the addition, it is also possible to select a peptide bond or other appropriate bonding method if desired.

**[0124]** As to the object peptide, it is possible to use a derivative which is a peptide having an amino acid sequence where one or some amino acid(s) is/are deleted, substituted and/or added in the amino acid sequence concerning the natural physiologically active peptide or the peptide and having the physiological activity of the peptide. Unless otherwise specifically mentioned in the present specification, there is no particular limitation for the numbers of amino acid(s) which is/are substituted, etc. in "one or more is/are substituted, deleted, inserted and/or added" concerning the amino acid provided that the peptide comprising the amino acid sequence or a derivative thereof has a desired function and, for example, the numbers are about 1 to 4 or about 1 to 2 and selection of plural sites is also possible. In the case of substitution, etc. to amino acid having the similar property (charge and/or polarity), it is likely that the desired function is not lost even when many amino acids are substituted. "Amino acid" in the present specification includes all amino acids such as L-amino acid, D-amino acid, α-amino acid, β-amino acid, γ-amino acid, natural amino acid or synthetic amino acid.

**[0125]** When the object peptide is a derivative of natural physiologically active peptide, its amino acid has a homology of preferably not less than 70%, more preferably not less than 80%, still more preferably not less than 90%, particularly preferably not less than 95% and, most preferably, not less than 97% as compared with the native amino acid sequence.

**[0126]** B may be solely bonded provided that the structure of the object peptide to which the half-life-extension peptide fulfills the structure requirement of A and/or C.

**[0127]** Examples of the suitable object peptide include natriuretic peptide or, preferably, atrial natriuretic peptide (ANP; although its origin is not limited, an example is human ANP of SEQ ID No: 100), brain natriuretic peptide (BNP; although its origin is not limited, an example is human BNP of SEQ ID No: 160), C type natriuretic peptide (CNP; although its origin is not limited, an example is CNP-22 of SEQ ID No: 101), motilin (although its origin is not limited, an example is human motilin of SEQ ID No: 102), glucagonic peptide-1 (GLP-1; although its origin is not limited, an example is human GLP-1 (7-36) amide of SEQ ID No: 103), parathyroid hormone (PTH; although its origin is not limited, an example is human PTH (1-34) of SEQ ID No: 104) and calcitonin (CT; although its origin is not limited, an example is human CT of SEQ ID No: 105) which are physiologically active peptide having short half-life or a derivative thereof.

**[0128]** The derivative is able to be selected by such a means that it is made to act on the incubated cells expressing a receptor concerning the original physiologically active peptide and then a marker substance in the culture liquid is measured. In the case of ANP or CNP for example, selection is possible by such a means that it is made to act on the incubated cells expressing GC-A or GC-B which is a receptor thereof and then the cyclic GMP in the culture liquid is measured. In the case of the active peptide acting on the cells expressing GPC-R such as motilin or GLP-1, it is possible to select by measuring the activation of a signal transduction system such as measurement of cyclic AMP in the culture liquid or measurement of intracellular calcium.

**[0129]** The present invention also includes a salt of chimeric peptide. Although any salt may be used provided that the salt is non-toxic, a pharmaceutically acceptable salt is preferred and its example includes salt with inorganic base, salt with organic base, salt with inorganic acid, salt with organic acid and salt with basic or acidic amino acid.

**[0130]** Preferred examples of the salt with inorganic base include alkali metal salt such as sodium salt or potassium salt; alkali earth metal salt such as calcium salt or magnesium salt; and salt such as aluminum salt or ammonium salt.

**[0131]** Preferred examples of the salt with organic base include salt with trimethylamine, triethylamine, pyridine, pi-

coline, ethanolamine, diethanolamine, triethanolamine, dicyclohexylamine or N,N'-dibenzylethylenediamine.

**[0132]** Preferred examples of the salt with inorganic acid include salt with hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid or phosphoric acid.

**[0133]** Preferred examples of the salt with organic acid include salt with formic acid, acetic acid, trifluoroacetic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid or p-toluenesulfonic acid.

**[0134]** Preferred examples of the salt with basic amino acid include salt with arginine, lysine or ornithine. Preferred examples of the salt with acidic amino acid include salt with aspartic acid or glutamic acid.

**[0135]** Among the above salts, preferred salts are those with inorganic base and sodium salt and potassium salt are most preferred.

**[0136]** A chimeric peptide or a pharmaceutically acceptable salt thereof where ANP and BNP are objects is effective for the treatment of acute cardiac insufficiency and acute worsening of chronic cardiac insufficiency based on a physiological action via NPRA (GCA) which is a receptor for ANP and BNP and, in recent years, its very high effect has been noted for an improvement of prognosis after myocardial infarction. Besides the above, it is able to be used as an effective ingredient of a treating agent for obliterative arteriosclerosis, ischemic cardiac disease, hypertension, edema disease, myocardial disease, retinitis, diabetic renal disease, nephrosclerosis, myocardial infarction, etc.

**[0137]** A chimeric peptide or a pharmaceutically acceptable salt thereof where CNP is an object peptide is effective for its application to prevent the restenosis after PTCA after coronary artery stenosis and atypical chondrogenesis insufficiency based on a physiological action via NPRB (GCB) which is a receptor for CNP and also to treat pulmonary hypertension, peripheral artery obliterative disease, osteoarthritis, rheumatoid arthritis, pulmonary fibrosis, hepatic fibrosis, renal fibrosis, myocardial infarction and myocarditis and it is able to be used as an effective ingredient for such a treating agent.

**[0138]** A chimeric peptide or a pharmaceutically acceptable salt thereof where motilin is an object peptide is effective for its application to functional dyspepsia, reflux esophagitis, diabetic gastric motility paralysis, constipation-type irritable bowel syndrome, chronic peudoileus, postoperative ileus, chronic gastritis and atrophic gastritis based on a physiological action via motilin-R which is a receptor for motilin and is able to be used as an effective ingredient of such a treating agent.

**[0139]** A chimeric peptide or a pharmaceutically acceptable salt thereof where GLP-1 is an object peptide is effective for its application to diabetes mellitus, etc. and is able to be used as an effective ingredient for such a treating agent. A chimeric peptide or a pharmaceutically acceptable salt thereof where PTH is an object peptide is effective for its application to hypoparathyroidism, etc. and is able to be used as an effective ingredient for such a treating agent.

(3) Process for producing a (chimeric peptide

**[0140]** The present invention also includes a process for producing a chimeric peptide where half-life in plasma is extended and the physiological activity of the object peptide is available in which the half-life-extension peptide of the present invention as mentioned above is bonded to N terminal side, C terminal side or both terminals of the object peptide.

**[0141]** The chimeric peptide according to the present invention is able to be produced by a conventional method (refer, for example, to J. Med. Chem. , 43, pp 4370-4376, 2000). For example, it is able to be produced by a recombinant DNA technique and/or chemical synthesis. In a producing process using a recombinant DNA technique for example, host cells transformed by an expression vector having the DNA which codes for the chimeric peptide of the present invention are incubated and the aimed peptide is collected from the cultured product whereupon the chimeric peptide of the present invention is able to be produced.

**[0142]** Examples of the vector into which gene is integrated include vector of *Escherichia coli* (pBR 322, pUC18, pUC19, etc.), vector of *Bacillus subtilis* (pUB110, pTP5, pC194, etc.), vector of yeast (Yep type, YRp type, Yip type, etc.) and vector of animal cells (retrovirus, vaccinia virus, etc.) although any other vector may be used provided that it is able to stably hold the object gene in the host cells. The vector is transduced into an appropriate host cells. As to a method for integrating the aimed object gene into a plasmid or a method for introducing it into the host cells, a method mentioned in Molecular Cloning (Sambrook, et al., 1989) may be utilized for example.

**[0143]** In expressing the chimeric peptide gene in the above plasmid, a promoter is connected to the upstream region of the gene to function.

**[0144]** As to a promoter used in the present invention, anything may be used provided that it is an appropriate promoter corresponding to the host cells used for expression of the object gene. When, for example, the host cell to be transformed is genus *Escherichia,* there may be used lac promoter, trp promoter, lpp promoter, λ PL promoter, recA promoter, etc. When it is genus *Bacillus,* there may be used SP01 promoter, SP02 promoter, etc. When it is yeast, there may be used GAP promoter, PH05 promoter, ADH promoter, etc. When it is animal cell, there may be used promoter derived from SV40, promoter derived from retrovirus, etc.

**[0145]** Host cells are transformed using a vector containing the object gene produced as above. As to the host cell, there may be used bacteria (such as genus *Escherichia* or genus *Bacillus*), yeast (such as genus *Sacaharomyces,*

genus *Pichia* or genus *Candida*), animal cell (such as CHO cell or COS cell), etc. As to a medium for the incubation, liquid medium is appropriate and it is particularly preferred that the medium contains carbon source, nitrogen source, etc. necessary for the growth of the transformed cells to be incubated. If desired, vitamins, growth promoting factor, serum, etc. may be added to the medium.

**[0146]** After the incubation, the chimeric peptide of the present invention is separated and purified by a conventional method from the incubated products. For example, in extracting the object substance from the incubated microbes or cells, the microbes or cells are collected after the incubation and suspended in a buffer containing a protein modifier (such as guanidine hydrochloride) and the microbes or cells are disintegrated by ultrasonic wave or the like followed by subjecting to centrifugal separation. In purifying the aimed substance from the supernatant liquid, separating and purifying means such as gel filtration, ultrafiltration, dialysis, SDS-PAGE or various chromatography are conducted by an appropriate combination thereof by taking molecular weight, solubility, charge (isotonic point), affinity, etc. of the object substance into consideration.

**[0147]** The chimeric peptide of the present invention is also able to be chemically synthesized by conventional methods. For example, amino acid having protective groups is condensed by a liquid phase method and/or a solid phase method to elongate the peptide chain, all protective groups are removed by an acid and the resulting crude product is purified to give the aimed product. With regard to a method which is able to be used for the production of the chimeric peptide, various methods have been known already and the chimeric peptide as the substance of the present invention is also able to be easily produced by known methods. For example, a classical peptide synthesis may be used or a solid phase method may be conducted whereby the product is able to be easily produced.

**[0148]** Further, a producing process in which a recombinant DNA technique and a chemical synthesis are combined may be used. Thus, when a half-life-extension peptide is added to side chain of an amino acid concerning the object peptide (such as amino group of lysine residue or carboxylic group of glutamic acid or aspartic acid), production is conducted by a chemical synthesis while, in other parts, production is conducted using a recombinant DNA technique and, after that, the fragments are fused to produce the product.

**[0149]** Such a method may also be grasped as a method where the half-life-extension peptide of the present invention mentioned hereinabove is bonded to N terminal side, C terminal side or both terminal sides of the object peptide whereby the half-life in plasma of the object peptide is elongated.

(4) Pharmaceutical preparations

**[0150]** The chimeric peptide of the present invention is able to be used as a drug for animals (individuals) including human. The chimeric peptide of the present invention or a pharmaceutically acceptable salt thereof is compounded with a pharmaceutically acceptable carrier whereupon a pharmaceutical composition (pharmaceutical preparation) is able to be manufactured. There is no particular limitation for the dosage form and, for example, the preparation is used as solid preparation or liquid preparation for oral administration or as an injection preparation for parenteral use.

**[0151]** Examples of the solid preparation for oral administration include tablets, pills, capsules, diluted powder and granules while examples of the liquid preparation for oral administration include syrup.

**[0152]** As to a pharmaceutically acceptable carrier, various organic or inorganic carrier substances which have been commonly used as materials for the preparations may be used and examples thereof in the solid preparation include excipient, lubricant, binder and disintegrating agent while examples thereof in the liquid preparation include solvent, solubilizing aid, suspending agent, isotonizing agent, buffer and analgesic agent. If necessary, additives for the preparation such as antiseptic, antioxidant, coloring agent or sweetener may also be used.

**[0153]** Appropriate examples of the excipient include lactose, sugar, D-mannitol, starch, crystalline cellulose and light silicic acid anhydride.

**[0154]** Appropriate examples of the lubricant include magnesium stearate, calcium stearate, talc and colloidal silica.

**[0155]** Appropriate examples of the binder include crystalline cellulose, sugar, D-mannitol, dextrin, hydroxypropyl cellulose, hydroxypropyl methyl cellulose and polyvinylpyrrolidone.

**[0156]** Appropriate examples of the disintegrating agent include starch, carboxymethyl cellulose, carboxymethyl cellulose calcium, croscarmellose sodium and carboxymethyl starch sodium.

**[0157]** Appropriate examples of the solvent include water for injection, alcohol, propylene glycol, Macrogol, sesame oil and corn oil.

**[0158]** Appropriate examples of the solubilizing aid include polyethylene glycol, propylene glycol, D-mannitol, benzyl benzoate, ethanol, trisaminomethane, cholesterol, triethanolamine, sodium carbonate and sodium citrate.

**[0159]** Appropriate examples of the suspending agent include surfactant such as stearyl triethanolamine, sodium lauryl sulfate, laurylaminopropionic acid, lecithin, benzalkonium chloride, benzethonium chloride and glycerol monostearate; and hydrophilic polymer such as polyvinyl alcohol, polyvinylpyrrolidone, carboxymethyl cellulose sodium, methyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose and hydroxypropyl cellulose.

**[0160]** Appropriate examples of the isotonizing agent include sodium chloride, glycerol and D-mannitol.

**[0161]** Appropriate examples of the buffer include a buffer liquid such as phosphate, acetate, carbonate or citrate.

**[0162]** Appropriate examples of the analgesic agent include benzyl alcohol.

**[0163]** Appropriate examples of the antiseptic include p-hydroxybenzoates, chlorobutanol, benzyl alcohol, phenethyl alcohol, dehydroacetic acid and sorbic acid.

**[0164]** Appropriate examples of the antioxidant include sulfite and ascorbic acid.

**[0165]** As to the dosage form of the drug of the present invention, a dosage form which is suitable for parenteral administration is preferred. Examples of the dosage form suitable for parenteral administration include injection preparation for intravenous administration, intracutaneous administration, subcutaneous administration or muscular administration, drip, suppository, percutaneous absorber, permucous absorber and inhaling agent. Among the above, the dosage form of an injection preparation is preferred and, particularly when the individual is human in the case of treatment at home, dosage form such as permucous absorber, inhaling agent or suppository is also preferred. Various dosage forms as such are known among persons skilled in the art whereby the persons skilled in the art are able to appropriately select the dosage form which is suitable for the desired administration route and use, if necessary, one or more pharmaceutical additive(s) being utilizable in the related field to manufacture the preparation in the form of a pharmaceutical composition.

**[0166]** For example, a drug in the form of injection or drip is able to be prepared and provided in such a manner that the chimeric peptide endowed with a long half-life which is an effective ingredient is dissolved in distilled water for injection together with one or more appropriate pharmaceutical additive(s) such as buffer, sugar solution, isotonizing agent, pH adjusting agent, analgesic agent or antiseptic agent, subjected to a sterilizing filtration (through a filter) and filled in ampoules or vials or the solution subjected to a sterilizing filtration is freeze-dried to prepare a freeze-dried preparation. Examples of the additive usable therefor include saccharide such as glucose, mannitol, xylitol or lactose: hydrophilic polymer such as polyethylene glycol; alcohol such as glycerol; amino acid such as glycine; protein such as serum albumin; salt such as NaCl and sodium citrate; acid such as acetic acid, tartaric acid, ascorbic acid or hyaluronic acid; surfactant such as Tween 80; and reducing agent such as sodium sulfite. The preparation as such is able to be used as injection preparation or drip preparation by addition of distilled water for injection or physiological saline solution thereto to dissolve in actual use. For a permucous administration, an intranasal preparation (preparation for administering via nose) such as intranasal drop or intranasal spray is also suitable. For a perpulmonary administration, inhaling agent is also suitable.

**[0167]** Amount of the chimeric peptide or a pharmaceutically acceptable salt thereof in the preparation varies depending upon the dosage form and it is usually about 0.001 to 1000 mg, preferably about 0.01 to 100 mg, more preferably about 0.1 to 100 mg and, particularly preferably, about 1 to 100 mg.

**[0168]** It is preferred that the above dose is administered one to three times a day or one to seven times a week and the period for the administration varies depending upon the type of the object peptide and is not particularly limited.

**[0169]** The dose of the chimeric peptide or a pharmaceutically acceptable salt thereof according to the present invention may be appropriately determined depending upon the type of the object peptide and also on the half-life of the chimeric peptide.

**[0170]** As such, the present invention includes a method for the treatment of the disease which is able to be treated by the object peptide contained in the pharmaceutical composition by, for example, administering the above-mentioned pharmaceutical composition of the present invention to individuals.

## EXAMPLES

**[0171]** The present invention will now be illustrated in more detail by way of the following Examples.

### Example 1 (Changes in ANP concentration of native ANP or chimeric ANP in plasma)

**[0172]** Changes in ANP immunoreactivity concentration in plasma when a native ANP and a chimeric peptide (chimeric ANP) to which a half-life-extension peptide was bestowed were administered into vein of rats were investigated.

**[0173]** The experiment was conducted using rats into which polyethylene tube (PE-50; manufactured by Clay Adams) was previously inserted into thigh artery under an anesthetized condition with Nembutal. As a test system, male rats of an SD strain of 7 weeks age (provided from Nippon Charles River) were subjected to the experiment where one group comprised three rats. Native ANP (α-hANP, SEQ ID No: 100) or a chimeric ANP (A, B and C) each was administered to the rat intravenously or subcutaneously (back) in a dose of 0.1 mg/kg and blood over the time from before the administration until 90 or 180 minutes after the administration was collected by a polyethylene tube inserted into thigh artery. A stabilizer and EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) as anticoagulants were added to the collected blood, plasma was separated by means of centrifugal separation and concentration in plasma was measured by a competitive radioimmunoassay (RIA) (Fig. 1). At that time, a rabbit polyclonal antibody (#8) recognizing a ring part of ANP was used as an antibody and $^{125}$I- [Tyr$^{28}$] α-hANP was used as a labeling compound.

**[0174]** Each amino acid sequence of the chimeric ANP (A, B and C) is as follows and all of them retained a human-type guanylate cyclase receptor A (GC-A receptor) agonist activity.

**[0175]** Chimeric ANP (A): CFGGRMDRIGAQSGLGCNSFRYVQQRKESKKPPAKLQPR (S-S bond between the underlined members) (SEQ ID No: 106)

**[0176]** Chimeric ANP (B): VQQRKESKKPPAKLQPRCFGGRMDRIGAQSGLGCNSFRY (S-S bond between the underlined members) (SEQ ID No: 107)

**[0177]** Chimeric ANP (C): RPQLKAPPKKSEKRQQVCFGGRMDRIGAQSGLGCNSFRY (S-S bond between the underlined members) (SEQ ID No: 108)

**[0178]** Pharmacokinetic parameters were calculated from the resulting changes in immunoreactivity concentrations in plasma. At that time, WinNonlin Professional Ver. 4.0.1 (manufactured by Pharsight Corporation, U. S. A.) was used as the analytical software. Method for the calculation of each parameter is mentioned as follows.

**[0179]** Concentration at time 0 (C0; extrapolated value, ng/mL), area under the curve for concentration in plasma vs. time (AUC; ng.min/mL) and quenching half-life (T1/2; minute(s)) were calculated as follows. C0 was calculated as a value at time 0 by means of extrapolation from the changes in immunoreactivity concentration in plasma at each blood collection period. AUC was calculated by a trapezoidal method using the immunoreactivity concentrations in plasma at all measuring points (t) and an extrapolated value until the infinitive time was determined. T1/2 was calculated by means of a least-squares method from the inclination of a straight line connecting several points in a disappearing phase of the immunoreactivity concentration in plasma. In the case of a subcutaneous administration, C0 was not determined but the maximum concentration in plasma (Cmax; ng/mL) and the time reaching the maximum concentration in plasma (Tmax; min) were determined. Cmax was the highest value in the immunoreactivity concentration in plasma at each of the blood collecting stages and, as to Tmax, a blood collecting time where the plasma showed Cmax was adopted.

**[0180]** The result is shown in the following Tables 8 and 9.

Table 8

Pharmacokinetic parameters when native ANP and chimeric ANP (A, B and C) thereof were intravenously administered for one time (0.1 mg/kg) to male rats (N = 3)

| Peptide | Dose (mg/kg) | | C0 (ng/mL) | AUC0 → ∞ (ng/min/mL) | T1/2 (min) | P value |
|---|---|---|---|---|---|---|
| native ANP | 0.1 | Mean Value ± Standard Deviation | 993 ± 208 | 1498 ± 90 | 2.88 ± 0.13 | - |
| Chimeric ANP (A) | 0.1 | Mean Value ± Standard Deviation | 1235 ± 71 | 11777 ± 1374 | 24.0 ± 2.5 * | [0.000] |
| Chimeric ANP (B) | 0.1 | Mean Value ± Standard Deviation | 1572 ± 361 | 10527 ± 969 | 40.0 ± 12.4 * | [0.007] |
| Chimeric ANP (C) | 0.1 | Mean Value ± Standard Deviation | 1533 ± 442 | 8867 ± 930 | 27.2 ± 3.8 * | [0.000] |

*: Significant difference was noted from the disappearing half-life of native ANP in the T-test ($P < 0.05$)

Table 9

Pharmacokinetic parameters when native ANP and chimeric ANP (A, B and C) thereof were subcutaneously administered (0.1 mg/kg) to male rats (N = 3)

| Peptide | Dose (mg/kg) | | Cmax (ng/mL) | Tmax (min) | AUC0 → ∞ (ng/min/mL) | T1/2 (min) | P value |
|---|---|---|---|---|---|---|---|
| Native ANP | 0.1 | Mean Value ± Standard Deviation | 22.3 ± 5.3 | 8.3 ± 2.9 | 535 ± 157 | 11.2 ± 2.0 | - |

(continued)

| Pharmacokinetic parameters when native ANP and chimeric ANP (A, B and C) thereof were subcutaneously administered (0.1 mg/kg) to male rats (N = 3) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Peptide | Dose (mg/kg) | | Cmax (ng/mL) | Tmax (min) | AUC0 → ∞ (ng/min/mL) | T1/2 (min) | P value |
| Chimeric ANP (A) | 0.1 | Mean Value ± Standard Deviation | 63.3 ± 31.8 | 5.0 ± 0.0 | 2041 ± 535 | 46.4 ± 8.0 * | [0.002] |
| Chimeric ANP (B) | 0.1 | Mean Value ± Standard Deviation | 64.5 ± 40.7 | 16.7 ± 5.8 | 3985 ± 2252 | 57.3 ± 7.1 * | [0.000] |
| Chimeric ANP (C) | 0.1 | Mean Value ± Standard Deviation | 55.1 ± 10.3 | 20.0 ±0.0 | 3402 ± 860 | 37.8 ± 7.8 * | [0.007] |
| *: Significant difference was noted from the disappearing half-life of native ANP in the T-test (P < 0.05) | | | | | | | |

**[0181]** When the chimeric ANP (A, B and C) was intravenously administered, any of those derivatives showed a rising concentration in plasma as compared with the native ANP showing a sustaining property.

**[0182]** Further, when the chimeric ANP (A, B and C) each was subcutaneously administered, any of those derivatives showed a rising concentration in plasma as compared with the native ANP showing a sustaining property.

Example 2 (Concentration changes in plasma and biological activity of native CNP-22 and chimeric CNP)

**[0183]** Changes in CNP immunoreactivity concentration in plasma of native CNP-22 and chimeric peptide (chimeric CNP) where a half-life-extension peptide was added to CNP were investigated.

**[0184]** The experiment was conducted in the same manner as in Example 1 that rats where polyethylene tube was previously inserted into thigh artery were used under an anesthetizing condition with Nembutal and male SD strain rats of 7 weeks age (purchased from Nippon Charles River) were used where one group comprised 3 rats. Native CNP-22 (SEQ ID No: 101) or a chimeric CNP (A and B) each was administered to the rat intravenously (tail) or subcutaneously (back) in a dose of 0.1 mg/kg and the blood over the time from before the administration until 90 or 180 minutes after the administration was collected by a polyethylene tube inserted into thigh artery. EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) were added as a stabilizer and an anti-coagulant, respectively to the collected blood, the plasma was separated by means of centrifugal separation and concentration in the plasma was measured by means of a competitive RIA. At that time, a rabbit polyclonal antibody (#2) recognizing a ring part of native CNP-22 was used as an antibody and $^{125}$I - [Tyr$^{0}$] CNP was used as a labeling compound. Pharmacokinetic analysis was carried out by the same method as in Example 1.

**[0185]** Amino acid sequences of the chimeric CNP (A and B) are as follows and both have a human type guanylate cyclase receptor B (GC-B receptor) agonist activity.

**[0186]** Chimeric CNP (A): RPQLKAPPKKSEKRQQVCFGLKLDRIGSMSGLGC (S-S bond between the underlined members) (SEQ ID No: 109)

**[0187]** Chimeric CNP (B): CFGLKLDRIGSMSGLGCVQQRKESKKPPAKLQPR (S-S bond between the underlined members) (SEQ ID No: 110)

**[0188]** The results are shown in Fig. 2 and the following Tables 10 and 11.

Table 10

| Pharmacokinetic parameters when native CNP-22 and (chimeric CNP (A and B) thereof were intravenously administered (0.1 mg/kg) to male rats (N = 3) | | | | | | |
|---|---|---|---|---|---|---|
| Peptide | Dose (mg/kg) | | C0 (ng/mL) | AUC0 → ∞ (ng/min/mL) | T1/2 (min) | P value |
| Native CNP-22 | 0.1 | Mean Value ± Standard Deviation | 1018 ± 273 | 1537 ± 602 | 2.93 ± 0.23 | - |
| Chimeric CNP (A) | 0.1 | Mean Value ± Standard Deviation | 1034 ± 358 | 6423 ± 553 | 21.7 ± 6.1* | [0.006] |
| Chimeric CNP (B) | 0.1 | Mean Value ± Standard Deviation | 1466 ± 749 | 9194 ± 557 | 362 ± 2.3 * | [0.000] |
| *: Significant difference was noted from the disappearing half-life of native CNP-22 in the T-test (P < 0.05) | | | | | | |

Table 11

| Pharmacokinetic parameters when native CNP-22 and chimeric CNP (A and B) thereof were subcutaneously administered (0.1 mg/kg) to male rats (N = 3) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Peptide | Dose (mg/kg) | | Cmax (ng/mL) | Tmax (min) | AUC0 → ∞ (ng/min/mL) | T1/2(min) | P value |
| Native CNP-22 | 0.1 | Mean Value ± Standard Deviation | 55.6 ± 4.5 | 5.0 ± 0.0 | 1063 ± 75 | 7,8 ± 0.9 * | - |
| Chimeric CNP (A) | 0.1 | Mean Value ± Standard Deviation | 27.9 ± 2.7 | 13.3 ± 5.8 | 1491 ± 493 | 20.7 ± 7.5 * | [0.042] |
| Chimeric CNP (B) | 0.1 | Mean Value ± Standard Deviation | 32.8 ± 7.9 | 10.0 ± 0.0 | 1688 ± 617 | 24.0 ± 9.7 * | [0.045] |
| *: Significant difference was noted from the disappearing half-life of native CNP-22 in the T-test (P < 0.05) | | | | | | | |

**[0189]** After the intravenous administration, the native CNP-22 quickly decreased from plasma in a short half-life while, in the chimeric CNP, about 100-fold higher concentration in plasma was retained as compared with the native CNP-22 even after 90 minutes from the administration.

**[0190]** On the other hand, when the native CNP-22 and the chimeric CNP were subcutaneously administered, the maximum concentration in plasma was about 1/2 in the administration of the chimeric CNP as compared with the native CNP-22 and disappearance from the plasma was slow. From the above result, it is now apparent that, in rats, the chimeric CNP shows a long half-life both in the intravenous administration and the subcutaneous administration as compared with the native CNP-22.

**[0191]** The native CNP-22 and the chimeric CNP (B) (structure was mentioned already) were subcutaneously administered and the cGMP concentration in plasma which reflects the biological activity of CNP was measured by a competitive RIA (documents). Administration of the drug and collection of the blood were made the same as those in the measurement of CNP immunoreactivity concentration in plasma. In measuring the cGMP concentration in plasma, Yamasa cGMP Assay Kit (manufactured by Yamasa Shoyu) was used as a measuring kit. The result is shown in Fig 3.

**[0192]** CNP immunoreactivity concentration in plasma after subcutaneous administration of the native CNP-22 quickly rose followed by quick decreasing and it almost disappeared after 60 minutes from the administration. The cGMP

concentration in plasma rose slightly slower than the CNP immunoreactivity concentration in plasma and, after reaching its peak after 15 to 30 minutes from the administration, it quickly decreased.

**[0193]** On the other hand, when the chimeric CNP was subcutaneously administered, the maximum value of the CNP immunoreactivity concentration in plasma was lower than the native CNP-22. The cGMP concentration in plasma was also about 60% of the case of the native CNP-22 and is retained in a predetermined level during 30 to 90 minutes after the administration.

Example 3 (Resistance of native CNP-22 and Chimeric CNP to peptidase)

**[0194]** Resistance of native CNP-22 and chimeric CNP to peptidase was investigated.

**[0195]** The experiment was conducted in N = 2 for each sample. Native CNP-22, chimeric CNP (A, B) (structure mentioned already; final concentration: 0.5 μg/mL) and 100 μL of reaction solution (medium: 20 mM MES, pH 6.5) of human type recombinant neutral endopeptidase (hNEP, manufactured by R&D Systems, Inc. , U. S. A.) were prepared. The initial value sample was boiled for 5 minutes immediately after preparation. Sample for the stability evaluation was subjected to an enzymatic reaction in a constant-temperature vessel set at 37°C for 1 hour and then boiled for 5 minutes. Distilled water (100 μL) was added to the sample after boiling followed by well mixing and 50 μL thereof was analyzed in a water-acetonitrile system using a high-performance liquid chromatography system LC-10A (manufactured by Shimadzu). The analytical data were analyzed by a Chromato-Pack (CRA-10A, manufactured by Shimadzu) and area of the peak zone of unchanged substance was calculated. The area of average zone of unchanged substance when made to react at 37°C was divided by the area of average zone of the boiled sample to calculate the residual rate of the unchanged substance after 1 hour. The result is shown in the following Table 12.

Table 12

| Stability of native CNP-22 and its chimeric CNP (A, B) to hNEP (mean value of N=2) | | |
|---|---|---|
| Peptide | Initial Value (%) | Residual Rate (%) |
| Native CNP-22 | 100% | 16.7% |
| Chimeric CNP (A) | 100% | 84.3% |
| Chimeric CNP (B) | 100% | 83.8% |

**[0196]** The residual rate of the unchanged native CNP-22 in the sample after the reaction of 1 hour in a constant-temperature vessel set at 37°C where hNEP was added to a native CNP-22 solution lowered to an extent of 16.7% of the initial value while the residual rates of the chimeric CNP (A and B) were 84.3% and 83.8%, respectively whereupon it was confirmed that each of them showed a resistance to metabolism to hNEP than the native CNP-22.

**[0197]** Further, NEP resistance *in vivo* of the native CNP-22 and chimeric CNP (B) was evaluated using the effect by the joint use with an NEP inhibitor affecting on changes in CNP immunoreactivity concentration in plasma after the intravenous administration as an index.

**[0198]** This test was also carried out using rats into which a polyethylene tube (PE-50, manufactured by Clay Adams, U. S. A.) was inserted into thigh artery for collection of blood and rats into which a polyethylene tube (PE-10, manufactured by Clay Adams, U. S. A.) was inserted into thigh vein for administration of an NEP inhibitor under an anesthetizing condition using Nembutal. As a test system, male SD strain rats of 7 weeks age (Nippon Charles River; body weight: ca. 250 g) were used for the experiment where one group comprised 3 rats. From the thigh vein, a medium (5% mannitol; 10 μL/min/body) or an NEP inhibitor (30 μg/100 μL/min/body) was intravenously administered at a constant speed. Time for the administration was from 10 minutes before the administration of the native CNP-22 or the chimeric CNP (B) to completion of blood collection which was 60 minutes after the administration. The native CNP-22 and the chimeric CNP (B) each was administered into the tail vein in a dose of 20 μg/kg and the blood before starting the administration of NEP inhibitor (-10 minutes), before the administration of CNP (0 minute) and until after 60 minutes from the administration was collected by a thigh artery cannula. After a stabilizer and an anticoagulant were added to the collected blood, it was centrifuged to give plasma and the immunoreactivity concentration in the plasma was measured by the above-mentioned competitive RIA. The pharmacokinetic analysis was carried out by the same method as in Example 1.

**[0199]** The result is shown in Fig. 4 and the following Table 13.

Table 13

| Influence of an NEP inhibitor on pharmacokinetic parameters when native CNP-22 and chimeric CNP (B) (ca 0.02 mg/kg) each was intravenously administered to male rats (N = 3) | | | | | |
|---|---|---|---|---|---|
| Peptide | Dose (mg/kg) | | C0 (ng/mL) | AUC0→∞ (ng·min/mL) | T1/2 (min) |
| Native CNP-22<br>NEP Inhibitor (-) | 0.02 | Mean Value ± Standard Deviation | 145 ±20 | 202 ±24 | 1.96 ±05 |
| Native CNP-22<br>NEP Inhibitor (+) | 0.02 | Mean Value ± Standard Deviation | 131 ±32 | 758 ±88 | 9.94 ±1.40 |
| Chimeric CNP (B)<br>NEP Inhibitor (-) | 0.02 | Mean Value ± Standard Deviation | 253 ±15 | 1470 ±3 | 14.9 ±3.1 |
| Chimeric CNP (B)<br>NEP Inhibitor (+) | 0.02 | Mean Value ± Standard Deviation | 203 ±71 | 1342 ±168 | 12.7 ±1.2 |

**[0200]** The CNP immunoreactivity concentration in plasma when the native CNP-22 was intravenously administered significantly increased when an NEP inhibitor was used together. The AUC when an NEP inhibitor was used together was about 3.7-fold and a half-life was about 5-fold as compared with the case where the inhibitor was not used.

**[0201]** On the other hand, changes in the CNP immunoreactivity concentration in plasma when the chimeric CNP was administered did not change even when the NEP inhibitor was used together and it was confirmed that, in the body of the rat, the chimeric CNP (B) was hardly decomposed by NEP as compared with the native CNP-22 (Table 13).

Example 4 (Extension-promoting action of native CNP-22 and chimeric CNP) (1)

**[0202]** An extension-promoting action of native CNP-22 and chimeric CNP was investigated using mice.

**[0203]** In the experiment, female S/VAF Crlj:CD1 (ICR) mice of three weeks age (Nippon Charles River) were used where one group comprised ten mice and 30 mice in total were used for the experiment. The animals (two weeks age) were purchased together with three mother mice and ten baby mice with one mother mouse were bred for one week as a group and then weaned. During the administering period, each group in 5 mice x two cages was bred in a see-through cage. As to water, tap water was made to freely drink and, as to a feed, a solid feed (CRF-1 manufactured by Oriental Yeast Industry) was made to freely ingest. Medium, native CNP-22 or chimeric CNP (B) (structure mentioned already) was repeatedly administered twice daily for 29 days subcutaneously from the back in a dose of 10 mL/kg, 0.25 mg/kg or 0.25 mg/kg, respectively and body length, tail length and body weight during the administered period were measured. The result is shown in Fig. 5.

**[0204]** As shown in Fig. 5, the body length of the mice to which a chimeric CNP (B) where a half-life-extension peptide was bonded to CNP was repeatedly administered by subcutaneous route significantly increased as compared with a group to which the medium was administered while the body length of the group to which the native CNP-22 was administered showed no difference from the group to which the medium was administered.

**[0205]** On the other hand, with regard to body weight, there was no difference among all administered groups.

Example 5 (Antigenicity of native CNP-22 and (chimeric CNP)

**[0206]** In order to investigate whether an antibody to native CNP-22 or chimeric CNP (B) was produced using serum collected from the mice after completion of repeated administration for four weeks in Example 4, antigenicity of each of them was evaluated by means of an indirect ELISA.

**[0207]** A peptide solution (100 μL) adjusted to 1.0 ng/μL using 50 mM of $NaCHO_3$ at pH 8.5 was added to a 96-well plate (manufactured by Nalge Nunc International; Denmark) treated with Maxisoap and subjected to a coating operation overnight at 4°C. A blocking was carried out at room temperature for 1 hour using a 2.0% Block Ace (manufactured by Snow Brand Milk). After the blocking, a serum sample diluted to an extent of $10^2$ to $10^5$ fold was added thereto and the

mixture was subjected to an antigen-antibody reaction at room temperature for 1 hour. After washing the wells, an anti-mouse IgG HRPAb (manufactured by Zymed Laboratories, U. S. A.) was added thereto. The antigen-antibody reaction was conducted for 1 hour at room temperature, then the wells were washed and a substrate ABTS (manufactured by KPL, U. S. A.) was added thereto. After the reaction at room temperature for 1 hour, absorbance of 405 nm was measured by Spectra Max 190 (manufactured by Molecular Devices, U. S. A.) and the degree of coloration was analyzed as an antibody value.

**[0208]** As a result, even when the native CNP-22 or the chimeric CNP (B) was repeatedly administered subcutaneously (back) in a dose of 0.25 mg/kg twice daily for 29 days, no rise of the antibody value to native CNP-22 and to chimeric CNP (B) was noted.

Example 6 (Half-life in plasma of native motilin and chimeric motilin) (1)

**[0209]** Changes in motilin immunoreactivity concentrations in plasma when native motilin (SEQ ID No: 102) and 13 kinds of chimeric motilins A to M (SEQ ID Nos: 111 to 123) half-life-extension peptide in various half lives comprising 5 to 22 amino acid residues were bonded to an amino acid sequence of amino acid numbers 1 to 12 of SEQ ID No: 102 which was the active center of native motilin were intravenously administered were investigated. The experiment was carried out using rats into which polyethylene tube (PE-50 manufactured by Clay Adams) was previously inserted into thigh artery under an anesthetized condition with Nembutal. As to a test system, male SD strain rats (Nippon Charles River) of 7 weeks age were used where one group comprised 3 rats. Human type motilin or a derivative thereof in a dose of 10 nmol/kg was administered into tail vein of the rat and the blood before the administration and until 60 minutes after the administration was collected by a polyethylene tube inserted into the thigh artery. To the collected blood were added EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) as a stabilizer and an anti-coagulant, respectively and the plasma was separated therefrom by centrifugal separation. Motilin immunoreactivity concentration in the plasma was measured by a competitive radioimmunoassay (RIA) (Fig. 6). At that time, a rabbit polyclonal antibody (#870623) which specifically recognizes the amino acid numbers 1 to 12 of native motilin shown by SEQ ID No: 102 was used as an antibody and, as a labeled compound, $^{125}$I- [Tyr$^{7}$] human type motilin was used.

**[0210]** Amino acid sequences, molecular weights and half-time for disappearance from the plasma for the native motilin and chimeric motilin (A to M) are shown in the following Table 14.

(Mean value ± standard deviation; N = 3)

**[0211]**

Table 14

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric motilin upon intravenous administration to rats

| Peptide | Amino Acid Sequence | Mol. Wt. | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±SD | |
| Native Motilin | FVPIFTYGELQRMQEKERNKGQ | 2699.05 | 3.26 | ±0.95 * | - |
| Chimeric **motilin (A)** | FVPIFTYGELQRSPEHQRVQQRKESKKPPAKLQPR 4204.79 | 4204.79 | 11.74 | ±3.36 * | 0.014 |
| **Chimeric motilin (B)** | FVPIFTYGELQREHQRVQQQRKESKKPPAKLQPR | 4020.60 | 11.14 | ±0.73 * | 0.000 |
| **Chimeric motilin (C)** | FVPIFTYGELQRQRVQQRKESKKPPAKLQPR | 3754.35 | 8.90 | ±0.53 * | 0.001 |
| **Chimeric** | FVPIFTYGELQRQRVQQRKESKKPPAKLQPR **Chimeric motilin (D)** | 3470.03 8.90 | 8.90 | ±0.50 * | 0.001 |

(continued)

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric motilin upon intravenous administration to rats

| Peptide | Amino Acid Sequence | Mol. Wt. | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±SD | |
| **Chimeric motilin (E)** | FVPIFTYGELQRQRVQQRKESKKPPAKLQPR | 3242.77<br>8.03 | 8.03 | ±0.73 *<br>±0.73 * | 0.002 |
| **Chimeric motilin (F)** | FVPIFTYGELQRQRVQQRKESKKPPAKLQPR | 2958.46<br>9.37 | 9.37 | ±0.58 * | 0.000 |
| **Chimeric motilin (G)** | FVPIFTYGELQRQRVQQRKESKKPPAKLQPR | 2701.17 | 5.25 | ±1.32<br>NS | 0.101 |
| **Chimeric motilin (H)** | FVPIFTYGELQRVQQRKESKKPPAKLQ | 3216.73<br>10.43 | 10.43 | ±0.14 * | 0.000 |
| **Chimeric motilin (I)** | FVPIFTYGELQRQRVQQRKESKKPPAK | 2975.45 | 5.76 | ±0.66 * | 0.018 |
| **Chimeric (J) motilin (J)** | FVPIFTYGELQRQRVQQRKESKKPP | 2776.20 | 6.34 | ±0.48 * | 0.008 |
| **Chimeric motilin (K)** | FVPIFTYGELQRQRVQQRKESKK | 2581.97 | 6.14 | ±2.37 * | 0.032 |
| **Chimeric motilin (L)** | FVPIFTYGELQRQRVQQRKES | 2325.62 | 4.89 | ±4.26<br>NS | 0.296 |
| **Chimeric motilin (M)** | FVPIFTYGELQRQRVQQRK | 2109.43 | 2.39 | ±2.37<br>NS | 0.272 |

*: In t-test, a significant difference was available from the disappearance half-time of native motilin ($p < 0.05$) NS: In t-test, a significant difference was not available from the disappearance half-time of native motilin ($p > 0.05$)

**[0212]** The result is shown in Fig. 6 as well.

**[0213]** All of the 13 types of chimeric motilins which were evaluated retained the human motilin receptor agonist activity. With regard to the chimeric motilin (G, L and M), more significant disappearance half-time extending action in vivo than native motilin was unable to be confirmed and it was confirmed that, when the structure of the amino acid sequence ESKK was lost, the action disappeared. With regard to other motives, all of them had an action of extending the half-life *in vivo* as compared with the native motilin and, among them, the fact whether the structure of RKESKK is held or not has the biggest influence on the extending action for the half-life.

Example 7 (Half-life of native CNP-22 and chimeric CNP in plasma) (1)

**[0214]** Changes in CNP immunoreactivity concentration in plasma when the native CNP-22 shown in SEQ ID No: 101 and seven kinds of chimeric CNPs (C to I) (SEQ ID Nos: 124 to 130) in which a half-life-extension peptide comprising 17 amino acid residues was bonded to N terminal, C terminal or both terminals of the peptide comprising the 6th to 22nd amino acid sequence from N terminal in SEQ ID No: 101 which was the active center of the native CNP-22 were intravenously administered were investigated.

**[0215]** The experiment was carried out using rats into which polyethylene tube (PE-50 manufactured by Clay Adams) was previously inserted into thigh artery under an anesthetized condition with Nembutal. As to a test system, male SD strain rats (Nippon Charles River) of 7 weeks age were used where one group comprised 3 rats. Native CNP-22 or chimeric CNP in a dose of 10 nmol/kg was administered into tail vein of the rat and the blood before the administration and until 60 minutes after the administration was collected by a polyethylene tube inserted into the thigh artery. To the collected blood were added EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) as a stabilizer and an anti-coagulant, respectively and the plasma was separated therefrom by centrifugal separation. CNP immunoreactivity concentration in plasma was measured by a competitive radioimmunoassay (RIA). At that time, a rabbit polyclonal antibody (#2) which specifically recognizes the ring partial structure of the native CNP-22 was used as an antibody and, as a labeled compound, $^{125}$I-[Tyr$^{0}$] CNP was used.

**[0216]** Amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric CNP are shown in the following Table 15.

(Mean value ± standard deviation; N = 3)

**[0217]**

Table 15

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for native CNP-22 and chimeric CNP (C to I) upon intravenous administration to rats in a dose of 10 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean | ±SD Value | |
| Native CNP-22 | GLSKGCFGLKLDRIGSMSGLGC | 2197.6 | 4.34 | ±0.20 | - |
| **Chimeric CNP(C)** | CFGLKLDRIGSMSGLGCVQQRKESKKPPAKLQPR | 3755.5 | 15.03 | ±4.31* | 0.013 |
| **Chimeric CNP(D)** | GLSKGFGLKLDRISMSGLGCVQQRKESKKPPAKLQPR | 4198.0 | 20.30 | ±3.09* | 0.001 |
| **Chimeric CNP(E)** | CFGLKLDRIGSMSGLGCVQQRKESKKPPAKLQPR ESKKPPAKLQPR | 5755.9 | 18,40 | ±2.05* | 0.000 |
| **Chimeric CNP(F)** | RPQLKAPPKKSEKRQQVCFGLKLDRIGSMSGLGCVQQ R KESKKPPAKLQPR | 5755.9 | 17.38 | ±1.00* | 0.000 |
| **Chimeric CNP (G)** | VQQRKESKKPPAKLQPRCFGLKLDRIGSMSGLGC | 3755.5 | 17.75 | ±3.99* | 0.004 |
| **CNP(H)** | **Chimeric** RPQLKAPPKKSEKRQQVCFGLKLDRIGSMSGLGC | 3755.5 | 14.86 | ±2.64* | 0.002 |
| **Chimeric CNP(I)** | CFGLKLDRIGSMSGLGCVQQRKESKKPPAKLQPR-amide | 3754.5 | 14.50 | ±0.85* | 0.000 |

In all of them, S-S bond was formed at the underlined italic cysteine residues to form a cyclic structure.
*: In t-test, a significant difference was available from the disappearance half-time of native CNP-22 ($p < 0.05$)

**[0218]** The result is shown in Fig. 7 as well.

**[0219]** All of the evaluated chimeric CNPs (C to I) retained the human CNP receptor agonist activity.

**[0220]** All of the chimeric CNPs evaluated at this time showed longer disappearance half-life than the native CNP-22. Therefore, it was found that the half-life extension peptide evaluated at this time showed an extending action for half-life when it was bonded to any of N terminal side and C terminal side or to both sides and that, even when the amino acid sequence was reversed, the property was not deteriorated. It was also found that, even when the C terminal structure was amidated, an extending action for the half-life was still available whereby, depending upon the structure of the aimed peptide, a bonding system was able to be selected.

Example 8 (Half-life of native motilin and chimeric motilin in plasma) (2)

**[0221]** Changes in motilin immunoreactivity concentrations in plasma when native motilin (SEQ ID No: 102) and 26 kinds of chimeric motilins (N to Z and I to XIII) (SEQ ID Nos: 131 to 156) where half-life-extension peptide in various half lives comprising 14 to 22 amino acid residues were bonded to an amino acid sequence of amino acid numbers 1 to 12 of SEQ ID No: 102 which was the active center of native motilin were intravenously administered were investigated.

**[0222]** The experiment was carried out using rats into which polyethylene tube (PE-50 manufactured by Clay Adams) was inserted into thigh artery under an anesthetized condition with Nembutal. As to a test system, male SD strain rats (Nippon Charles River) of 7 weeks age were used where one group comprised 3 rats. Human motilin or a derivative thereof in a dose of 10 nmol/kg was administered into tail vein of the rat and the blood before the administration and until 60 minutes after the administration was periodically collected by a polyethylene tube inserted into the thigh artery. To the collected blood were added EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) as a stabilizer and an anti-coagulant, respectively and the plasma was separated therefrom by centrifugal separation. Motilin immunoreactivity concentration in plasma was measured by a competitive radioimmunoassay (RIA) (Fig. 8). At that time, a rabbit polyclonal antibody (#870623) which specifically recognizes the amino acid numbers 1 to 12 of native motilin shown by SEQ ID No: 102 was used as an antibody and, as a labeled compound, $^{125}$I-[Tyr$^{7}$] human motilin was used.

**[0223]** Amino acid sequences, molecular weights and half-time for disappearance from the plasma for the native motilin and chimeric motilins (N to Z and I to XIII) are shown in the following Table 16 and Fig. 8.

(Mean value ± standard deviation; N = 3)

**[0224]**

Table 16

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric motilins upon intravenous administration to rats in a dose of 10 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±SD | |
| Native Motilin | FVPIFTYGELQRMQEKERNKGQ | 2699.1 | 3.26 | ±0.95 | - |
| **Chimeric motilin (D)** | FVPIFTYGELQRVQQRKESKKPPAKLQPR | 3470.0 | 8.90 | ±0.50* | 0.001 |
| **Chimeric motilin (N) (MG-dS)** | FVPIFTYGELQRVQQRKEKKPPAKLQPR | 3383.0 | 9.12 | ±1.22* | 0.003 |
| **Chimeric motilin (O) (MG-18S/F)** | FVPIFTYGELQRVQQRKEFKKPPAKLQPR | 3530.2 | 7.73 | ±1.14* | 0.007 |
| **Chimeric motilin (P) (MG-18S/T)** | FVPIFTYGELQRVQQRKETKKPPAKLQPR | 3484.1 | 9.67 | ±0.13* | 0.000 |
| **Chimeric motilin (Q) (MG-18S/P)** | FVPIFTYGELQRVQQRKEPKKPPAKLQPR | 3480.1 | 9 65 | ±0.96* | 0.001 |
| **Chimeric motilin (R) (MG-18S/L)** | FVPIFTYGELQRVQQRKELKKPPAKLQPR | 3496.2 | 9.13 | ±0.67* | 0.001 |
| **Chimeric motilin (S) (MG-18S/A)** | FVPIFTYGELQRVQQRKEAKKPPAKLQPR | 3454.1 | 9.29 | ±0.30* | 0.000 |

(continued)

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric motilins upon intravenous administration to rats in a dose of 10 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±SD | |
| **Chimeric motilin (T) (MG-17E/N)** | FVPIFTYGELORVQQRKNSKKPPAKLQPR | 3455.1 | 5.47 | ±0.22* | 0.017 |
| **Chimeric motilin (U) (MG-17E/Q)** | FVPIFTYGELQRVQQRKQSKKPPAKLQPR | 3469.1 | 5.66 | ±0.66* | 0.023 |
| **Chimeric motilin (V) (MG-ES/DS)** | FVPIFTYGELQRVQQRKDSKKPPAKLQPR | 3456.1 | 10.14 | ±0,92* | 0.001 |
| **Chimeric motilin (W) (MG-dES)** | FVPIFTYGELQRVQQRKKKPPAKLQPR | 3253.9 | 5.39 | ±1.23$^{NS}$ | 0.077 |
| **Chimeric motilin (X) (MG-17E/D)** | FVPIFTYGELQRVQQKRDSRRPPAKLQPR | 3512.1 | 7.89 | ±0.34* | 0.001 |
| **Chimeric motilin (Y) (MG-BR)** | FVPIFTYGELQRVQQKKSEKRPPAKLQPR | 3470.1 | 11.60 | ±1.18* | 0.001 |
| **Chimeric motilin (Z) (MG-i17G)** | FVPIFTYGELQRVQQRKGESKKPPAKLQPR | 3527.1 | 10.53 | ±1.66* | 0.003 |
| **Chimeric motilin (I) (MG-i19G)** | FVPIFTYGELQRQQRKESGKKPPAKLQPR | 3527.1 | 11.55 | ±2.00* | 0.003 |
| **Chimeric motilin (II) (MG-i17G2)** | FVPIFTYGELQRVQQRKGGESKKPPAKLQPR | 3584.2 | 11.50 | ±0.64* | 0.000 |
| **Chimeric motilin (III) (MG-i17G2-19iG)** | FVPIFTYGELQRVQQRKGGESGKKPPAKLQPR | 3641.2 | 10.65 | ±0.72* | 0.000 |
| **Chimeric motilin (IV) (MG-dPP)** | FVPIFTYGELQRVQQRKESKKAKLQPR | 3196.8 | 7.73 | ±0.52* | 0.002 |
| **Chimeric motilin (V) (MG-dPPH1)** | FVPIFTYGELQRVQQRKESKKAKLAALKA | 3349.0 | 8.94 | ±0.68* | 0.001 |
| **Chimeric motilin (VI) (MG-H1)** | FVPIFTYGELQRVQQRKESKKPPAKLAALKA | 3543.2 | 9.94 | ±0.30* | 0.000 |
| **Chimeric motilin (VII) (MG-H3)** | FVPIFTYGELQRVQQRKESKKPPAELAALEA | 3545.1 | 12.78 | ±0.27* | 0.000 |

(continued)

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric motilins upon intravenous administration to rats in a dose of 10 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±SD | |
| **Chimeric motilin (VIII) (MG-H4)** | FVPIFTYGELQRVQQRKESKKPPAELAALKA | 3544.2 | 11.56 | ±0.93* | 0.000 |
| **Chimeric motilin** | FVPIFTYGELQRVQQRKESKKMITIR (IX)(MG-dPPS) | 3196.8 | 4.39 | ±0.38$^{NS}$ | 0.170 |
| **Chimeric motilin** (X) (MG-S) | FVPIFTYGELQRVQQRKESKKPPMITIR | 3391.0 | 5.25 | ±0.45* | 0.031 |
| Chimeric motilin (XI) (MG-d12/14) | FVPIFTYGELQRKESKKPAKLAALKA | 3031.6 | 10.29 | ±1.00* | 0.001 |
| **Chimeric motilin** (XII) (MGP1) | FVPIFTYGELQRVQQKKAYSPDKERKPPALQPR | 3916.5 | 13.03 | ±0.98* | 0.000 |
| **Chimeric motilin** (XIII) (MGP2) | FVPIFTYGELQRVQQKKAYSPDKERKPPAKLQPR | 4044.7 | 13.09 | ±2.90* | 0.005 |

*: In t-test, a significant difference was available from the disappearance half-time of native motilin ($p < 0.05$)
NS: In t-test, a significant difference was not available from the disappearance half-time of native motilin ($p > 0.05$)

**[0225]** From the result for the chimeric motilin (XI) (MG-d12/14) together with the result for the chimeric motilins (A to M) concerning Example 6, it was shown that, with regard to A in the formula of the half-life-extension peptide of the present invention, that may not be present or that may have a sequence of any amino acid numbers. As to the length when the sequence is present, it was shown to be the length of about 1 to 9 amino acid(s), preferably 3 to 9 amino acids, more preferably 3 amino acid and, most preferably, Val-Gln-Gln.

**[0226]** From the result for the chimeric motilins (T) (MG-17E/N), (U) (MG-17E/Q) and (W) (MG-Des), it was shown that E(Glu) in RKESKK in the structure (core sequence: RKESKK sequence part) necessary for extension of half-life concerning the present invention is an amino acid which is necessary for the action of half-life extension and that, from the result for the chimeric motilins (V) (MG-ES/DS) and (X) (MG-17E/D), the amino acid is able to be substituted with D (Asp). Thus, it was shown that the amino acid at the position of the above E(Glu) may be any acidic amino acid.

**[0227]** From the result for the chimeric motilins (O) (MG-18S/F), (P) (MG-18S/T), (Q) (MG-18S/P), (R) (MG-18S/L) and (S) (MG-18S/A), it was shown that S(Ser) in RKESKK is able to be substituted with T(Thr), P(Pro), L(Leu), F(Phe) or A(Ala). Thus, it was shown that the amino acid at the position of the above S(Ser) is able to be substituted with an amino acid having any side-chain structure such as aromatic amino acid, hydrophobic amino acid or polar non-charged amino acid. It was also shown that, from the result for the chimeric motilin (N) (MG-dS), the amino acid at the position of the above S(Ser) may not be present.

**[0228]** From the result for the chimeric motilin (X) (MG-17E/D), it was shown that each of R(Arg) and K(Lys) in RKESKK is able to be substituted with any basic amino acid.

**[0229]** It is characteristic that the RKESKK sequence part contains a basic amino acid cluster (a continued sequence of two basic amino acids) and an acidic amino acid and, from the result for the chimeric motilins (Z) (MG-i17G), (I) (MG-i19G) , (II) (MG-i17G2) and (III) (MG-i17G2-i19G), it was shown that the distance in the cluster is able to be expanded by insertion of any amino acid.

**[0230]** From the result for each chimeric peptide mentioned in Table 16, it was shown that the distance in the basic amino acid cluster is 1 to 5 amino acids.

**[0231]** From the result for the chimeric motilin (Y) (MG-BR), it was shown that the alignment of RKESKK may be reversed (KKSEKR).

**[0232]** From the result for the chimeric motilins (XII) (MGP1) and (XIII) (MGP2), it was shown that the action for

extending the half-life is potentiated even when two sequences corresponding to the core sequence are aligned in tandem and it was shown that the presence of plural core sequences in a molecule is also an effective means for achieving the longer extension of half-life in blood.

**[0233]** The chimeric motilins (VI) (MG-H1), (VII) (MG-H3) and (VIII) (MG-H4) are peptides where, at the part concerning C in the formula of the half-life-extension peptide according to the present invention, a sequence which is able to be predicted to form an α-helix structure by a known method for predicting the secondary structure (Chou-Fasman method: Biochemistry. 1974 Jan 15; 13(2):222-45 Prediction of protein conformation. Chou PY, Fasman GD, or Garnier method: J Mol Biol. 1978 Mar 25;120(1):97-120. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. Garnier J, Osguthorpe DJ, Robson B.) is aligned in connection to the two Pro's (Pro-Pro sequence). From the result for those chimeric peptides, it was shown that, as to C in the formula of the half-life-extension peptide of the present invention, it is preferred to align any amino acid sequence which is able to form an α-helix structure.

**[0234]** Further, as shown by the comparison of the chimeric motilin (IV) (MG-dPP) with the chimeric motilin D, the comparison of the chimeric motilin (V) (MG-dPPH1) with the chimeric motilin (VI) (MG-H1) and the comparison of the chimeric motilin (IX) (MG-dPPS) with the chimeric motilin (X) (MG-S), it was shown that, when no P(Pro) is present at the N terminal of the amino acid sequence corresponding to the formula C of the half-life-extension peptide concerning the present invention, it is preferred to align a P(Pro) sequence (in Pro numbers within such a range of making the amino acid numbers of C 2 to 14 or, preferably, Pro-Pro) at the site.

Example 9 (Half-life of native CNP-22 and chimeric CNP in plasma) (2)

**[0235]** Changes in CNP immunoreactivity concentration in plasma when the native CNP-22 shown in SEQ ID No: 101 and chimeric CNPs (D, J and K) (J of the chimeric CNP is SEQ ID No: 157 and K of the chimeric CNP is SEQ ID No: 158) in which a half-life-extension peptide comprising 17 or 20 amino acid residues was bonded to C terminal of the peptide comprising the 6th to 22nd amino acid sequence from N terminal in SEQ ID No: 101 which was the active center of the native CNP-22 were intravenously administered were investigated.

**[0236]** The experiment was carried out using rats into which polyethylene tube (PE-50 manufactured by Clay Adams) was inserted into thigh artery under an anesthetized condition with Nembutal. As to a test system, male SD strain rats (Nippon Charles River) of 7 weeks age were used where one group comprised 3 rats. Native CNP-22 or chimeric CNP in a dose of 20 nmol/kg was administered into tail vein of the rat and the blood before the administration until 90 minutes after the administration was collected by a polyethylene tube inserted into the thigh artery. To the collected blood were added EDTA (manufactured by Dojin Laboratories) and aprotinin (manufactured by Bayer) as a stabilizer and an anti-coagulant, respectively and the plasma was separated therefrom by centrifugal separation. The concentration in plasma was measured by a competitive radioimmunoassay (RIA). At that time, a rabbit polyclonal antibody (#2) which specifically recognizes the ring partial structure of the native CNP-22 was used as an antibody and, as a labeled compound, $^{125}$I-[Tyr$^{0}$] CNP was used.

**[0237]** Amino acid sequences, molecular weights and half-time for disappearance from the plasma for chimeric CNP are shown in the following Table 17 and Fig. 9.

(Mean value ± standard deviation; N = 3)

**[0238]**

Table 17

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for native CNP-22 and chimeric CNPs (D, J and K) upon intravenous administration to rats in a dose of 20 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±S.D. | |
| Native CNP-22 | GLSKGCFGLKLDRIGSMSGLGC | 2198 | 1.60 | ±0.50 | - |
| Chimeric CNP (D) | GLSKGCFGLKLDRIGSMSGLGCVQQRKESKKPPAKLQPR | 4198 | 43.59 | ±10.58* | 0.002 |

(continued)

List of amino acid sequences, molecular weights and half-time for disappearance from the plasma for native CNP-22 and chimeric CNPs (D, J and K) upon intravenous administration to rats in a dose of 20 nmmol/kg

| Peptide | Amino Acid Sequence | Mol Wt | T1/2 (min) | | P Value |
|---|---|---|---|---|---|
| | | | Mean Value | ±S.D. | |
| Chimeric CNP (J) (ED Substituted Product) | GLSKGCFGLKLDRIGSMSGLGCVQQRKDSKKPPAKLQPR | 4184 | 31.24 | ±4.79* | 0.000 |
| Chimeric CNP (K) | CFGLKLDRIGSMSGLGCAGSVDHKGKQRKWDHPKR (CO1-KR) | 3880 | 18.03 | ±4.72* | 0-004 |

*: In t-test, a significant difference was available from the disappearance half-time of native CNP-22 ($p < 0.05$)

**[0239]** All of the chimeric CNPs evaluated at this time showed a significantly longer half-life for disappearance than the native CNP-22.

**[0240]** From the result of the chimeric CNP (J), it was shown that, with regard to E(Glu) in RKESKK concerning B (core sequence) of the formula of the half-life-extension peptide concerning the present invention, D(Asp) is acceptable instead of E.

**[0241]** Further, from the result of the chimeric CNP (K), it was shown that any amino acid (for example, Val (which may be in plural), H or P) may be present between E and basic amino acid cluster (RK, KK) in RKESKK concerning B (core sequence) of the formula of the half-life-extension peptide of the present invention. Furthermore, it was shown that the part concerning A of the formula of the half-life-extension peptide of the present invention does not particularly require a specific amino acid sequence but any amino acid may be used. For example, the amino acid numbers for the part concerning A will be acceptable provided that they are within 10.

Example 10 (Extension-promoting action of chimeric CNP) (2)

**[0242]** Dose-dependency of the extension-elongation action of the chimeric CNPs (D, J) was investigated using mice.

**[0243]** In the experiment, female S/VAF Crlj:CD1 (ICR) mice of three weeks age (Nippon Charles River) were used where one group comprised ten mice and 30 mice were used for the experiment in total. The animals (two weeks age) were purchased together with three mother mice and ten baby mice with one mother mouse were bred for one week as a group and then weaned. During the administering period, each group in 5 mice x two cages was bred in a see-through cage. As to water, tap water was made to freely drink and, as to a feed, a solid feed (CRF-1 manufactured by Oriental Yeast Industry) was made to freely ingest. Medium or the chimeric CNP (D, J) (structure mentioned already) was repeatedly administered once daily for 56 days subcutaneously from the back in a dose of 5 mL/kg, 50 nmol/kg or 200 nmol/kg, respectively and body length, tail length and body weight during the administered period were measured. The result is shown in Fig. 10 and the picture of full body length of the mice on the next day of the completion of the chimeric CNP J administration is shown in Fig. 11.

**[0244]** As shown in Fig. 10, when the chimeric CNPs (D, J) each where a half-life-extension peptide is bonded to native CNP-22 was repeatedly administered subcutaneously, body length and tail length of the mice significantly increased in a dose-dependent manner.

Example 11 (Extension-promoting action of Chimeric CNP) (3)

**[0245]** Extension-promoting action of chimeric CNP was investigated using rats.

**[0246]** In the experiment, female SD (IGS) rats of three weeks age (Nippon Charles River) were used where one group comprised five rats and 20 rats in total were used for the experiment. The animals (17 days age) were purchased and ten baby rats with one mother rat were subjected to a preliminary breeding and then weaned. During the administering period, each group was bred in an aluminum hanging cage. As to water, tap water was made to freely drink and, as to a feed, a solid feed (CRF-1 manufactured by Oriental Yeast Industry) was made to freely ingest. Medium or the chimeric CNP (D) (structure mentioned already) was repeatedly administered once daily for 56 days subcutaneously from the back in a dose of 5 mL/kg, 50 nmol/kg or 200 nmol/kg, respectively and body length, tail length and body weight during

the administered period were measured. The result is shown in Fig. 12.

**[0247]** As shown in Fig. 12, body length of the rats to which a chimeric CNP (D) where a half-life-extension peptide was bonded to native CNP-22 was repeatedly administered subcutaneously in a dose of 12.5, 50 and 200 nmol/l mL/kg significantly increased as compared with the group to which a medium was administered.

Example 12 (Comparison of half-life in plasma of native CNP and CNP-53 and (chimeric CNP)

**[0248]** Changes in CNP immunoreactivity concentration in plasma when the native CNP-22 shown by SEQ ID No: 101 was administered either solely or jointly with neutral endo-peptidase (NEP) inhibitor and compared with changes in CNP immunoreactivity concentration in plasma when native CNP-53 (SEQ ID No: 159) and the chimeric CNP (D) (structure mentioned already) were intravenously administered.

**[0249]** The experiment was conducted using rats where polyethylene tube (PE-50, manufactured by Clay Adams) was previously inserted into thigh artery under the anesthetized condition with Nembutal. In the group where the native CNP-22 was administered either solely or jointly with an NEP inhibitor, the polyethylene tube (PE-10, manufactured by Clay Adams) was also inserted into the thigh vein. As to a test system, male SD rats (Nippon Charles River) of 7 weeks age were used where one group comprised three rats. As to the NEP inhibitor, DL-Thiorphan (manufactured by Sigma) was used and, during the period from 10 minutes before the initiation of administration of the native CNP-22 until 60 minutes after the initiation of the administration where collection of the blood finished, 5% mannitol (100 μL/min/body) or NEP inhibitor (30 μg/100 μL/min/body) was subjected to a constant-speed intravenous administration for 70 minutes using an Infusion Pump (CFV 2100, manufactured by Nippon Koden K. K.). Native CNP-22, native CNP-53 or chimeric CNP (D) was administered into tail vein in a dose of 20 nmol/kg each and blood over the time from before administration and 60 minutes after administration was collected from a polyethylene tube inserted into the thigh artery. To the collected blood were added EDTA (Dojin Laboratories) and aprotinin (manufactured by Bayer) as a stabilizer and an anti-coagulant, respectively and the plasma was separated by means of centrifugal separation. CNP immunoreactivity concentration in the plasma was measured by a competitive radioimmunoassay (RIA). At that time, a rabbit polyclonal antibody (#2) which specifically recognizes a ring part structure which is the common structure for native CNP-22 and native CNP-53 was used as an antibody while, as a labeling compound, $^{125}$I- [Tyr$^{0}$] CNPs-22 was used.

**[0250]** Amino acid sequence, molecular weight and half-life for disappearance from the plasma for the native CNP-22, the native CNP-53 and the chimeric CNP (D) are shown in Table 18 and flg. 13.

(mean value ± standard devision, N = 3)

**[0251]**

Table 18

List of amino acid sequence of native CNP-22, native CNP-53 and chimeric CNP (D) and half-life for disappearance from plasma when they were intravenously administered in a dose of 10 nmmol/kg

| Peptide | Amino Acid Sequence | Mol. Wt. | Joint Use with NEP Inhibitor | T1/2 (min) Mean ±S.D. Value | P Value |
|---|---|---|---|---|---|
| Native CNP-22 | GLSKGCFGLKLDRIGSMSGLGC | 2198 | no | 1.11 ±0.16 | - |
| | | | yes | 16.25 ±2.22* | 0.000 |
| Native CNP-53 | DLRVDTKSRAAWARLLQEHPNARKYKGANKKG L SKGGLSKGCFGLKLDRIGSMSGLGC | 5802 | no | 15.72 ±1.49* | 0.000 |
| Chimeric CNP(D) | GLSKGCFGLKLDRIGSMSGLGCVQQRKESKKPP AKLQPR | 4198 | no | 20.30 ±3.09* | 0.000 |

In all of them, S-S bond was formed at the underlined italic cysteine residues to form a cyclic structure.
*: In t-test, a significant difference was available from the disappearance half-time of native CNP (without NEP inhibitor) ($p < 0.05$)

**[0252]** From Table 18 and Fig. 13, it was shown that the chimeric CNP (D) had longer half-life than any of the native CNP-22, the native CNP-22 together with NEP inhibitor and the native CNP-53. Since the native CNP-53 has a NEP resistance, its half-life is longer than the native CNP-22 and, since the pharmacokinetic pattern when the native CNP-22 was used together with the NEP inhibitor was nearly the same in both, it is likely that an amino acid sequence resulting in an NEP resistance is present in 1 to 21 positions of the native CNP-53. In addition, in the 1 to 21 positions of the native CNP-53, there is a sequence (RKYKGANKK) similar to the formula B (corresponding to a core sequence) of the present invention.

**[0253]** Since the chimeric CNP (D) showed better pharmacokinetic pattern than the native CNP-53, it was shown that the alignment of acidic amino acid between the basic amino acid clusters represented by the formula B is necessary for exhibiting the characteristic of longer half-life.

INDUSTRIAL APPLICABILITY

**[0254]** When the half-life-extension peptide according to the present invention is added to the object peptide having a short half-life, the pharmacokinetics *in vivo* is improved and the product has a practical half-life as a drug.

SEQUENCE LISTING

<110> Asubio Pharma.Co.,Ltd.

<120> Peptide having an extending action for half-life of object peptide in plasma

<130> S1216 EP

<140> EP 09 75 0669.5
<141> 2009-05-22

<150> JP 2008-136106
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20 25

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1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

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1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

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1 5 10 15

Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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1 5 10 15

Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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20 25

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20 25

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Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 25

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Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
20 25

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<400> 22

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Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met Asn Arg Arg
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Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met Asn
20 25

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1 5 10 15

Arg Lys Pro Pro Arg Val Gly Arg Arg
20 25

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1 5 10 15

Arg Lys Pro Pro Arg Val Gly
20

<210> 32
<211> 22
<212> PRT
<213> Catfish

<220>
<221> MOD_RES
<222> (22)..(22)
<223> AMIDATION of the C-terminus residue

<400> 32

Gly Ser Ser Phe Leu Ser Pro Thr Gln Lys Pro Gln Asn Arg Gly Asp
1 5 10 15

Arg Lys Pro Pro Arg Val
20

<210> 33
<211> 26
<212> PRT
<213> Shark

<400> 33

Gly Val Ser Phe His Pro Arg Leu Lys Glu Lys Asp Asp Asn Ser Ser
1 5 10 15

Gly Asn Ser Arg Lys Ser Asn Pro Lys Arg
20 25

<210> 34
<211> 17
<212> PRT
<213> Homo sapiens

<400> 34

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg

<210> 35
<211> 16
<212> PRT

<213> Homo sapiens

<400> 35

Val Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
1 5 10 15

<210> 36
<211> 17
<212> PRT
<213> Simian

<400> 36

Ala Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg

<210> 37
<211> 16
<212> PRT
<213> Bovine

<400> 37

Leu Gln Arg Lys Glu Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 38
<211> 16
<212> PRT
<213> Bovine

<400> 38

Leu Gln Arg Lys Glu Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 39
<211> 17
<212> PRT
<213> Equine

<400> 39

Val Gln His Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Lys Pro
1 5 10 15

Arg

<210> 40
<211> 17
<212> PRT
<213> Porcine

<400> 40

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Ala Ala Lys Leu Lys Pro

1 5 10 15

Arg

<210> 41
<211> 17
<212> PRT
<213> Canine

<400> 41

Leu Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg

<210> 42
<211> 16
<212> PRT
<213> Cervidae

<400> 42

Leu Gln Arg Lys Glu Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 43
<211> 16
<212> PRT
<213> Cervidae

<400> 43

Leu Gln Arg Lys Glu Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 44
<211> 16
<212> PRT
<213> Ovine

<400> 44

Leu Gln Arg Lys Glu Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 45
<211> 16
<212> PRT
<213> Goat

<400> 45

Leu Gln Arg Lys Glu Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 46
<211> 16

<212> PRT
<213> Feline

<400> 46

Val Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
1 5 10 15

<210> 47
<211> 17
<212> PRT
<213> Rabbit

<400> 47

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Ala Ala Lys Leu Lys Pro
1 5 10 15

Arg

<210> 48
<211> 17
<212> PRT
<213> Murine

<400> 48

Ala Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg

<210> 49
<211> 16
<212> PRT
<213> Murine

<400> 49

Ala Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
1 5 10 15

<210> 50
<211> 17
<212> PRT
<213> Murine

<400> 50

Thr Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg

<210> 51
<211> 15
<212> PRT

<213> Suncus murinus

<400> 51

Gly Pro Lys Lys Asp Pro Arg Lys Pro Pro Lys Leu Gln Pro Arg
1 5 10 15

<210> 52
<211> 16
<212> PRT
<213> Cetacea

<400> 52

Leu Gln Arg Lys Glu Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg
1 5 10 15

<210> 53
<211> 17
<212> PRT
<213> Meleagrididae

<400> 53

Ile Gln Gln Gln Lys Asp Thr Arg Lys Pro Thr Ala Arg Leu His Pro
1 5 10 15

Arg

<210> 54
<211> 17
<212> PRT
<213> Chicken

<400> 54

Ile Gln Gln Gln Lys Asp Thr Arg Lys Pro Thr Ala Arg Leu His Arg
1 5 10 15

Arg

<210> 55
<211> 15
<212> PRT
<213> Chicken

<400> 55

Ile Gln Gln Gln Lys Asp Thr Arg Lys Pro Thr Ala Arg Leu His
1 5 10 15

<210> 56
<211> 13
<212> PRT
<213> Chicken

<400> 56

Ile Gln Gln Gln Lys Asp Thr Arg Lys Pro Thr Ala Arg

1 5 10

<210> 57
<211> 16
<212> PRT
<213> Turtle

<400> 57

Thr Gln Gln Arg Lys Asp Pro Lys Lys His Thr Lys Leu Asn Arg Arg
1 5 10 15

<210> 58
<211> 20
<212> PRT
<213> Frog

<400> 58

Gln Lys Ile Ala Glu Arg Gln Ser Gln Asn Lys Leu Arg His Gly Asn
1 5 10 15

Met Asn Arg Arg
20

<210> 59
<211> 18
<212> PRT
<213> Frog

<400> 59

Gln Lys Ile Ala Glu Arg Gln Ser Gln Asn Lys Leu Arg His Gly Asn
1 5 10 15

Met Asn

<210> 60
<211> 17
<212> PRT
<213> Frog

<400> 60

Gln Lys Ile Ala Glu Arg Gln Ser Gln Asn Lys Leu Arg His Gly Asn
1 5 10 15

Met

<210> 61
<211> 13
<212> PRT
<213> Eel

<400> 61

Gln Gly Lys Asp Lys Lys Pro Pro Arg Val Gly Arg Arg
1 5 10

<210> 62
<211> 10
<212> PRT
<213> Eel

<400> 62

Gln Gly Lys Asp Lys Lys Pro Pro Arg Val
1 5 10

<210> 63
<211> 14
<212> PRT
<213> Catfish

<400> 63

Gln Asn Arg Gly Asp Arg Lys Pro Pro Arg Val Gly Arg Arg
1 5 10

<210> 64
<211> 12
<212> PRT
<213> Catfish

<400> 64

Gln Asn Arg Gly Asp Arg Lys Pro Pro Arg Val Gly
1 5 10

<210> 65
<211> 11
<212> PRT
<213> Catfish

<400> 65

Gln Asn Arg Gly Asp Arg Lys Pro Pro Arg Val
1 5 10

<210> 66
<211> 18
<212> PRT
<213> Shark

<400> 66

Lys Glu Lys Asp Asp Asn Ser Ser Gly Asn Ser Arg Lys Ser Asn Pro
1 5 10 15

Lys Arg

<210> 67
<211> 17
<212> PRT
<213> Homo sapiens

<400> 67

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val

<210> 68
<211> 16
<212> PRT
<213> Homo sapiens

<400> 68

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Val
1 5 10 15

<210> 69
<211> 17
<212> PRT
<213> Simian

<400> 69

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Ala

<210> 70
<211> 16
<212> PRT
<213> Bovine

<400> 70

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Ala Glu Lys Arg Gln Leu
1 5 10 15

<210> 71
<211> 16
<212> PRT
<213> Bovine

<400> 71

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Pro Glu Lys Arg Gln Leu
1 5 10 15

<210> 72
<211> 17
<212> PRT
<213> Equine

<400> 72

Arg Pro Lys Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg His Gln
1 5 10 15

Val

<210> 73
<211> 17
<212> PRT
<213> Porcine

<400> 73

Arg Pro Lys Leu Lys Ala Ala Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val

<210> 74
<211> 17
<212> PRT
<213> Canine

<400> 74

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Leu

<210> 75
<211> 16
<212> PRT
<213> Cervidae

<400> 75

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Pro Glu Lys Arg Gln Leu
1 5 10 15

<210> 76
<211> 16
<212> PRT
<213> Cervidae

<400> 76

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Pro Glu Lys Arg Gln Leu
1 5 10 15

<210> 77
<211> 16
<212> PRT
<213> Ovine

<400> 77

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Pro Glu Lys Arg Gln Leu
1 5 10 15

<210> 78
<211> 16

<212> PRT
<213> Goat

<400> 78

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Pro Glu Lys Arg Gln Leu
1 5 10 15

<210> 79
<211> 16
<212> PRT
<213> Feline

<400> 79

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Val
1 5 10 15

<210> 80
<211> 17
<212> PRT
<213> Rabbit

<400> 80

Arg Pro Lys Leu Lys Ala Ala Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val

<210> 81
<211> 17
<212> PRT
<213> Murine

<400> 81

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Ala

<210> 82
<211> 16
<212> PRT
<213> Murine

<400> 82

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Ala
1 5 10 15

<210> 83
<211> 17
<212> PRT
<213> Murine

<400> 83

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Thr

<210> 84
<211> 15
<212> PRT
<213> Suncus murinus

<400> 84

Arg Pro Gln Leu Lys Pro Pro Lys Arg Pro Asp Lys Lys Pro Gly
1 5 10 15

<210> 85
<211> 16
<212> PRT
<213> Cetacea

<400> 85

Arg Pro Lys Leu Arg Gly Ser Pro Lys Lys Ala Glu Lys Arg Gln Leu
1 5 10 15

<210> 86
<211> 17
<212> PRT
<213> Meleagrididae

<400> 86

Arg Pro His Leu Arg Ala Thr Pro Lys Arg Thr Asp Lys Gln Gln Gln
1 5 10 15

Ile

<210> 87
<211> 17
<212> PRT
<213> Chicken

<400> 87

Arg Arg His Leu Arg Ala Thr Pro Lys Arg Thr Asp Lys Gln Gln Gln
1 5 10 15

Ile

<210> 88
<211> 15
<212> PRT
<213> Chicken

<400> 88

His Leu Arg Ala Thr Pro Lys Arg Thr Asp Lys Gln Gln Gln Ile

1 5 10 15

<210> 89
<211> 13
<212> PRT
<213> Chicken

<400> 89

Arg Ala Thr Pro Lys Arg Thr Asp Lys Gln Gln Gln Ile
1 5 10

<210> 90
<211> 16
<212> PRT
<213> Turtle

<400> 90

Arg Arg Asn Leu Lys Thr His Lys Lys Pro Asp Lys Arg Gln Gln Thr
1 5 10 15

<210> 91
<211> 20
<212> PRT
<213> Frog

<400> 92

Arg Arg Asn Met Asn Gly His Arg Leu Lys Asn Gln Ser Gln Arg Glu
1 5 10 15

Ala Ile Lys Gln
20

<210> 92
<211> 18
<212> PRT
<213> Frog

<400> 92

Asn Met Asn Gly His Arg Leu Lys Asn Gln Ser Gln Arg Glu Ala Ile
1 5 10 15

Lys Gln

<210> 93
<211> 17
<212> PRT
<213> Frog

<400> 93

Met Asn Gly His Arg Leu Lys Asn Gln Ser Gln Arg Glu Ala Ile Lys
1 5 10 15

Gln

<210> 94
<211> 13
<212> PRT
<213> Eel

<400> 94

Arg Arg Gly Val Arg Pro Pro Lys Lys Asp Lys Gly Gln
1 5 10

<210> 95
<211> 10
<212> PRT
<213> Eel

<400> 95

Val Arg Pro Pro Lys Lys Asp Lys Gly Gln
1 5 10

<210> 96
<211> 14
<212> PRT
<213> Catfish

<400> 96

Arg Arg Gly Val Arg Pro Pro Lys Arg Asp Gly Arg Asn Gln
1 5 10

<210> 97
<211> 12
<212> PRT
<213> Catfish

<400> 97

Gly Val Arg Pro Pro Lys Arg Asp Gly Arg Asn Gln
1 5 10

<210> 98
<211> 11
<212> PRT
<213> Catfish

<400> 98

Val Arg Pro Pro Lys Arg Asp Gly Arg Asn Gln
1 5 10

<210> 99
<211> 18
<212> PRT
<213> Shark

<400> 99

Arg Lys Pro Asn Ser Lys Arg Ser Asn Gly Ser Ser Asn Asp Asp Lys

1 5 10 15

Glu Lys

<210> 100
<211> 28
<212> PRT
<213> Homo sapiens

<220>
<221> DISULFID
<222> (7)..(23)
<223> Cyclic peptide : disulfide bond from Cys at position 7 to Cys at position 23

<400> 100

Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly
1 5 10 15

Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

<210> 101
<211> 22
<212> PRT
<213> Homo sapiens

<220>
<221> DISULFID
<222> (6)..(22)
<223> Cyclic peptide : disulfide bond from Cys at position 6 to Cys at position 22

<400> 101

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

<210> 102
<211> 22
<212> PRT
<213> Homo sapiens

<400> 102

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Met Gln Glu Lys
1 5 10 15

Glu Arg Asn Lys Gly Gln
20

<210> 103
<211> 30

<212> PRT
<213> Homo sapiens

<220>
<221> MOD_RES
<222> (30)..(30)
<223> AMIDATION of the C-terminus residue

<400> 103

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
20 25 30

<210> 104
<211> 34
<212> PRT
<213> Homo sapiens

<400> 104

Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn
1 5 10 15

Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His
20 25 30

Asn Phe

<210> 105
<211> 32
<212> PRT
<213> Homo sapiens

<220>
<221> MOD_RES
<222> (32)..(32)
<223> AMIDATION of the C-terminus residue

<400> 105

Cys Gly Asn Leu Ser Thr Cys Met Leu Gly Thr Tyr Thr Gln Asp Phe
1 5 10 15

Asn Lys Phe His Thr Phe Pro Gln Thr Ala Ile Gly Val Gly Ala Pro
20 25 30

<210> 106
<211> 39
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>

<221> DISULFID
<222> (1)..(17)
<223> Cyclic peptide : disulfide bond from Cys at position 1 to Cys at position 17

<400> 106

Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
1 5 10 15

Cys Asn Ser Phe Arg Tyr Val Gln Gln Arg Lys Glu Ser Lys Lys Pro
20 25 30

Pro Ala Lys Leu Gln Pro Arg
35

<210> 107
<211> 39
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 107

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu
20 25 30

Gly Cys Asn Ser Phe Arg Tyr
35

<210> 108
<211> 39
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys 18 at position to Cys at position 34

<400> 108

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu
20 25 30

Gly Cys Asn Ser Phe Arg Tyr
35

<210> 109
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 109

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> 110
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (1)..(17)
<223> Cyclic peptide : disulfide bond from Cys at position 1 to Cys at position 17

<400> 110

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln
20 25 30

Pro Arg

<210> 111
<211> 35
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 111

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Ser Pro Glu His
1 5 10 15

Gln Arg Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu
20 25 30

Gln Pro Arg
35

<210> 112
<211> 33
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 112

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Glu His Gln Arg
1 5 10 15

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
20 25 30

Arg

<210> 113
<211> 31
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 113

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Gln Arg Val Gln
1 5 10 15

Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25 30

<210> 114
<211> 29
<212> PRT
<213> Artificial

<220>

<223> Synthetic

<400> 114

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

<210> 115
<211> 27
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 115

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Gln Arg Lys Glu
1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

<210> 116
<211> 25
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 116

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Lys Glu Ser Lys
1 5 10 15

Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

<210> 117
<211> 23
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 117

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Ser Lys Lys Pro
1 5 10 15

Pro Ala Lys Leu Gln Pro Arg
20

<210> 118
<211> 27

<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 118

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln
20 25

<210> 119
<211> 25
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 119

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser Lys Lys Pro Pro Ala Lys
20 25

<210> 120
<211> 23
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 120

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser Lys Lys Pro Pro
20

<210> 121
<211> 21
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 121

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser Lys Lys
20

<210> 122
<211> 19
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 122

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Ser

<210> 123
<211> 17
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 123

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys

<210> 124
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (1)..(17)
<223> Cyclic peptide : disulfide bond from Cys at position 1 to Cys a
position 17

<400> 124

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln
20 25 30

Pro Arg

<210> 125

<211> 39
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (6)..(22)
<223> Cyclic peptide : disulfide bond from Cys 6 at position to Cys at position 22

<400> 125

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro
20 25 30

Pro Ala Lys Leu Gln Pro Arg
35

<210> 126
<211> 51
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 126

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu
35 40 45

Gln Pro Arg
50

<210> 127
<211> 51
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 127

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu
35 40 45

Gln Pro Arg
50

<210> 128
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 128

Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro
1 5 10 15

Arg Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> 129
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<400> 129

Arg Pro Gln Leu Lys Ala Pro Pro Lys Lys Ser Glu Lys Arg Gln Gln
1 5 10 15

Val Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu
20 25 30

Gly Cys

<210> 130
<211> 34
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<220>
<221> DISULFID
<222> (18)..(34)
<223> Cyclic peptide : disulfide bond from Cys at position 18 to Cys at position 34

<220>
<221> MOD_RES
<222> (34)..(34)
<223> AMIDATION of the C-terminus residue

<400> 130

Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly
1 5 10 15

Cys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln
20 25 30

Pro Arg

<210> 131
<211> 28
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 131

Phe Val Pro Ile Phe Thr Tyr Gly Glu Leu Gln Arg Val Gln Gln Arg
1 5 10 15

Lys Glu Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
20 25

<210> 132
<211> 29
<212> PRT
<213> Artificial

<220>
<223> synthesis

<400> 132

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20 25 30

## Claims

1. A peptide of the following (I) or (II).

(I) a peptide represented by the formula B, A-B, B-C or A-B-C in which A, B and C each is represented by the following (1), (2) and (3) and, when it is bonded to other object peptide, it is able to extent the half-life in plasma as compared with the object peptide where the physiological activity of the object peptide is still retained.
(II) a peptide comprising a reversed sequence of the peptide of (I); a sequence which is represented by A-B in (I) and A or B is reversed; a sequence which is represented by B-C in (I) and B or C is reversed; or a sequence which is represented by A-B-C in (I) and A, B, C, A and B, B and C or A and C is reserved.

(1) A is a peptide comprising 1 to 14 of any amino acid(s)
(2) B is a peptide represented by the formula 1:
(Wk-Xl-Y-Zm-Wn)-(Wo-Xp-Y-Zq-Wr)s
(In the formula 1, W is a basic amino acid; X and Z are any amino acids; Y is an acidic amino acid; k is 1 or 2; 1 is an integer of $4 \geq 1 \geq 0$; m is an integer of $2 \geq m \geq 0$; $4 \geq 1 + m \geq 0$; n is 1 or 2; o is 1 or 2; p is an integer of $4 \geq p \geq 0$; q is an integer of $2 \geq q \geq 0$; $4 \geq p + q \geq 0$ ; r is 1 or 2; and s is 0 or 1.)
(3) C is a peptide comprising 2 to 14 of any amino acids.

2. The peptide according to claim 1, wherein B is a peptide where s is 1 in the formula 1.

3. The peptide according to claim 2, wherein o is 0, p is 0, q is 0 and r is 2 in the formula 1.

4. The peptide according to claim 1, wherein C contains 4 to 9 amino acid sequences being able to take $\alpha$ helix structure.

5. The peptide according to claim 4, wherein C has a Pro sequence between its terminal and the amino acid sequence being able to take $\alpha$ helix structure.

6. The peptide according to claim 1, wherein A, B and C each is the following (1) or (2).

(1) A is an amino acid sequence of one or more amino acid numbers 1 to 4 continuing to B in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;
B is an amino acid sequence of amino acid numbers 5 to 9 in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and
C is an amino acid sequence of one or more amino acid numbers 10 to 17 continuing to B in SEQ ID No: 34 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added.
(2) A is an amino acid sequence of one or more amino acid numbers 1 to 8 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added;
B is an amino acid sequence of amino acid numbers 9 to 13 in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added; and
C is an amino acid sequence of one or more amino acid numbers 14 to 17 continuing to B in SEQ ID No: 67 or an amino acid sequence where one or some amino acid(s) in the above amino acid sequence is/are deleted, substituted and/or added.

7. A chimeric peptide where a peptide selected from the group consisting of the peptides mentioned in any of claims 1 to 6 is bonded to N terminal, C terminal or both of the object peptide.

8. The chimeric peptide according to claim 7, wherein the object peptide is an atrial natriuretic peptide, a C type natriuretic peptide or motilin or derivatives thereof.

9. A pharmaceutical composition in which the chimeric peptide mentioned in claim 7 or a pharmaceutically acceptable salt thereof is an effective ingredient.

10. A process for producing a chimeric peptide where half-life in plasma is extended as compared with the object peptide and a physiological activity of the object peptide is available, **characterized in that**, a peptide selected from the group consisting of the peptides mentioned in any of claims 1 to 6 is bonded to N terminal, C terminal or both of the object peptide.

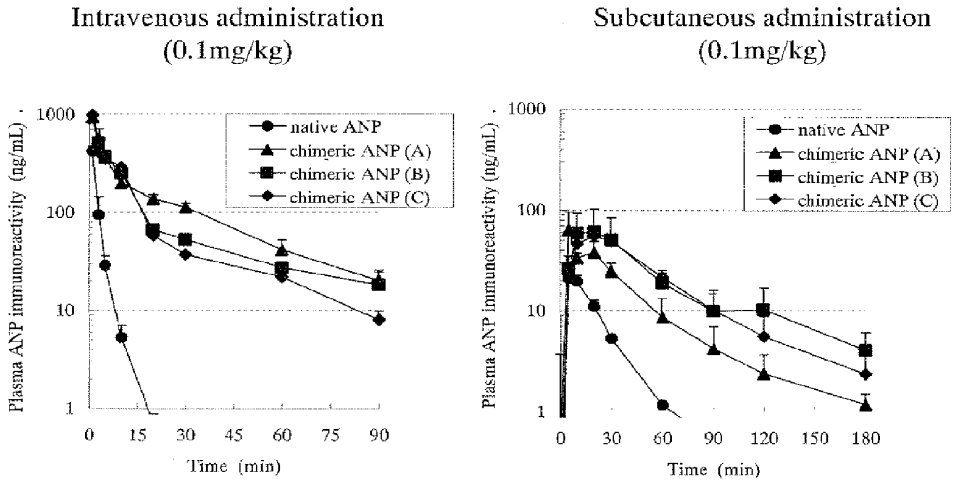
11. The process according to claim 10, wherein the object peptide is a natriuretic peptide or motilin or derivatives thereof.

12. A method for extension of half-life in plasma of the object peptide, **characterized in that**, the peptide selected from the peptides mentioned in any of claims 1 to 6 is bonded to N terminal, C terminal or both of the object peptide.

13. The method according to claim 12, wherein the object peptide is an atrial natriuretic peptide, a C type natriuretic peptide or motilin or derivatives thereof.

14. A method for the treatment of a disease which is able to be treated by the object peptide contained in a pharmaceutical composition, comprising administration of the pharmaceutical composition mentioned in claim 9 to an individual.

[Fig. 1]

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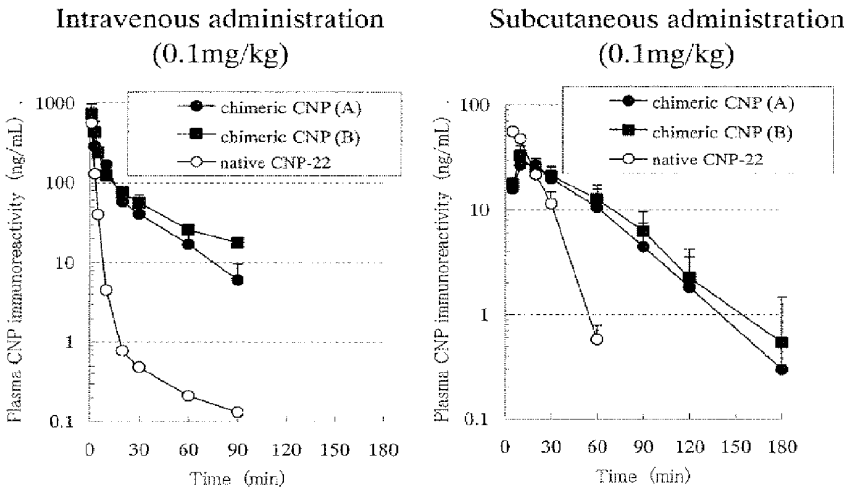
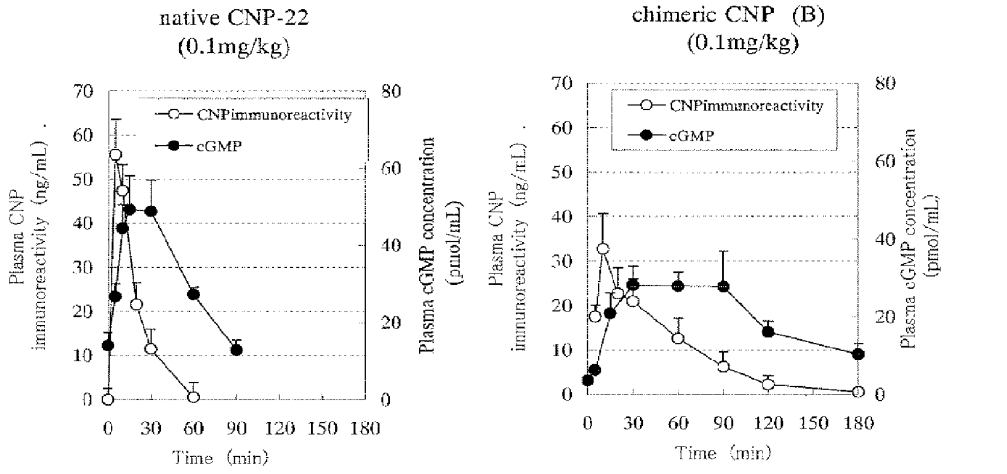
[Fig. 2]



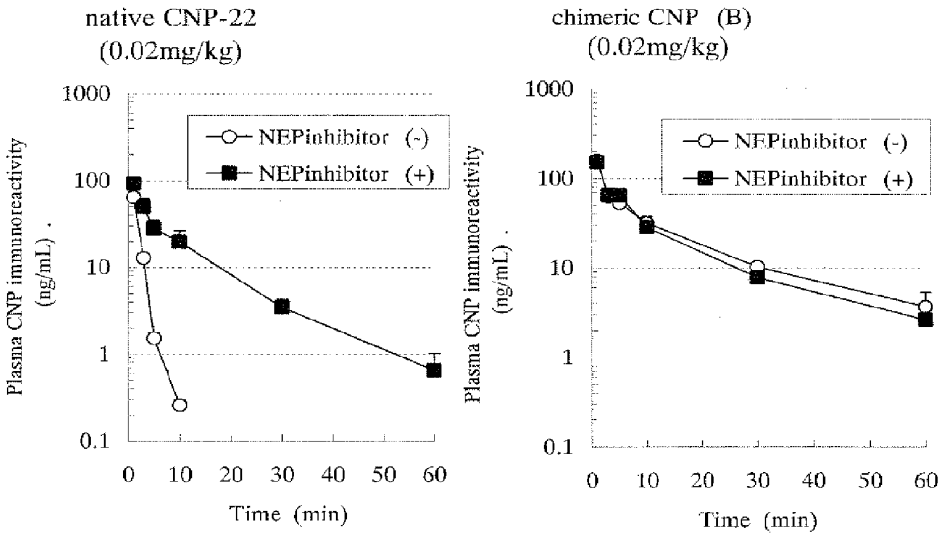

(Mean Value/Standard Deviation, N=3)

[Fig. 3]



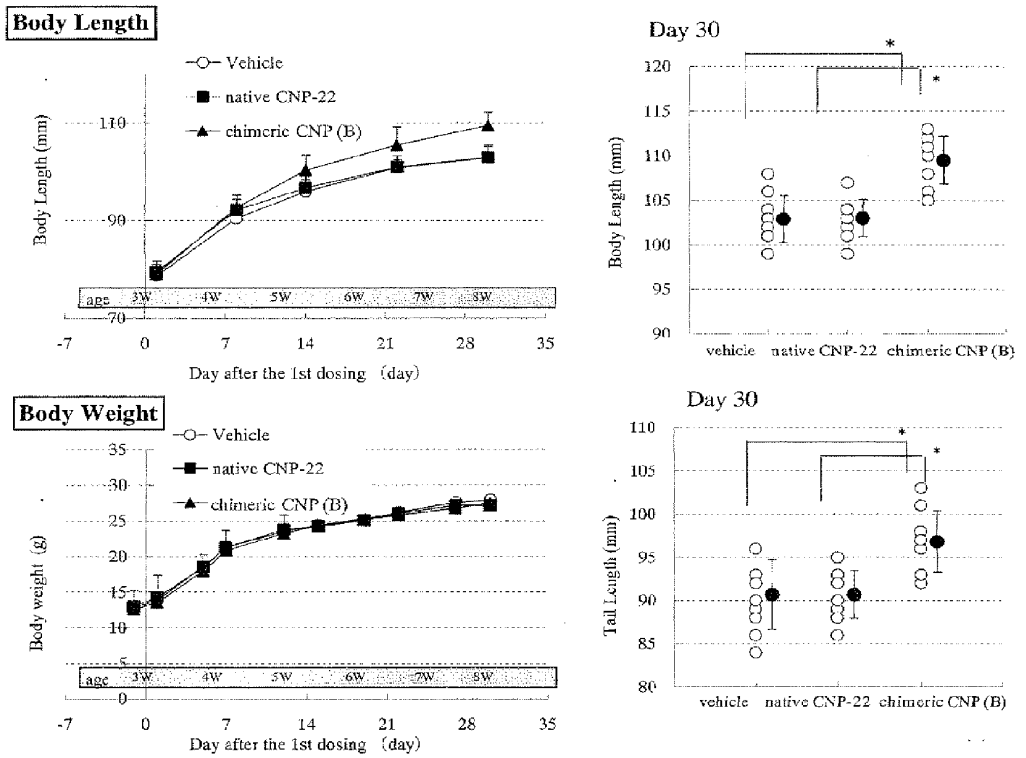

(Mean Value/Standard Deviation, N=3)

[Fig. 4]




(Mean Value/Standard Deviation, N=3)

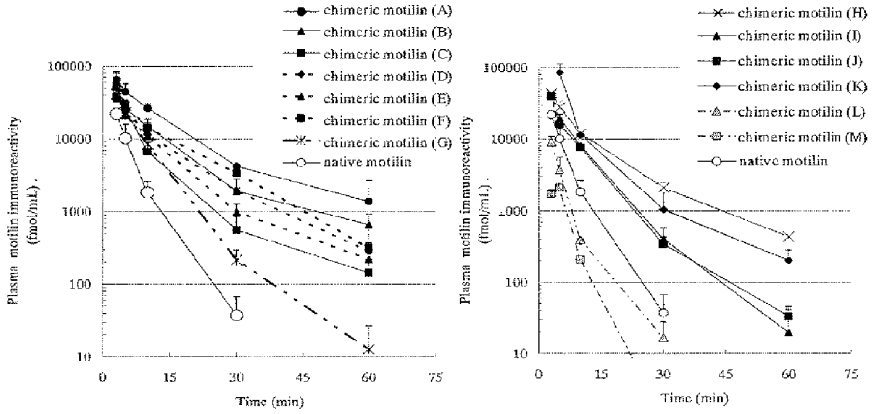
[Fig. 5]




Day 30: the final dosing day
* p<0.05 by Tukey-Kramer HSD test

(Mean Value/Standard Deviation, N=10)

[Fig. 6]




(Mean Value/Standard Deviation, N=3)

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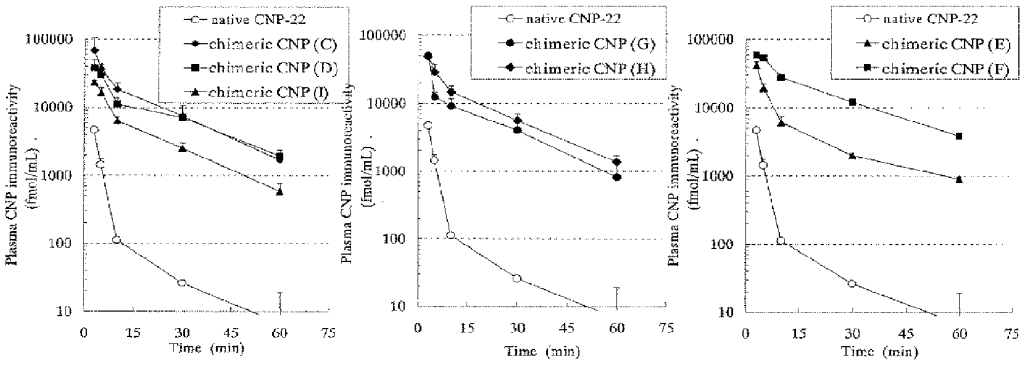


(Mean Value/Standard Deviation, N=3)

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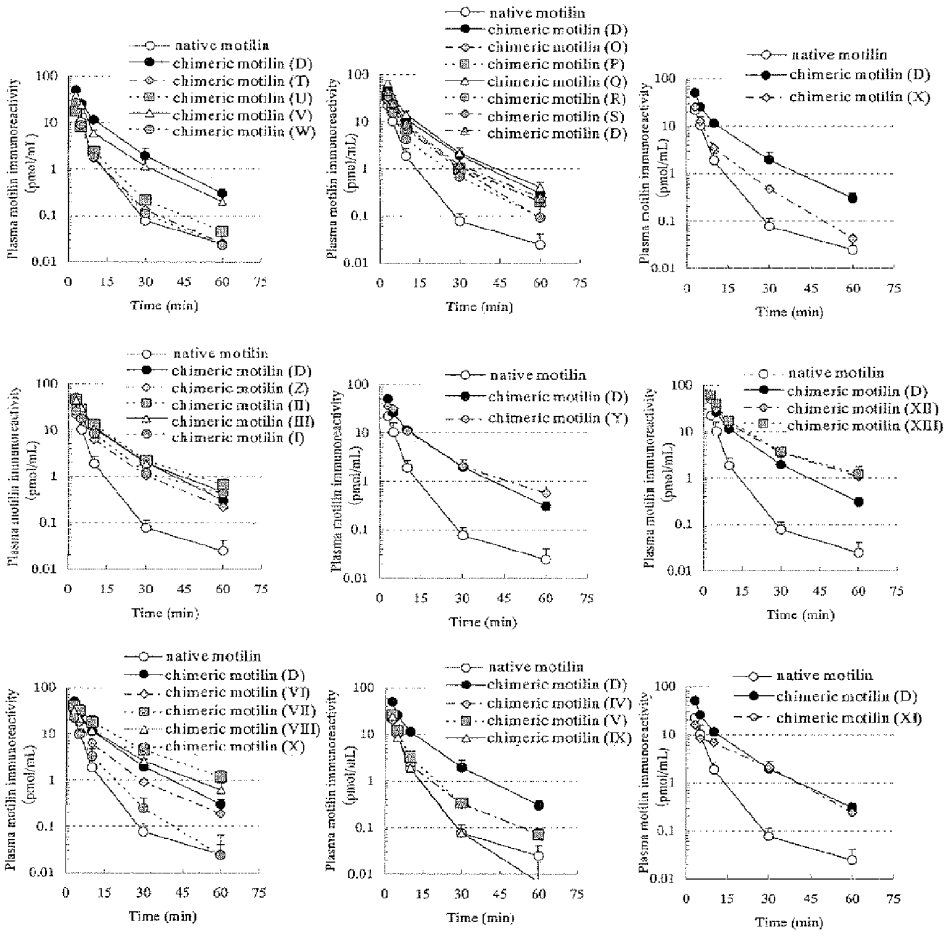


(Mean Value/Standard Deviation, N=3)

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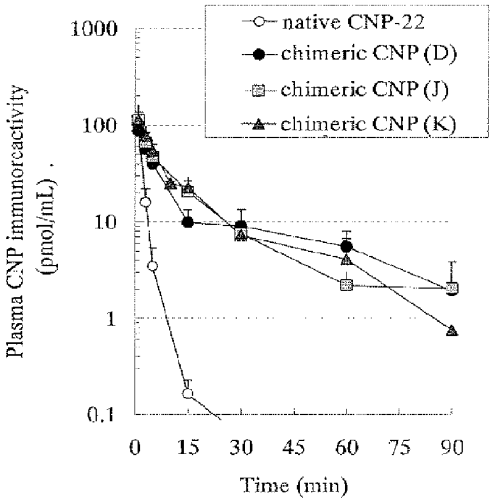
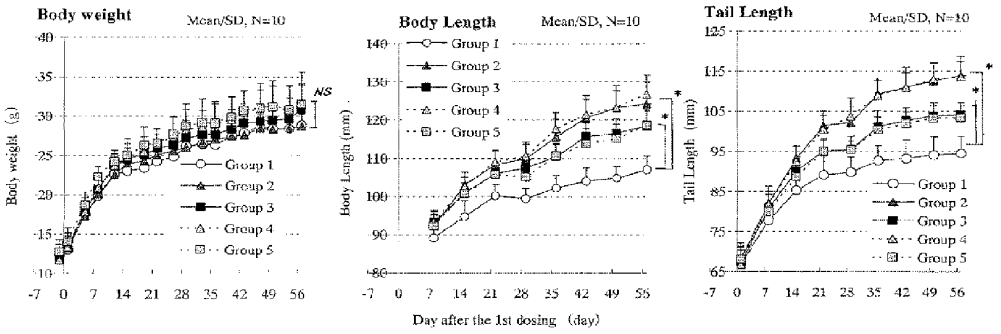



(Mean Value/Standard Deviation, N=3)

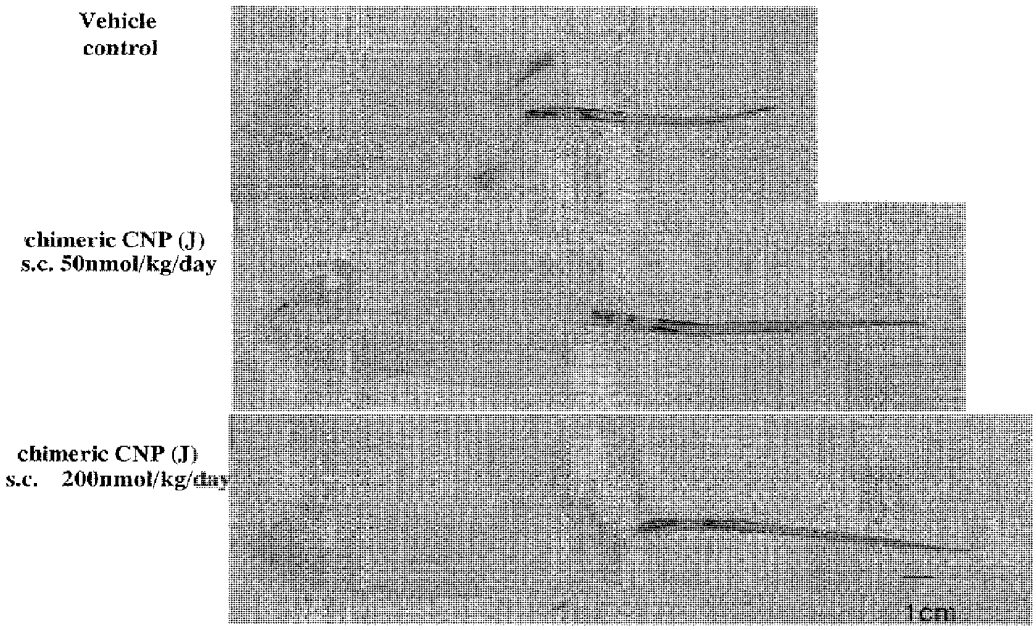
[Fig. 10]




Group 1: Vehicle control Group 2: chimeric CNP (D), 50nmol/kg/day Group 4: chimeric CNP (J), 50nmol/kg/day
Group 3: chimeric CNP (D), 200nmol/kg/day Group 5: chimeric CNP (J), 200nmol/kg/day

NS : Not significant by Dunnet test(p>0.05)
* : Significant by Dunnet test(p<0.01)

[Fig. 11]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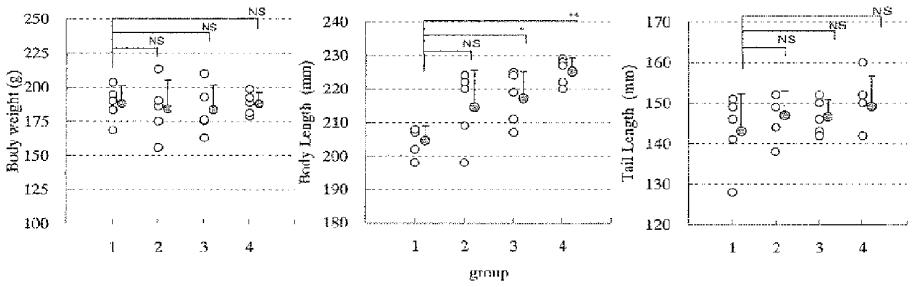
chimeric CNP (J) s.c. 200nmol/kg/day
1cm

[Fig. 12]

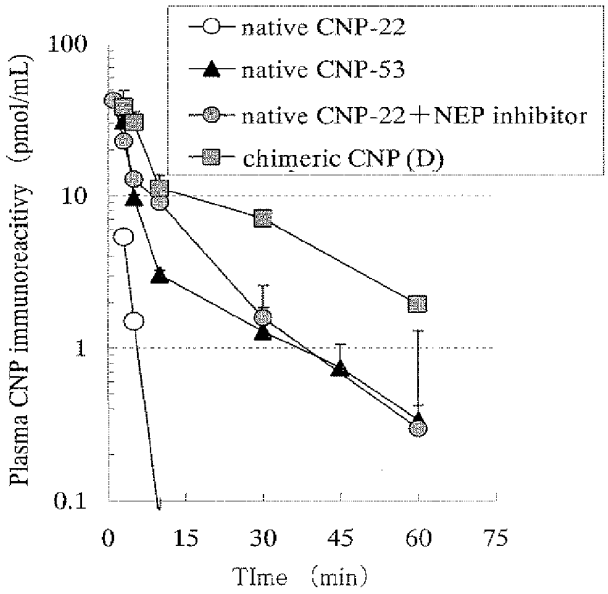



Group 1: Vehicle control
Group 2: chimeric CNP (D), 12.5nmol/kg/day
Group 3: chimeric CNP (D), 50nmol/kg/day
Group 4: chimeric CNP (D), 200nmol/kg/day

○ : Individual data, N=5, ● : Mean Value/Standard Deviation, N=5

NS : Not significant by Dunnet test(p>0.05)
* : Significant by Dunnet test(p<0.05)
** : Significant by Dunnet test(p<0.01)

[Fig. 13]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native CNP-53
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chimeric CNP (D)
Plasma CNP immunoreacitivy (pmol/mL)
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10
1
0.1
0
15
30
45
60
75
TIme (min)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2009/059464

A. CLASSIFICATION OF SUBJECT MATTER
*C07K7/08*(2006.01)i, *A61K47/48*(2006.01)i, *C07K14/58*(2006.01)i, *C07K14/63*(2006.01)i, *C07K19/00*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/02*(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K7/08, A61K47/48, C07K14/58, C07K14/63, C07K19/00, C12N15/09, C12P21/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2009
Kokai Jitsuyo Shinan Koho 1971-2009 Toroku Jitsuyo Shinan Koho 1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/BIOSIS/MEDLINE/WPIDS(STN), CA/REGISTRY(STN), JSTPlus/JMEDPlus/JST7580(JDreamII), PubMed, Science Direct

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DONG, J. Z. et al., GHS-1a receptor agonists that are highly effective in stimulating body weight gain and food intake., Biopolymers, 2005, Vol.80, No.4, pages 578 to 579, full text | 1-13 |
| A | WO 2008/018600 A1 (University of Miyazaki, Asubio Pharma Co., Ltd., Japan as represented by President of National Cardiovascular Center), 14 February, 2008 (14.02.08), Full text & EP 2052738 A1 | 1-13 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 June, 2009 (25.06.09) | 07 July, 2009 (07.07.09) |
| Name and mailing address of the ISA/ Japanese Patent Office | Authorized officer |
| Facsimile No. | Telephone No. |

Form PCT/ISA/210 (second sheet) (April 2007)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2009/059464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 2008/018597 A1 (University of Miyazaki, Asubio Pharma Co., Ltd., Japan as represented by President of National Cardiovascular Center), 14 February, 2008 (14.02.08), Full text & EP 2038374 A1 | 1-13 |
| A | WO 2007/058359 A1 (University of Miyazaki, President of National Cardiovascular Center, Asubio Pharma Co., Ltd.), 24 May, 2007 (24.05.07), Full text & EP 1967208 A1 & KR 10-2008-0075530 A & CN 101312745 A | 1-13 |
| A | WO 2007/058360 A1 (University of Miyazaki, President of National Cardiovascular Center, Asubio Pharma Co., Ltd.), 24 May, 2007 (24.05.07), Full text & EP 1967201 A1 & KR 10-2008-0082651 A & CN 101312739 A | 1-13 |
| A | PEMBERTON, C. J. & Richards A. M., Biochemistry of Ghrelin Precursor Peptides, Vitamins and Hormones, 2007.12, Vol.77, pages 13 to 30, page 15,1st paragraph | 1-13 |
| A | Koji MAGOTA, "Idenshi Kumikae ni yoru Peptide Iyakuhin no Chosei", Gene & medicine MOOK, 2007.07, No.8, pages 232 to 238, page 235, right column to page 236, left column, passage 4 | 1-13 |

Form PCT/ISA/210 (continuation of second sheet) (April 2007)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2009/059464

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 14 pertains to method for treatment of the human body by therapy and thus relates to a subject matter which this International Searching Authority is not required, under the provisions of Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

the

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/059464

<Subject of search>

Claims 1-13 include great many peptides. However, those peptides which are disclosed in the meaning within PCT Article 5 are limited to an extremely small part of the claimed peptides. Therefore, these claims are not fully supported in the meaning within PCT Article 6.

Such being the case, the search was made on a part which are disclosed in and supported by the description, i.e., peptides which are used in the working examples and a carboxy terminal region of ghrelin.

Form PCT/ISA/210 (extra sheet) (April 2007)

## REFERENCES CITED IN THE DESCRIPTION

*This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.*

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(71) Applicants *(for all designated States except US)*: **THE UNIVERSITY OF QUEENSLAND** [—/AU]; St Lucia, Queensland 4072 (AU). **BAKER HEART RESEARCH INSTITUTE** [—/AU]; Commercial Road, Melbourne, Victoria 3004 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ALEWOOD, Paul** [AU/AU]; 145 O'Brien Road, Pullenvale, Queensland 4069 (AU). **HEAD, Geoffrey, A.** [AU/AU]; 10 Elaroo Avenue, Camberwell, Victoria 3125 (AU). **FRY, Bryan, Grieg** [US/AU]; 64 Inverness Road, Kalorama, Victoria 3766 (AU).

(74) Agents: **ARGAET, Victor, Peter** et al.; Davies Collison Cave, Level 3, 303 Coronation Drive, Milton, Queensland 4064 (AU).

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(54) Title: PROTEINACEOUS COMPOUNDS AND USES THEREFOR

(57) Abstract: The present invention discloses proteinaceous compounds that comprise at least a biologically active portion of a taipan natriuretic peptide (TNP) or a variant or derivative thereof. The invention also relates to the use of these compounds in methods for stimulating vasodilation, natriuresis, diuresis, renin-suppression, bactericidal activity, weight-loss or bone growth in a mammalian host.

WO 2006/005140 A2

## TITLE OF THE INVENTION

"PROTEINACEOUS COMPOUNDS AND USES THEREFOR"

## FIELD OF THE INVENTION

**[0001]** This invention relates generally to proteinaceous compounds and more particularly to proteinaceous compounds that comprise at least a biologically active portion of a taipan natriuretic peptide (TNP) or a variant or derivative thereof. The invention also relates to the use of these compounds in methods for stimulating vasodilation, natriuresis, diuresis, renin-suppression, bactericidal activity, weight-loss or bone growth in a mammalian host.

## BACKGROUND OF THE INVENTION

**[0002]** Natriuretic peptides (NPs) are known for their role in cardiovascular homeostasis, diuresis, natriuresis and vasodilation. They act in the body to oppose the activity of the renin-angiotensin system and enhance excretion of sodium and water. There are four known types of natriuretic peptides in humans: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), S-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP). Isoforms of these occur in nature across species.

**[0003]** ANP is secreted by atrial myocytes in response to increased intravascular volume. Once it is in the circulation, its effects are primarily on the kidney, vascular tissue and adrenal gland, in which its actions lead to the excretion of sodium and water by the kidneys and a decrease in intravascular volume and blood pressure (Atlas *et al.*, 1987, *In Atrial Hormones and Other Natriuretic Factors*, P.J. Mulrow *et al.*, Eds, *Am. Physiol. Soc.*, Bethesda, MD, pp. 53-76).

**[0004]** BNP is of myocardial cell origin and, like ANP, circulates in human plasma (de Bold *et al.*, 1981, *Life Science*, **28**(1): 89-94; Burnett *et al.*, 1986, *Science*, **231**(4742): 1145-1147). BNP is natriuretic, renin-inhibiting, vasodilating and lusitropic (Mukoyama *et al.*, 1991, *J. Clin. Invest.*, **87**(4): 1402-1412; Yamamoto *et al.*, 1996, *Am. J. Physiol.*, **271**(6 Pt 2):R1529-1534; Grantham *et al.*, 1997, *Natriuretic Peptides in Health and Disease*, Samson W.K. 11e.r., eds, Humana Press, pp. 309-326).

**[0005]** CNP is of endothelial cell origin and functions as a vasodilating and growth-inhibiting peptide (Suga *et al.*, 1992, *J. Clin. Invest.*, **90**(3): 1145-1149; Stingo *et al.*, 1992, *Am. J. Physiol.*, 262(1 Pt 2):H308-312; Koller *et al.*, 1991, *Science*, **252**(5002): 120-123).

**[0006]** DNP possesses structural similarity to ANP, BNP and CNP. It was originally found in the venom of *Dendroaspis angusticeps* and has been shown to vasorelax rodent aorta and isolated canine coronary arteries with potency comparable to that of ANP (Schweitz *et al.*, 1992, *J. Biol. Chem.*, **267**: 13928-13932; Wenberg *et al.*, 1997, *Am. Coll. Cardiol.*, **29**: 305a). Recently DNP

immunoreactivity has been demonstrated in human circulation, and its plasma levels are elevated in congestive heart failure (CHF) (Schirger *et al.*, 1999, *Mayo Clin. Proc.*, **74**: 126-130). A NP-like peptide, Lebetin 2 isoform alpha, was found in the venom of *Vipera lepitera* (Barbouche *et al.*, 1996, *FEBS Lett.* **392**:6-10). This peptide contains the consensus natriuretic peptide signature which indicates that it belongs to the same general family. Illustrative examples of known natriuretic peptides are given in Table 1.

**[0007]** Natriuretic peptides effect their biological role through three receptors: NPR-A, NPR-B and NPR-C. NPR-A and NPR-B have cytoplasmic guanylyl cyclase domains, which are activated upon ligand binding and lead to accumulation of intracellular cGMP. The tissue distribution of each receptor is different. While NPR-A is expressed in vasculature, kidney and adrenal glands, NPR-B is mainly expressed in the brain. NPR-C, is devoid of the kinase and cytoplasmic GC domains and is generally considered to be a clearance receptor for removing natriuretic peptides from the circulation, although other biological functions for it have been postulated (Murthy *et al.*, 1999, *J. Biol. Chem.*, **274**(25): 17587-17592). U.S. Patent Nos. 5,846,932 and 6,028,055 disclose potent ANP and BNP variants having decreased affinity for the human clearance or C-receptor (NPR-C). DNP has been shown to produce relaxation through both the NPR-A and NPR-C receptors.

**[0008]** ANP and BNP each bind to both NPR-A and NPR-C with high affinity, but only bind to NPR-B with low affinity, whereas CNP binds to NPR-B and NPR-C, but not appreciably to NPR-A (Potter LR and Hunter T, 2001, *J. Biol. Chem.* **276**: 6057 – 6060). It is generally considered that the beneficial effects (natriuresis/diuresis or effects on renin/angiotensin system) of natriuretic peptides occur *via* the synthesis of the intracellular messenger cyclic-GMP (cGMP). NPRA is thought to be the primary ANP/BNP signalling molecule and has been suggested as the principal mediator of natriuretic peptide activities. Conclusive studies have determined that NPR-A is the only NP binding receptor that regulates natriuresis/diuresis and regulates the renin/angiotensin system (Kishimoto I, *et al.*, 1996, *Proc Natl Acad Sci USA* **93**: 6215-6219). In this study it was demonstrated that ANP does not bind to tissues from animals that are homozygous null mutants for NPR-A. These animals do not undergo natriuresis or diuresis in response to ANP and their aortic rings do not relax in response to that peptide. Shi *et al.* (2003, AJP - Renal **285**:694-702) established that NPRA is critical in mediating the natriuresis, diuresis, and renal hemodynamic responses to acute blood volume expansion. From these studies it is apparent that, for a NP peptide to be of use in the treatment of CHF, it must act at the NPR-A receptor.

**[0009]** The ability for a NP to bind human NPR-A cannot be predicted from an analysis of primary sequence alone, nor can analysis of primary sequence predict the affinity with which a NP can bind to NPR-A. The sequences of both the hormones and their receptors vary across species. A consensus NP sequence has been identified for NPs but this is loosely defined and provides no information on whether the peptide acts as an NP, and no guidance as to whether the peptide is active

at NPR-A in particular (and hence potentially useful for treating CHF), NPR-B and/or NPR-C. Schweitz *et al.* (1992 *J. Biol. Chem.* **267**: 13928 – 13932) identified DNP as a ligand for NPR-A on the basis that the peptide relaxes aortic rings. In an early attempt to define features important for NPR-A binding, they proposed a conservative tripeptide sequence in the C-terminal part of the as then known natriuretic peptides consisting of (i) a small non-hydrophilic residue; (ii) a large hydrophobic residue and (iii) an arginine residue, as highlighted in Figure 2. However, as is evident in later studies and in the present specification, this generalisation does not hold and provides little guidance as to whether a natriuretic peptide has NPR-A activity, or otherwise.

**[0010]** ANP, BNP, CNP and DNP are synthesised from large precursors and the mature, active peptides have a 17-amino acid ring structure formed by an intramolecular disulphide linkage. In the human peptides, ten of these amino acids are identical, whereas the N-terminal head and C-terminal tail vary in both length and composition (see Kambayashi *et al.*, 1990, *FEBS Lett*, **259**: 341-345; Tawaragi *et al.*, 1991, *Biochem. Biophys. Res. Commun.* **175**: 645-651).

**[0011]** Due to their diverse actions on both the cardiovascular system and the kidney, ANP, BNP, CNP and DNP and their analogues have been the subject of great interest for developing novel therapeutics. For example, reference may be made to Lewicki *et al.* (U.S. Pat. Nos. 5,114,923, 4,804,650 and 4,757,048), Johnson *et al.* (U.S. Pat. No. 5,047,397), Johnson *et al.* (U.S. Pat. No. 4,935,492), and Wei *et al.* (U.S. Pat. No. 5,583,108). U.S. Pat. No. 5,583,108 discloses a chimera of ANP and CNP, termed vasonatrin peptide (VNP). VNP, which includes 22 amino acids of CNP and the 5 amino acids at the carboxy-terminus of ANP, has arterial and venous vasodilating and natriuretic effects. U.S. Patent No. 6,407,211 discloses chimeric compounds comprising the C-terminal tail of DNP with the core ring structure of BNP or CNP.

**[0012]** The above natriuretic peptides and their analogues are known to be useful for treating a variety of different conditions including: oedematous states such as congestive heart failure (CHF), nephrotic syndrome and hepatic cirrhosis; hypertension; pulmonary hypertension; and renal disorders as well as diseases such as renal failure due to ineffective renal perfusion or reduced glomerular filtrate rate; bacterial infections; weight loss; asthma; inflammatory-related diseases; erectile dysfunction; hypercholesterolemia; skeletal dysplasias and as a protectant for toxicity of anti-cancer drugs, as describe4d for example in WO 2004/011498.

**[0013]** Typically, heart failure patients receive several chronic oral therapies, primarily including diuretics, angiotensin-converting enzyme (ACE) inhibitors, and beta-blockers. Often, these patients have an acute episode of CHF, requiring hospitalisation. Following an acute episode, survivors are required to continue management of the chronic condition. The gold standard for the treatment of more advanced CHF is quadruple therapy with an ACE inhibitor (or an ARB), a beta-blocker, a diuretic and digoxin. However, each of these is associated with a number of deleterious side-effects such as neurohumoral activation, ototoxicity, hypokalemia, renal impairment and an

increased risk of arrhythmic death. It has been observed that enhancement of the NP system may provide a better strategy to treat the sodium and water retention that increases cardiac preload leading to progressive cardiac dysfunction in CHF patients (Costello-Boerrigter *et al.*, 2003, *Medical Clinics of North America* **87**(2)). The therapeutic potential of the NPs is underscored by the role played by ANP and BNP in overcoming sodium retention in the early phase of CHF. As CHF evolves, the balance between the antinatriuretic-vasoconstrictive systems (i.e., RAAS, sympathetic nervous system, vasopressin and endothelin) and natriuretic-vasodilatory systems (i.e., NPs, nitrous oxide, prostaglandins and adrenomedulin), which has a profound effect on sodium and water homeostasis, moves towards the former system. The current hypothesis is that there is a state of relative deficiency of NP in CHF. Consequently, enhancement of NPs is considered a beneficial strategy either *via* administration of exogenous NPs, through agonists of NP-A, or by inhibition of the enzyme responsible for NP degradation, NEP 24.11. In tribute to the growing recognition of NP therapy, nesiritide citrate or Natrecor®, a BNP citrate salt, has been launched commercially for the intravenous treatment of acute CHF patients in the United States. A recombinant form of hANP, termed carperitide, has been launched in Japan for the same indication and is currently in clinical trials in the USA (Pharmaprojects, 2005).

**[0014]** An advantage of NPs over currently used diuretics is that they do not activate the RAAS system (which is associated with the progression of CHF). Furthermore, they inhibit the sympathetic nervous system (which is associated with heart failure progression, myocyte necrosis and apoptosis, and arrhythmias). It has been demonstrated that NPs exhibit fewer arrhythmic events, such as tachycardia. In trials, BNP was reported to be associated with less arrhythmic events than dobutamine (Burger *et al.*, 2001, *Am J Cardiol 88*, 35-39). NPs and their derivatives can be administered alone or in combination with one or more of the following compounds: beta blockers, diuretics, ACE inhibitors, digoxin, spironolactone, anticoagulation and antiplatelet agents and angiotension receptor blockers, as disclosed for example in WO03081246.

**[0015]** A disadvantage of NPs is that they are notoriously fragile *in vivo*, being degraded rapidly by neutral endopeptidases (NEPs) and through the clearance receptor (NPR-C), thus limiting their bioavailability. A corollary of this is that NPs, in particular Nesritide, is only useful in the treatment of acute CHF requiring intravenous infusion. To access the chronic CHF market, new stabilized NPs are sought. Such NPs have the requirements of significantly improved half lives so that more convenient routes of administration such as subcutaneous injection or oral administration can be adopted and with less frequent dosing than would be needed for current NP therapies such as ANP or BNP, both which have short half lives.

**[0016]** Attempts to increase resistance to proteolytic degradation of NPs through use of PEGylation and other conjugates (see, for example,WO2004/047871), resulted in improved resistance to trypsin, oral availability and in increased plasma levels as compared to native BNP in some

examples.. Interestingly, analysis of animal plasma assays for its lead compound reveal that their oral delivery technology is successful, in that the hBNP survives the GI tract and reaches the blood stream. However, once in the blood stream, it is degraded and cleared quite quickly (within 30 minutes the plasma levels of the compound are almost zero), demonstrating no superiority over Natrecor's 18 minute half-life. In short, hBNP is too unstable, and therefore not amenable to oral administration. An alternative approach is bioconjugation of the NP with blood components for example human serum albumin. This technology has been demonstrated to increase NP half life (see, for example, WO2004011498). However, the corresponding large size of the resulting complex makes it unsuitable for most forms of administration. Additionally, it is not clear that the activated NP does not conjugate to other biological species *in vivo* with the consequence that regulatory and safety issues may arise. Other studies have targeted the stability of NPs by selecting for analogues that favour the NPR-A receptor over the NPR-C receptor (see, for example U.S. Patent Nos. 6,028,055 and 5,846,932), thus reducing clearance mechanisms or by stabilizing the sequence against NEP protease digestion, for example by replacing the susceptible Phe with Cha. All these studies demonstrate the general amenability of NPs, without significant loss of activity, to substitution and derivatisation provided that the primary binding moieties are not disturbed.

**[0017]** DNP has been found to possess higher resistance to NEPs than previously known NPs, putatively due to its extended tail region. The tail region is roughly correlated to stability, with CNP, the least stable having the shortest tail. Indeed, the significantly higher potency of BNP over ANP in elevating cGMP levels in mice kidney is attributed to the greater stability of the former peptide. Correspondingly, there still exists a need for compounds with NP-like activity that are better able to withstand proteolytic enzyme attack, particularly that of NEP 24.11.

**[0018]** It is not possible to determine the stability of NP peptides from an examination of their primary sequences alone. The program "Peptide Cutter" available on the suite of protein analysis programs, Swissprot (http://us.expasy.org/sprot/) predicts that all known peptides have multiple regions of susceptibility to proteolytic attack. For instance, ANP is predicted to contain 59 cleavage sites for 12 enzymes, whereas BNP is predicted to contain 55 cleavage sites for a total of 13 enzymes. The peptides of the present invention, are predicted to contain 59 cleavage sites for a total of 16 enzymes. Yet, as is demonstrated herein, these latter peptides are significantly more stable than their human counterparts.

## SUMMARY OF THE INVENTION

**[0019]** In work leading up to the present invention, the inventors isolated three novel peptides of between 35 and 39 amino acid residues in length from the venom of *Oxyuranus microlepidotus* (Inland taipan). The amino acid sequences of these taipan natriuretic peptides (TNPs) (see Table 2) display significant similarity to ANP and BNP within the core structure (i.e., between the cysteine residues) but no similarity at either the N-terminal head or C-terminal tail (see Figure 2). Of interest, one of these peptides, designated TNPc, contains the consensus natriuretic signature (Table 1), whilst the other two peptides, designated, TNPa and TNPb, differ from the consensus signature by only a single residue,(i.e., both have a His at position 16, whereas the consensus signature has a conservative Arg at the same position), although this is a conservative substitution.

**[0020]** Based upon their structural similarity to mammalian natriuretic peptides, the isolated TNPs were predicted to have at least some of the properties associated with their mammalian homologues (e.g., vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing and/or bone growth plate size-increasing activities). However, despite their suspected activities, the TNPs were not considered to be particularly useful in therapeutic or prophylactic applications in mammals because they differed substantially with the known mammalian natriuretic peptides in the N-terminal head and C-terminal tail, and were suspected, therefore, to elicit significant immune responses when administered to mammals. It was proposed that these immune responses would render the snake venom peptides ineffective, especially in the context of chronic administration.

**[0021]** Surprisingly, the inventors have discovered that these TNPs do not produce significant anti-peptide immune responses when administered to mammals and that only TNPc is useful in therapeutic or prophylactic applications. In particular, it was found that TNPc has high affinity for the NPR-A receptor as well as vasodilatory, natriuretic and diuretic properties characteristic of the NP family, with improved *in vitro* and in *vivo* stability. By contrast, both TNPa and TNPb were found to have poor activity at the NPR-A receptor as well as insignificant vasodilatory activity, despite their structural similarity to TNPc.

**[0022]** In studies exploring the structure/function relationship of the TNPs, it was found that substitution of Arg16 or Ile28 in TNPc with His and Pro, respectively, which are found at the same positions in TNPa/b, reduces the activity markedly. These results suggested that the reduced activity of TNPa and TNPb arises, at least in part, by the departure (despite its apparent conservative nature) from the consensus natriuretic signature and the presence of a Pro in the C-terminal tail where a hydrophobic residue is found consistently in other natriuretic peptides.

**[0023]** Since the C-termini of natriuretic peptides are believed to be important for binding to NP receptors, and for stability, the inventors propose that the C-terminus of TNPc as well as its variants and derivatives can be linked to the core structures of other natriuretic peptides to form

chimeric compounds with useful profiles of bioactivity. These chimeric compounds may optionally further comprise a N-terminal portion of TNPc or of another natriuretic peptide. The inventors also propose that the N-terminal portion and core ring structure of TNPc and their variants and derivatives can be linked in a similar fashion to the core ring structure and C-terminal portion, respectively, of other natriuretic peptides to form useful chimeric compounds.

**[0024]** Thus, in one aspect, the present invention provides isolated proteinaceous compounds comprising at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals. The compounds generally comprise the formula:

**[0025]** $\Delta$–$\Theta$–$\Omega$ (I)

**[0026]** wherein:

**[0027]** $\Delta$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of that portion;

**[0028]** $\Theta$ is the core ring structure of a natriuretic peptide or a variant or derivative of the structure;

**[0029]** $\Omega$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of that portion; and

**[0030]** at least one of $\Delta$, $\Theta$ and $\Omega$ comprises a biologically active portion of TNPc or a variant or derivative thereof.

**[0031]** In some embodiments, the biologically active portion comprises a C-terminal portion of TNPc, e.g., NRIMQNPPKK [SEQ ID NO:1], NRIMQNPPKKF [SEQ ID NO:2], NRIMQNPPKKFS [SEQ ID NO:3], NRIMQNPPKKFSG [SEQ ID NO:4] or NRIMQNPPKKFSGE, [SEQ ID NO:5], or a variant or derivative thereof. In other embodiments, the biologically active portion comprises the TNPc core ring structure sequence CFGFPLDRIGSVSGLGC [SEQ ID NO:6], or a variant or derivative thereof. In still other embodiments, the biologically active portion comprises a N-terminal portion of TNPc, e.g., SDSKIGNG [SEQ ID NO:7], or a variant or derivative thereof.

**[0032]** In certain embodiments, the variant or derivative of the biologically active portion of TNPc comprises a sequence having at least 80, 85, 90, 96, 97, 98 or 99% sequence similarity with, or differs at no more than 1, 2, 3, 4, 5 or 10 amino acid residues from, the corresponding portion represented by any one of SEQ ID NO:1-7, with the proviso that: (a) when the variant or derivative corresponds to a C-terminal portion of TNPc, it comprises a hydrophobic amino acid residue, e.g., I, L or F, at position 28 relative to the amino acid sequence of TNPc; and (b) when the variant or derivative corresponds to the core ring structure of TNPc, it comprises a basic amino acid residue, e.g., R or K at position 16 relative to the amino acid sequence of TNPc.

[0033] In specific embodiments, the variant or derivative of the biologically active portion of TNPc shares at least 90% sequence identity with any one of SEQ ID NO:1-7.

[0034] When $\Delta$, $\Theta$ or $\Omega$ comprise a biologically active portion of a natriuretic peptide other than TNPc or its variants or derivatives, the natriuretic peptide is suitably selected from ANP, BNP, CNP, DNP, VNP, PNP or an *Oxyuranus* natriuretic peptide such as a natriuretic peptide from *Oxyuranus microlepidotus*, other than TNPc, or its variants or derivatives.

[0035] In some embodiments, the compounds generally comprise the formula:

[0036] $$\Delta_1\text{–}\Theta_1\text{–}\Omega_1 \qquad \text{(II)}$$

[0037] wherein:

[0038] $\Delta_1$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of the portion;

[0039] $\Theta_1$ is the core ring structure of a natriuretic peptide or a variant or derivative of the structure; and

[0040] $\Omega_1$ is a C-terminal portion comprising an amino acid sequence having the formula:

[0041] $$\mathbf{X_1\ X_2\ \Phi_1\ X_3\ X_4\ X_5\ X_6\ \Sigma_1\ X_7\ X_8\ \Lambda} \qquad \text{(III)}$$

[0042] wherein:

[0043] $X_{1\text{-}8}$ are independently selected from any amino acid residue, or modified form thereof;

[0044] $\Phi_1$ is selected from any hydrophobic amino acid residue, or modified form thereof; and

[0045] $\Sigma_1$ is a small amino acid residue, or modified form thereof; and

[0046] $\Lambda$ is absent or is a peptide comprising an amino acid sequence having the formula:

[0047] $$\mathbf{X_9\ \Sigma_2\ \Sigma_3\ X_{10}\ X_{11}} \qquad \text{(IV)}$$

[0048] wherein:

[0049] $X_9$ is selected from any amino acid residue, or modified form thereof;

[0050] $\Sigma_2$ is absent or is selected from any small amino acid residue, or modified form thereof;

[0051] $\Sigma_3$ is absent or is selected from any small amino acid residue, or modified form thereof, when $\Sigma_2$ is present;

**[0052]** $X_{10}$ is selected from any amino acid residue, or modified form thereof, when $\Sigma_3$ is present; and

**[0053]** $X_{11}$ is selected from any amino acid residue, or modified form thereof, when $X_{10}$ is present,

**[0054]** with the proviso that formula III excludes:

**[0055]** NSFRY [SEQ ID NO:8] when $\Delta_1$–$\Theta_1$ consists of SLRRSSCFGGRMDRIGAQSGLGC [SEQ ID NO:187];

**[0056]** NSFRYRR [SEQ ID NO:9] when $\Delta_1$–$\Theta_1$ consists of SLRRSSCFGGRIDRIGAQSGLGC [SEQ ID NO:188];

**[0057]** KVLRRH [SEQ ID NO:10] when $\Delta_1$–$\Theta_1$ consists of SPKMVQGSGCFGRKMDRISSSSGLGC [SEQ ID NO:189];

**[0058]** DGLRLF [SEQ ID NO:11] when $\Delta_1$–$\Theta_1$ consists of IQERLRNSKMAHSSSCFGQKIDRIGAVSRLGC [SEQ ID NO:190];

**[0059]** PSLRDPRPNAPSTSA [SEQ ID NO:12] when $\Delta_1$–$\Theta_1$ consists of EVKYDPCFGHKIDRINHVSNLGC [SEQ ID NO:191]; and

**[0060]** NKVDDNKG [SEQ ID NO:13] when $\Delta_1$–$\Theta_1$ consists of GDNKPPKKGPPNGCFGHKIDRIGSHSGLGC [SEQ ID NO:192].

**[0061]** In some embodiments, $X_1$ is selected from a basic amino acid residue, e.g., K, a neutral/polar amino acid residue, especially a polar/large amino acid residue, e.g., N, or a small amino acid residue, e.g., N, or modified form thereof. In some embodiments, $X_2$ is selected from a hydrophobic amino acid residue, e.g., V, a basic amino acid residue, e.g., R, or a small amino acid residue, e.g., S, or modified form thereof. In some embodiments, $\Phi_1$ is an amino acid residue that comprises an aliphatic side chain, e.g., I or L ,or an aromatic side chain, e.g., F, or modified form thereof. In some embodiments, $X_3$ is selected from a basic amino acid residue, e.g., R, or a hydrophobic amino acid residue, e.g., M or V, or modified form thereof. In some embodiments, $X_4$ is selected from a neutral/polar, especially a polar/large, amino acid residue, e.g., Q, a basic amino acid residue, e.g., R, a hydrophobic amino acid residue, especially one with an aromatic side chain, e.g, Y or an acidic amino acid residue, e.g., D, or modified form thereof. In some embodiments, $X_5$ is selected from a neutral/polar, especially a polar/large, amino acid residue, e.g., Q or N, a basic amino acid residue, e.g., H, or a small amino acid residue, e.g., A or P, or modified form thereof. In some embodiments, $X_6$ is selected from a basic amino acid residue, e.g., R, or a small amino acid residue, e.g., P, or modified form thereof. In some embodiments, $\Sigma_1$ is P, or modified form thereof. In some embodiments, $X_7$ is selected from a basic amino acid residue, e.g., K, or a neutral/polar, especially a polar/large, amino acid residue, e.g., N, or modified form thereof. In some embodiments, $X_8$ is

selected from a basic amino acid residue, e.g., K, or a small amino acid residue, e.g., A, or modified form thereof.

**[0062]** In some embodiments, $X_9$ is selected from a hydrophobic amino acid residue, especially one with an aromatic side chain, e.g, F, or a small amino acid residue, e.g., P, or modified form thereof. In some embodiments, $\Sigma_2$ is S, or modified form thereof. In some embodiments, $\Sigma_3$ is selected from G or T, or modified form thereof. In some embodiments, $X_{10}$ is selected from an acidic amino acid residue, e.g., E, or a small amino acid residue, e.g., S, or modified form thereof. In some embodiments, $X_{11}$ is A, or modified form thereof.

**[0063]** In certain embodiments, formula (III) is represented by:

**[0064]** $$\Pi_1\ \Psi_1\ \Phi_2\ \Phi_3\ \Pi_2\ \Pi_3\ X_{12}.\Sigma_4\ \Psi_2\ \Psi_3\ \Gamma \qquad \text{(V)}$$

**[0065]** wherein:

**[0066]** $\Pi_1$, $\Pi_2$ and $\Pi_3$ are independently selected from any neutral/polar amino acid residue, or modified form thereof;

**[0067]** $\Psi_1$ $\Psi_2$ and $\Psi_3$ are independently selected from any basic amino acid residue, or modified form thereof;

**[0068]** $\Phi_2$ and $\Phi_3$ are independently selected from any hydrophobic amino acid residue, or modified form thereof;

**[0069]** $X_{12}$ is selected from any amino acid residue, or modified form thereof;

**[0070]** $\Sigma_4$ is a small amino acid residue, or modified form thereof; and

**[0071]** $\Gamma$ is absent or is a peptide of 1-10 amino acid residues.

**[0072]** In some embodiments, $\Pi_1$ is a polar/large amino acid residue, e.g., N, or modified form thereof. In some embodiments, $\Psi_1$ is R, or modified form thereof. In some embodiments, $\Phi_2$ is an amino acid residue that comprises an aliphatic side chain, e.g., I or L ,or an aromatic side chain, e.g., F, or modified form thereof. In some embodiments, $\Phi_3$ is selected from M, Nle or V, or modified form thereof. In some embodiments, $\Pi_2$ is a polar/large amino acid residue, e.g., Q, or modified form thereof. In some embodiments, $\Pi_3$ is a polar/large amino acid residue, e.g., N, or modified form thereof. In some embodiments, $X_{12}$ is a small or basic amino acid residue, e.g., P or R, or modified form thereof. In some embodiments, $\Sigma_4$ is P, or modified form thereof. In some embodiments, $\Psi_2$ is K, or modified form thereof. In some embodiments, $\Psi_3$ is K, or modified form thereof.

**[0073]** In certain embodiments, $\Pi_1$ is N, or modified form thereof, $\Psi_1$ is R, or modified form thereof, $\Phi_2$ is I, L or F, or modified form thereof, $\Phi_3$ is M or V, or modified form thereof, $\Pi_2$ is Q, or modified form thereof, $\Pi_3$ is N, or modified form thereof, $X_{12}$ is selected from R or P, or

modified form thereof, $\Sigma_4$ is P, or modified form thereof, and each of $\Psi_2$ and $\Psi_3$ are K or modified form thereof.

**[0074]** In certain embodiments, $\Gamma$ is present and comprises the formula:

**[0075]** $$\Phi_4\,\Sigma_5\,\Sigma_6\,\Xi \qquad \text{(VI)}$$

**[0076]** wherein:

**[0077]** $\Phi_4$ is a hydrophobic amino acid residue, e.g., comprising an aromatic side chain such as but not limited to F;

**[0078]** $\Sigma_5$ is small amino acid residue, e.g., S, or modified form thereof;

**[0079]** $\Sigma_6$ is small amino acid residue, e.g., G, or modified form thereof; and

**[0080]** $\Xi$ is an acidic amino acid residue, e.g., E, or modified form thereof.

**[0081]** Suitably, formula (VI) is represented by the sequence FSGE [SEQ ID NO:14] or a variant or derivative thereof.

**[0082]** Illustrative examples of C-terminal portions (i.e., $\Omega_1$) include: NRIVQNRPKK [SEQ ID NO:15]; NRLVQNRPKK [SEQ ID NO:16]; NRIMQNPPKKFSGE [SEQ ID NO:17]; NRFMQNRPKKFSGE [SEQ ID NO:18]; NRILQNPPKKFSGE [SEQ ID NO:19]; NRPMNNPPKKFSGE [SEQ ID NO:20]; NRIVQNPPKKFSGE [SEQ ID NO:21]; NRIMQNRPKKFSGE [SEQ ID NO:22]; KRIMQNPPKKFSGE [SEQ ID NO:23]; NSIMQNPPKKFSGE [SEQ ID NO:24]; NRLMQNPPKKFSGE [SEQ ID NO:25]; NRFMQNPPKKFSGE [SEQ ID NO:26]; KRIVQNPPKKFSGE [SEQ ID NO:27]; NRIMQQPPKKFSGE [SEQ ID NO:28]; NRInQNPPKKFSGE [SEQ ID NO:29]; NRIMQAPPKKFSGE [SEQ ID NO:30]; NRLMQQPPKKFSGE [SEQ ID NO:31]; NRIMQAOPKKFSGE [SEQ ID NO:32]; NRIMQQOPKKFSGE [SEQ ID NO:33]; NRIMQNPPKKFSGE [SEQ ID NO:34]; NRInQAPPKKFSGE [SEQ ID NO:35]; NRVnQAPPKKFSGE [SEQ ID NO:36]; NRIMQNRPKKFSGE [SEQ ID NO:37]; NRVMQNPPKKFSGE [SEQ ID NO:38]; NRIMQQRPKKFSGE [SEQ ID NO:39]; KRIMQNPPKKFSGE [SEQ ID NO:40]; NRIMRQPPKKFSGE [SEQ ID NO:41]; NRVMRQPPKKFSGE [SEQ ID NO:42]; NRIMQNPPKKFSTE [SEQ ID NO:43]; PSLRDPRPNAPSTSA [SEQ ID NO:44]; PVLRDPRPNAPSTSA [SEQ ID NO:45]; NRLVQNPPKKFSGE [SEQ ID NO:46]; NRIMQNRPKKFSGE [SEQ ID NO:47]; and PRLRDPRPNAPSTSA [SEQ ID NO:48].

**[0083]** Suitably, $\Delta_1$ and $\Theta_1$ are independently selected from N-terminal portions and core structures, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP, VNP, PNP or an

*Oxyuranus* natriuretic peptide such as a natriuretic peptide from *Oxyuranus microlepidotus*, or their variants or derivatives.

**[0084]** In certain embodiments, $\Delta_1$ is present and is represented by the formula:

**[0085]** $$O\ \Sigma_1\ \vartheta\ X_1\ X_2\ X_3\ X_4\ X_5\ \Sigma_2 \qquad \textbf{(VII)}$$

**[0086]** wherein:

**[0087]** O is absent or is a peptide of 1-10 amino acid residues;

**[0088]** $\Sigma_{1-2}$ are independently selected from any small amino acid residue, or modified form thereof;

**[0089]** $\vartheta$ is selected from any charged amino acid residue, or modified form thereof; and

**[0090]** $X_{1-5}$ are independently selected from any amino acid residue, or modified form thereof.

**[0091]** In some embodiments, O is selected from a small amino acid residue, e.g., S, or modified form thereof, or a protecting moiety. In some embodiments, $\Sigma_1$ is selected from P or S, or modified form thereof. In some embodiments, $\vartheta$ is selected from an acidic amino acid residue, e.g., D, or a basic amino acid residue, e.g., K, or modified form thereof. In some embodiments, $X_1$ is selected from a hydrophobic amino acid residue, e.g., M, a small amino acid residue, e.g., P or S, or an acidic amino acid residue, e.g., E, or modified form thereof. In some embodiments, $X_2$ is selected from a basic amino acid residue, e.g., K, or a hydrophobic amino acid residue, e.g., V or L, or modified form thereof. In some embodiments, $X_3$ is selected from a hydrophobic amino acid residue, e.g., I, a neutral/polar, especially a polar/large, amino acid residue, e.g., Q, or a basic amino acid residue, e.g., R or K, or modified form thereof. In some embodiments, $X_4$ is selected from a small amino acid residue, e.g., G, a basic amino acid residue, e.g., R, or a hydrophobic amino acid residue, especially one with an aromatic side chain, e.g., Y, or modified form thereof. In some embodiments, $X_5$ is selected from a neutral/polar, especially a polar/large, amino acid residue, e.g., N or Q, a small amino acid residue, e.g., A or S, a basic amino acid residue, e.g., K, or an acidic amino acid residue, e.g., D, or modified form thereof. In some embodiments, $\Sigma_2$ is selected from P, S or G, or modified form thereof.

**[0092]** In certain embodiments, $\Sigma_1$ is S, or modified form thereof, $\vartheta$ is D, or modified form thereof, $X_1$ is selected a small amino acid residue, e.g., P or S, or modified form thereof, $X_2$ is K, or modified form thereof, $X_3$ is I, or modified form thereof, $X_4$ is G, or modified form thereof, $X_5$ is selected from D or N, or modified form thereof, and $\Sigma_2$ is G, or modified form thereof.

**[0093]** Illustrative examples of peptide sequences falling within the scope of formula (VII) include SDPKIGDG [SEQ ID NO:49], SDSKIGDG [SEQ ID NO:50], SDSKIGNG [SEQ ID NO:51],

SDSKIGQG [SEQ ID NO:52], SDSKIGAG [SEQ ID NO:53], SDSKIGKG [SEQ ID NO:54], SDSKISKG [SEQ ID NO:55], SDSKIGNG [SEQ ID NO:56], ZSDSKIGNG [SEQ ID NO:57], SDSKIGAG [SEQ ID NO:58], ZSDSKIGAG [SEQ ID NO:59], SPKMVQGSG [SEQ ID NO:60], SPKMVQGSG [SEQ ID NO:61], SDSKIGNG [SEQ ID NO:62], SPKMVQGSG [SEQ ID NO:63], SDSKIGNG [SEQ ID NO:64], SDSKIGNG [SEQ ID NO:65], SLRRSS [SEQ ID NO:66] and SDSKIGNG [SEQ ID NO:67].

**[0094]** In some embodiments, $\Theta_1$ is represented by the formula:

**[0095]** $$\mathbf{CFGX_1X_2\Phi_1DRIX_3X_4X_5SX_6LGC} \qquad \text{(VIII)}$$

**[0096]** wherein:

**[0097]** $X_{1\text{-}6}$ are each independently selected from any amino acid residue, or modified form thereof;

**[0098]** $\Phi_1$ is selected from a hydrophobic amino acid residue, or modified form thereof; and

**[0099]** the two C residues are connected by a bridging bond.

**[0100]** In some embodiments, $X_1$ is selected from a basic amino acid residue, e.g., H or R, a hydrophobic amino acid residue, e.g., F or L, a neutral/polar, especially a polar/large, amino acid residue, e.g., Q, or a small amino acid residue, e.g., G, or modified form thereof. In some embodiments, $X_2$ is selected from a small amino acid residue, e.g., P or a basic amino acid residue, e.g., R or K, or modified form thereof. In some embodiments, $\Phi_1$ is selected from M, L or I, or modified form thereof. In some embodiments $X_3$ is selected from a small amino acid residue, e.g., G or S, a neutral/polar, especially a polar/large, amino acid residue, e.g., N, or a basic amino acid residue, e.g., R, H or K, or modified form thereof. In some embodiments $X_4$ is selected from a small amino acid residue, e.g., A or S, or a basic amino acid residue, e.g., H, or modified form thereof. In some embodiments $X_5$ is selected from a small amino acid residue, e.g., S, a basic amino acid residue, e.g., H, a neutral/polar, especially a polar/large, amino acid residue, e.g., Q, or a hydrophobic amino acid residue, e.g., M or V, or modified form thereof. In some embodiments $X_6$ is selected from a small amino acid residue, e.g., G, a basic amino acid residue, e.g., R, or a neutral/polar, especially a polar/large, amino acid residue, e.g., N, or modified form thereof.

**[0101]** Illustrative examples of peptide sequences falling within the scope of formula (VIII) include: CFGFPLDRIGSVSGLGC [SEQ ID NO:68], CFGLPLDRIGSVSGLGC [SEQ ID NO:69], CFGIPLDRIGSVSGLGC [SEQ ID NO:70], CFGHPLDRIGSVSGLGC, [SEQ ID NO:71], CFGQPLDRIGSVSGLGC [SEQ ID NO:72], CFGRPLDRIGSVSGLGC [SEQ ID NO:73], CFGFKLDRIGSVSGLGC [SEQ ID NO:74], CFGFRLDRIGSVSGLGC [SEQ ID NO:75], CFGFPIDRIGSVSGLGC [SEQ ID NO:76], CFGFPMDRIGSVSGLGC [SEQ ID NO:74],

CFGFPLDRINSVSGLGC [SEQ ID NO:75], CFGFPLDRIGHVSGLGC [SEQ ID NO:76], CFGFPLDRIGAVSGLGC [SEQ ID NO:77], CFGFPLDRIGSSSGLGC[SEQ ID NO:78], CFGFPLDRIGSQSGLGC [SEQ ID NO:79], CFGFPLDRIGSVSNLGC [SEQ ID NO:80] and CFGFPLDRIGSVSRLGC [SEQ ID NO:81].

**[0102]** In other embodiments, the compounds generally comprise the formula:

**[0103]** $$\Delta_2\text{–}\Theta_2\text{–}\Omega_2 \qquad \textbf{(IX)}$$

**[0104]** wherein:

**[0105]** $\Delta_2$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of that portion;

**[0106]** $\Omega_2$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of that portion; and

**[0107]** $\Theta_2$ comprises an amino acid sequence having the formula:

**[0108]** $$\textbf{CFG}\Phi_1\textbf{PLDRIX}_1\textbf{SVSGLGC} \qquad \textbf{(X)}$$

**[0109]** wherein:

**[0110]** $\Phi_1$ is selected from any hydrophobic amino acid residue, or modified form thereof.; and

**[0111]** $X_1$ is selected from any small or a basic amino acid residue, or modified form thereof.

**[0112]** In some embodiments, $\Phi_1$ is selected from an amino acid residue having an aromatic side chain, e.g., F, or one having an aliphatic side chain, e.g., L, or modified form thereof.

**[0113]** In some embodiments, $X_1$ is selected from G, S, or a basic residue such as R or K, or modified form thereof.

**[0114]** Suitably, $\Delta_2$ and $\Omega_2$ are independently selected from N- and C-terminal portions, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP or an *Oxyuranus* natriuretic peptide such as a natriuretic peptide from *Oxyuranus microlepidotus*, or their variants or derivatives.

**[00100]** In still other embodiments, the compounds generally comprise the formula:

**[0115]** $$\Delta_3\text{–}\Theta_3\text{–}\Omega_3 \qquad \textbf{(XI)}$$

**[0116]** wherein:

**[0117]** $\Theta_3$ is a core ring structure of a natriuretic peptide or a variant or derivative of the structure;

**[0118]** $\Omega_3$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of the portion; and

**[0119]** $\Delta_3$ comprises an amino acid sequence having the formula:

**[0120]** $$\mathbf{SD\Sigma_1KGX_1G} \quad \text{(XII)}$$

**[0121]** wherein:

**[0122]** $\Sigma_1$ is selected from any small amino acid residue, or modified form thereof; and

**[0123]** $X_1$ is selected from any amino acid residue, or modified form thereof.

**[0124]** In some embodiments, $\Sigma_1$ is S or P, or modified form thereof. In some embodiments, $X_1$ is an acidic amino acid residue, e.g., D, or a neutral polar, especially polar/large, amino acid residue, e.g., N, or modified form thereof.

**[0125]** Suitably, $\Theta_3$ and $\Omega_3$ are independently selected from core structures and C-terminal portions, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP or an *Oxyuranus* natriuretic peptide such as a natriuretic peptide from *Oxyuranus microlepidotus*, or their variants or derivatives.

**[0126]** If desired, the proteinaceous compounds broadly described above are provided in the form of pharmaceutically acceptable salts.

**[0127]** Yet another aspect of the present invention provides isolated proteinaceous compounds having at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals, wherein the compounds have the formula:

**[0128]** $$\mathbf{Z_1SD\Sigma_1KIGX_1GCFG\Phi_1PLDB_1IX_2SVSGLGCNRX_3\Phi_2QNX_4PKKZ_2} \quad \text{(XIII)}$$

**[0129]** wherein:

**[0130]** $Z_1$ is absent or is a peptide from about 1 to about 50 amino acids or is a protecting moiety, (e.g., an N-terminal blocking residue such as pyroglutamate);

**[0131]** $\Sigma_1$ is a small amino acid residue, or modified form thereof;

**[0132]** $X_1$ is an acidic or neutral/polar amino acid residue, or modified form thereof;

**[0133]** $\Phi_1$ is a hydrophobic amino acid residue, or modified form thereof;

**[0134]** $B_1$ is a basic amino acid residue, or modified form thereof;

**[0135]** $X_2$ is a basic or small amino acid residue, or modified form thereof;

**[0136]** $X_3$ is a small or hydrophobic amino acid residue, or modified form thereof;

**[0137]** $\Phi_2$ is a hydrophobic amino acid residue, or modified form thereof;

**[0138]** $X_4$ is a basic or small amino acid residue, or modified form thereof; and

**[0139]** $Z_2$ is absent or is a peptide of from about 1 to about 10 amino acids,

**[0140]** the two C residues are connected by a bridging bond,

**[0141]** or a pharmaceutically acceptable salt thereof.

**[0142]** In some embodiments, $\Sigma_1$ is selected from P or S, or modified form thereof. In some embodiments, $X_1$ is selected from Q, K, A, S, D or N, or modified form thereof. In some embodiments, $\Phi_1$ is selected from L or F, or modified form thereof. In some embodiments, $B_1$ is selected from H, K or R, or modified form thereof. In some embodiments, $X_2$ is selected from G, S, R or K or modified form thereof. In some embodiments, $X_3$ is selected from P or I, or modified form thereof. In some embodiments, $\Phi_2$ is selected from V or M, or modified form thereof. In some embodiments, $X_4$ is selected from R or P, or modified form thereof. In some embodiments, the sequence Asn-Pro is replaced by a sequence selected from Ala-Pro, Gln-Pro, Ala-Hyp or Gln-Hyp. In some embodiments, the sequence Asn-Gly is replaced by a sequence selected from Gln-Gly or Ala-Gly. In some embodiments the Met residue is replaced by Nor or Val. In some embodiments, one or both of the C residues are replaced by selenocysteine. In some embodiments the N-terminus is blocked by pGlu, Pro, Hyp or any N-acetylated residue. Alternatively, or in addition,, the C-terminus is blocked by amidation or by other means, or the compounds are cyclised (e.g., end to end).

**[0143]** When the peptide $Z_2$ is present, it suitably comprises the sequence FSGE [SEQ ID NO:14] or a variant or derivative thereof.

**[0144]** Generally, the bridging bond that connects the two C residues is selected from the group consisting of a disulphide bond, a diselenide or mixed sulphide-selenide bond, a methylene bond, a sulphide/methylene bond, an amide bond and an ester bond. In specific embodiments, the bridging bond is a disulphide bond.

**[0145]** In some embodiments, the compounds comprise any one of the sequences set forth in SEQ ID NO: 90, 91, 94-114, 117, 118, 120, 123, 124, 126-130, 132-134, 150, 154, 160, 166-169, 173, 176 and 178-192 (see Table 2)

**[0146]** Still other aspects of the present invention provide isolated polynucleotides that comprise a nucleotide sequence encoding a compound as broadly described above, and vectors that comprise such polynucleotides optionally operably connected to a regulatory sequence, as well as host cells containing such vectors. In a related aspect, the invention provides processes for producing a compound as broadly described above, including a process that comprises culturing a host cell containing a vector under conditions sufficient to produce a compound of the invention from a polynucleotide as broadly described above and isolating the compound.

[0147] In a further aspect, the invention provides antigen-binding molecules that are immuno-interactive with a compound as broadly described above. The molecules are useful in screening expression libraries for compounds of the invention as well as isolating or detecting such compounds.

[0148] In still another aspect, the present invention provides a pharmaceutical composition comprising a compound as broadly described above, and a pharmaceutically acceptable carrier, excipient and/or diluent. These compositions and the aforementioned compounds are useful, either singly or in combination, for inducing, stimulating or otherwise promoting at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals. Accordingly, in a further aspect, the invention provides methods for inducing, stimulating or otherwise promoting at least one of these activities in a subject, wherein the methods generally comprise administering to the subject an effective amount of a compound or composition as broadly described above.

[0149] Advantageously, these activities are useful for treating or preventing a variety of conditions including heart failure, nephrotic syndrome, cirrhosis of the liver, hypertension, kidney failure and other oedematous states, bacterial infections, weight loss, asthma, inflammatory-related diseases, erectile dysfunction, hypercholesterolemia, skeletal dysplasias and as a protectant for toxicity of anti-cancer drugs. Accordingly, in still a further aspect, the invention provides methods for treating or preventing at least one of these conditions in a subject, wherein the methods generally comprise administering to the subject an effective amount of a compound or composition as broadly described above.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0150]** Figure 1 is a graphical representation showing an LC-MS profile of <10 kDa centrifuge fraction of *Oxyuranus microlepidotus* (Inland taipan) venom. Mass in Daltons is given above each major peak. On-line LC-MS of venoms was performed on a Vydac C18 analytical column (2.1 x 35 mm, 5μ particle size, 300 Å) with Solvent A (0.05% TFA, $H_2O$) and Solvent B (90% acetonitrile in 0.045% TFA) at a turbospray flow rate of 130 μL/min. The solvent delivery and gradient formation was achieved using an Applied Biosystems 140 B solvent delivery system. The variable gradient was 0-20% in the first two minutes and then 20-45% over the next 12 minutes followed by 45-80% over the next minute. Electrospray mass spectra were acquired on a PE-SCIEX triple quadrupole mass spectrometer that was equipped with an Ionspray atmospheric pressure ionisation source. Samples (10 μL) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data was acquired at an orifice potential of 80V over the mass range 400-2100 Da with a step size of 0.2 amu.

**[0151]** Figure 2 is a diagrammatic representation showing a comparison of the isolated *Oxyuranus microlepidotus* natriuretic peptides with the invariant residues of the natriuretic peptide family and previously isolated venom natriuretic peptides. Many other sequences predicted from the full gene sequences are available on Swissprot.

**[0152]** Figure 3 is a graphical representation showing an ESI-TOF MS and RP-HPLC profile of synthetic TNPa, TNPb and TNPc. Reconstructured Mass in Daltons is given. RP-HPLC was performed on a C18 phenomenex 4.6x250mm analytical column, with a 1%B /min linear gradient.

**[0153]** Figure 4 is a graphical representation showing comparative effects of venom derived natriuretic peptides(0.1 μM) on rat (A) endothelium-intact aortae and (B) endothelium-denuded aortae pre-contracted with 40 mM KCl (N = 5). (C) 0.1 μM comparative cCMP production in 293 cells over-expressing GC-A. N = 3.

**[0154]** Figure 5 is a graphical representation showing that TNPa and TNPb lack the ability to competitively inhibit the binding of TNPc to either the endothelium intact or endothelium denuded aortic rings. TNPa (10 nM; t = 0) followed by TNPc (0.1 μM; t = 42) on (A) endothelium-denuded and (B) endothelium-intact aortae pre-contracted with 40 mM KCl. TNPb (0.1 μM; t = 0) followed by TNPc (0.1 μM) on (C) endothelium-denuded (t = 5) and (D) endothelium-intact aortae pre-contracted with 40 mM KCl (t = 27). N = 3. Note that TNPc is able to cause complete inhibition of contraction, while 10-fold higher doses of TNPa and TNPb cause only partial or no inhibition of contraction.

**[0155]** Figure 6 is a graphical representation showing the comparative stability of (a) TNPc and Wt-hANP in human and rat plasma; (b) TNPc, BNP, ANP and DNP against NEP enzyme; and (c) TNPc, DNP, ANP and BNP against plasmin enzyme.

## DETAILED DESCRIPTION OF THE INVENTION

### *1. Definitions*

**[0156]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

**[0157]** The articles "*a*" and "*an*" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

**[0158]** By "*antigen-binding molecule*" is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

**[0159]** By "*biologically active portion*" is meant a portion of a full-length parent peptide or polypeptide which portion retains an activity of the parent compound. For example, a biologically active portion of a natriuretic peptide of the invention will retain at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in mammal. As used herein, the term "*biologically active portion*" includes deletion mutants and small peptides, for example of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 contiguous amino acids, which comprise an activity of a parent compound. Portions of this type may be obtained through the application of standard recombinant nucleic acid techniques or synthesised using conventional liquid or solid phase synthesis techniques. For example, reference may be made to solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications. Alternatively, peptides can be produced by digestion of a peptide or polypeptide of the invention with proteinases such as endoLys-C, endoArg-C, endoGlu-C and staphylococcus V8-protease. The digested fragments can be purified by, for example, high performance liquid chromatographic (HPLC) techniques. Recombinant nucleic acid techniques can also be used to produce such portions.

**[0160]** The term "*biological sample*" as used herein refers to a sample that may be extracted, untreated, treated, diluted or concentrated from an animal. The biological sample may include a biological fluid such as whole blood, serum, plasma, saliva, urine, sweat, ascitic fluid, peritoneal fluid, synovial fluid, amniotic fluid, cerebrospinal fluid, tissue biopsy, and the like. In certain embodiments, the biological sample is blood, especially peripheral blood.

**[0161]** Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

**[0162]** By "*corresponds to*" or "*corresponding to*" is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein. This phrase also includes within its scope a peptide or polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference peptide or protein.

**[0163]** As used herein, the terms "*culturing*," "*culture*" and the like refer to the set of procedures used *in vitro* where a population of cells (or a single cell) is incubated under conditions that have been shown to support the growth or maintenance of the cells *in vitro*. The art recognises a wide number of formats, media, temperature ranges, gas concentrations etc., which need to be defined in a culture system. The parameters will vary based on the format selected and the specific needs of the individual who practices the methods herein disclosed. However, it is recognised that the determination of culture parameters is routine in nature.

**[0164]** By "*derivative*" is meant a peptide or polypeptide that has been derived from the basic sequence by modification, for example by conjugation or complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art.

**[0165]** By "*effective amount*", in the context of modulating an activity or of treating or preventing a condition is meant the administration of that amount of active to an individual in need of such modulation, treatment or prophylaxis, either in a single dose or as part of a series, that is effective for modulation of that effect or for treatment or prophylaxis of that condition. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

**[0166]** By "*expression vector*" is meant any autonomous genetic element capable of directing the transcription of a polynucleotide contained within the vector and suitably the synthesis of a peptide or polypeptide encoded by the polynucleotide. Such expression vectors are known to practitioners in the art.

**[0167]** The term "*gene*" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. The gene is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-

coding nucleotide sequences involved in the regulation of expression. In this regard, the gene may further comprise control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals. The DNA sequences may be cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

**[0168]** "*Hybridisation*" is used herein to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA U pairs with A and C pairs with G. In this regard, the terms "match" and "mismatch" as used herein refer to the hybridisation potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridise efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides that do not hybridise efficiently.

**[0169]** Reference herein to "*immuno-interactive*" includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the molecules is, or mimics, a component of the immune system.

**[0170]** By "*isolated*" is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an "isolated polynucleotide", as used herein, refers to a polynucleotide, which has been purified from the sequences which flank it in a naturally-occurring state, e.g., a DNA fragment which has been removed from the sequences that are normally adjacent to the fragment. Alternatively, an "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, i.e., it is not associated with *in vivo* substances.

**[0171]** By "*marker gene*" is meant a gene that imparts a distinct phenotype to cells expressing the marker gene and thus allows such transformed cells to be distinguished from cells that do not have the marker. A selectable marker gene confers a trait for which one can 'select' based on resistance to a selective agent (*e.g.*, a herbicide, antibiotic, radiation, heat, or other treatment damaging to untransformed cells). A screenable marker gene (or reporter gene) confers a trait that one can identify through observation or testing, *i.e.*, by 'screening' (*e.g.* β-glucuronidase, luciferase, or other enzyme activity not present in untransformed cells).

**[0172]** As used herein, a "*naturally-occurring*" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature. For example a naturally-occurring nucleic acid molecule can encode a natural protein.

**[0173]** The term "*oligonucleotide*" as used herein refers to a polymer composed of a multiplicity of nucleotide residues (deoxyribonucleotides or ribonucleotides, or related structural

variants or synthetic analogues thereof, including nucleotides with modified or substituted sugar groups and the like) linked *via* phosphodiester bonds (or related structural variants or synthetic analogues thereof). Thus, while the term "oligonucleotide" typically refers to a nucleotide polymer in which the nucleotide residues and linkages between them are naturally-occurring, it will be understood that the term also includes within its scope various analogues including, but not restricted to, peptide nucleic acids (PNAs), phosphorothioate, phosphorodithioate, phophoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoroamidate, methyl phosphonates, 2-O-methyl ribonucleic acids, and the like. The exact size of the molecule can vary depending on the particular application. Oligonucleotides are a polynucleotide subset with 200 bases or fewer in length. Preferably, oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g., for probes; although oligonucleotides may be double stranded, e.g., for use in the construction of a variant nucleic acid sequence. Oligonucleotides of the invention can be either sense or antisense oligonucleotides.

**[0174]** The term "*operably connected*" or "*operably linked*" as used herein means placing a structural gene under the regulatory control of a promoter, which then controls the transcription and optionally translation of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the genetic sequence or promoter at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting; i.e. the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting; i.e. the genes from which it is derived.

**[0175]** By "*pharmaceutically acceptable carrier*" is meant a solid or liquid filler, diluent or encapsulating substance that can be safely used in topical or systemic administration to a mammal.

**[0176]** The term "*polynucleotide*" or "*nucleic acid*" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

**[0177]** "*Polypeptide*," "*peptide*" and "*protein*" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally-occurring amino acid, such as a chemical analogue of a corresponding naturally-occurring amino acid, as well as to naturally-occurring amino acid polymers.

**[0178]** By "*primer*" is meant an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerising agent. The primer is preferably single-stranded for maximum efficiency in amplification but can alternatively be double-stranded. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerisation agent. The length of the primer depends on many factors, including application, temperature to be employed, template reaction conditions, other reagents, and source of primers. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15 to 35 or more nucleotide residues, although it can contain fewer nucleotide residues. Primers can be large polynucleotides, such as from about 35 nucleotides to several kilobases or more. Primers can be selected to be "substantially complementary" to the sequence on the template to which it is designed to hybridise and serve as a site for the initiation of synthesis. By "substantially complementary", it is meant that the primer is sufficiently complementary to hybridise with a target polynucleotide. Preferably, the primer contains no mismatches with the template to which it is designed to hybridise but this is not essential. For example, non-complementary nucleotide residues can be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary nucleotide residues or a stretch of non-complementary nucleotide residues can be interspersed into a primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridise therewith and thereby form a template for synthesis of the extension product of the primer.

**[0179]** "*Probe*" refers to a molecule that binds to a specific sequence or sub-sequence or other moiety of another molecule. Unless otherwise indicated, the term "probe" typically refers to a polynucleotide probe that binds to another polynucleotide, often called the "target polynucleotide", through complementary base pairing. Probes can bind target polynucleotides lacking complete sequence complementarity with the probe, depending on the stringency of the hybridisation conditions. Probes can be labelled directly or indirectly.

**[0180]** The term "*recombinant polynucleotide*" as used herein refers to a polynucleotide formed *in vitro* by the manipulation of nucleic acid into a form not normally found in nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleotide sequence.

**[0181]** By "*recombinant polypeptide*" is meant a polypeptide made using recombinant techniques, i.e., through the expression of a recombinant or synthetic polynucleotide.

**[0182]** By "*regulatory sequence*" is meant nucleic acid sequences (e.g., DNA) necessary for expression of an operably linked coding sequence in a particular host cell. The regulatory sequences that are suitable for prokaryotic cells for example, include a promoter, and optionally a *cis*-acting sequence such as an operator sequence and a ribosome binding site. Control sequences that are

suitable for eukaryotic cells include promoters, polyadenylation signals, transcriptional enhancers, translational enhancers, leader or trailing sequences that modulate mRNA stability, as well as targeting sequences that target a product encoded by a transcribed polynucleotide to an intracellular compartment within a cell or to the extracellular environment.

**[0183]** The term "*sequence identity*" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "*percentage of sequence identity*" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "*sequence identity*" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software.

**[0184]** "*Similarity*" refers to the percentage number of amino acids that are identical or constitute conservative substitutions as defined in Table 5 *infra*. Similarity may be determined using sequence comparison programs such as GAP (Deveraux *et al.* 1984, *Nucleic Acids Research* **12**, 387-395). In this way, sequences of a similar or substantially different length to those cited herein might be compared by insertion of gaps into the alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

**[0185]** Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". A "*reference sequence*" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e., only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "*comparison window*" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (i.e., gaps) of

about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e., resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, *Nucl. Acids Res.* **25**:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15.

**[0186]** The term "*subject*" refers to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, primates, avians, livestock animals (*e.g.*, sheep, cows, horses, donkeys, pigs), laboratory test animals (*e.g.*, rabbits, mice, rats, guinea pigs, hamsters), companion animals (*e.g.*, cats, dogs) and captive wild animals (*e.g.*, foxes, deer, dingoes). A preferred subject is a human in need of treatment or prophylaxis of a disease or condition, as defined herein. However, it will be understood that the aforementioned terms do not imply that symptoms are present.

**[0187]** As used herein, the term "*substantially free*" means that a preparation of a natriuretic peptide of the invention is at least 10% pure. In certain embodiments, the preparation of natriuretic peptide has less than about 30%, 20%, 10% and preferably less than about 5% (by dry weight), of non-natriuretic peptide (also referred to herein as a "contaminating protein"), or of chemical precursors or non- natriuretic peptide chemicals. When the natriuretic peptide or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and more preferably less than about 5% of the volume of the protein preparation. The invention includes isolated or purified preparations of at least 0.01, 0.1, 1.0, and 10 milligrams in dry weight.

**[0188]** By "*substantially purified*" is meant a compound that has been separated from components that naturally accompany it. Typically, a compound is substantially pure when at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., by chromatography or HPLC analysis. For those compounds prepared by synthetic procedures or derivatization of a naturally-occurring compound, "*substantially purified*" refers to a compound that has been separated from the reagents and solvents used in the synthetic procedure.

**[0189]** The term "*transformation*" means alteration of the genotype of an organism, for example a bacterium, yeast or plant, by the introduction of a foreign or endogenous nucleic acid.

**[0190]** The term "*variant*" refers to polypeptides in which one or more amino acids have been replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions) as described hereinafter. These terms also encompass polypeptides in which one or more amino acids have been added or deleted, or replaced with different amino acids.

**[0191]** By "*vector*" is meant a polynucleotide molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, yeast or virus, into which a polynucleotide can be inserted or cloned. A vector preferably contains one or more unique restriction sites and can be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector can be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector can contain any means for assuring self-replication. Alternatively, the vector can be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. A vector system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. In the present case, the vector is preferably a viral or viral-derived vector, which is operably functional in animal and preferably mammalian cells. Such vector may be derived from a poxvirus, an adenovirus or yeast. The vector can also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are known to those of skill in the art.

### *2. Proteinaceous compounds of the invention*

**[0192]** The present invention is based, in part, on the isolation and characterisation of novel natriuretic peptides from *Oxyuranus microlepidotus* (Inland taipan), reputedly the world's most venomous snake. These peptides, which are also referred to herein as TNPa, TNPb and TNPc, share similarity within the 17-membered ring structure, which contains a natriuretic signature consensus sequence, with known natriuretic peptides at the amino acid level (see Figure 2 and compare Tables 1 and 2) and are proposed, therefore to have at least one activity selected from natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing and/or bone growth plate size-increasing activities, which are known activities of natriuretic peptides. For example, the ability of TNPc to reduce blood

pressure without increasing heart rate, as demonstrated in the Examples *infra*, provides evidence that this peptide and its variants are useful in the treatment of congestive heart failure.

**[0193]** The natriuretic peptide TNPc disclosed herein, whose amino acid sequences is set forth in SEQ ID NO:90, as well as its fragments, variants and derivatives, illustrative examples of which are set forth in SEQ ID NO: 1-7, 15-81 *supra* and in SEQ ID NO: 91, 94-114, 117, 118, 120, 123, 124, 126-130, 132-134, 150, 154, 160, 166-169, 173, 176 and 178-182 (Table 2) and are collectively referred to as "compounds, peptides or natriuretic peptides of the invention" or as "*Oxyuranus* natriuretic peptides" or as "TNPs." Nucleic acid molecules encoding such molecules are collectively referred to as "nucleic acids of the invention" or as "*Oxyuranus* nucleic acids." Thus, in accordance with the present invention, proteinaceous compounds comprising at least one of these activities are provided, comprising at least a biologically active portion of the *Oxyuranus* natriuretic peptide TNPc, or a variant or derivative thereof.

**[0194]** The sequence of amino acid residues of these compounds, and preferred embodiments thereof, are defined in terms of amino acids of certain characteristics or subclasses. Amino acid residues can be generally sub-classified into major subclasses as follows:

**[0195]** Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH. Amino acids having an acidic side chain include glutamic acid and aspartic acid.

**[0196]** Basic: The residue has a positive charge due to association with H ion at physiological pH or within one or two pH units thereof (e.g., histidine) and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH. Amino acids having a basic side chain include arginine, lysine and histidine.

**[0197]** Charged: The residues are charged at physiological pH and, therefore, include amino acids having acidic or basic side chains (i.e., glutamic acid, aspartic acid, arginine, lysine and histidine).

**[0198]** Hydrophobic: The residues are not charged at physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a hydrophobic side chain include tyrosine, valine, isoleucine, leucine, methionine, phenylalanine and tryptophan.

**[0199]** Neutral/polar: The residues are not charged at physiological pH, but the residue is not sufficiently repelled by aqueous solutions so that it would seek inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a neutral/polar side chain include asparagine, glutamine, cysteine, histidine, serine and threonine.

**[0200]** This description also characterises certain amino acids as "small" since their side chains are not sufficiently large, even if polar groups are lacking, to confer hydrophobicity. With the exception of proline, "small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not. Amino acids having a small side chain include glycine, serine, alanine and threonine. The gene-encoded secondary amino acid proline is a special case due to its known effects on the secondary conformation of peptide chains. The structure of proline differs from all the other naturally-occurring amino acids in that its side chain is bonded to the nitrogen of the α-amino group, as well as the α-carbon. Several amino acid similarity matrices (e.g., PAM120 matrix and PAM250 matrix as disclosed for example by Dayhoff *et al.* (1978) A model of evolutionary change in proteins. Matrices for determining distance relationships *In* M. O. Dayhoff, (ed.), Atlas of protein sequence and structure, Vol. 5, pp. 345-358, National Biomedical Research Foundation, Washington DC; and by Gonnet *et al.*, 1992, *Science* **256**(5062): 144301445), however, include proline in the same group as glycine, serine, alanine and threonine. Accordingly, for the purposes of the present invention, proline is classified as a "small" amino acid.

**[0201]** The degree of attraction or repulsion required for classification as polar or nonpolar is arbitrary and, therefore, amino acids specifically contemplated by the invention have been classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behaviour.

**[0202]** Amino acid residues can be further sub-classified as cyclic or noncyclic, and aromatic or nonaromatic, self-explanatory classifications with respect to the side-chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon, provided an additional polar substituent is present; three or less if not. Small residues are, of course, always nonaromatic. Dependent on their structural properties, amino acid residues may fall in two or more classes.

**[0203]** For the naturally-occurring protein amino acids, sub-classification according to the foregoing scheme is presented in Table 3.

**[0204]** The amino acids in the proteinaceous compounds of the invention may be those encoded by genes or their analogues or D-isomers thereof. Compounds within the scope of the present invention can be obtained by modifying the disclosed formulae in numerous ways, while preserving the activity of the natriuretic compounds thus obtained. For example, while the amino acids of these compounds are normally in the natural L optical isomer form, one or more, usually two or less and preferably one amino acid may be replaced with the optical isomer D form, or a D,L-racemic mixture can be provided in the molecules comprising the peptide compound. In one embodiment, the compounds of the invention are in a form wherein all of the residues are in the D-configuration thus conferring resistance to protease activity while retaining at least one activity associated with parent

compounds. The resulting molecules are themselves enantiomers of the native L-amino acid-containing forms.

**[0205]** The nomenclature used to describe proteinaceous molecules of the present invention follows the conventional practice wherein the amino group is presented to the left and the carboxy group to the right of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- (N-) and carboxy- (C-) terminal groups, although not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by a single letter designation, corresponding to the trivial name of the amino acid, in accordance with the Table 4, in which the three-letter designations for each residue is also shown:

**[0206]** "Modified" amino acids that may be included in the compounds of the invention can be gene-encoded amino acids which have been processed after translation of the gene, e.g., by the addition of methyl groups or derivatization through covalent linkage to other substituents or oxidation or reduction or other covalent modification. The classification into which the resulting modified amino acid falls will be determined by the characteristics of the modified form. For example, if lysine were modified by acylating the ε-amino group, the modified form would not be classed as basic but as polar/large.

**[0207]** Certain commonly encountered amino acids, which are not encoded by the genetic code, include, for example, β-alanine (β-Ala), or other omega-amino acids, such as 3-aminopropionic, 2,3-diaminopropionic (2,3-diaP), 4-aminobutyric and so forth, α-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine (2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β-2-thienylalanine (Thi); methionine sulfoxide (MSO); and homoarginirie (Har). These also fall conveniently into particular categories.

**[0208]** Based on the above definitions, Sar, β-Ala and Aib are small; t-BuA, t-BuG, N-MeIle, Nle, Mvl, Cha, Phg, Nal, Thi and Tic are hydrophobic; 2,3-diaP, Orn and Har are basic; Cit, Acetyl Lys and MSO are neutral/polar/large. The various omega-amino acids are classified according to size as small (β-Ala and 3-aminopropionic) or as large and hydrophobic (all others).

**[0209]** Other amino acid substitutions for those encoded in the gene can also be included in proteinaceous molecules within the scope of the invention and can be classified within this general scheme according to their structure.

**[0210]** In the compounds of the invention, one or more amide linkages (–CO–NH–) may optionally be replaced with another linkage which is an isostere such as $–CH_2NH–$, $–CH_2S–$, $–CH_2CH_2–$, –CH=CH– (*cis* and *trans*), $–COCH_2–$, $–CH(OH)CH_2–$ and $–CH_2SO–$. This replacement can

be made by methods known in the art. The following references describe preparation of peptide analogues which include these alternative-linking moieties: Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Spatola, A. F., in "Chemistry and Biochemistry of Amino Acids Peptides and Proteins", B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J. S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D., *et al.*, *Int J Pept Prot Res* (1979) **14**:177-185 (–$CH_2NH$–, –$CH_2CH_2$–); Spatola, A. F., *et al.*, *Life Sci* (1986) **38**:1243-1249 (–$CH_2$–S); Hann, M. M., *J Chem Soc Perkin Trans I* (1982) 307-314 (–CH–CH–, cis and trans); Almiquist, R. G., *et al.*, *J Med Chem* (1980) **23**:1392-1398 (–$COCH_2$–); Jennings-White, C., *et al.*, *Tetrahedron Lett* (1982) **23**:2533 (–$COCH_2$–); Szelke, M., *et al.*, European Application EP 45665 (1982) CA:**97**:39405 (1982) (–$CH(OH)CH_2$–); Holladay, M. W., *et al.*, *Tetrahedron Lett* (1983) **24**:4401-4404 (–$C(OH)CH_2$–); and Hruby, V. J., *Life Sci* (1982) **31**: 189-199 (–$CH_2$–S–).

**[0211]** Amino acid residues contained within the compounds, and particularly at the carboxy- or amino-terminus, can also be modified by amidation, acetylation or substitution with other chemical groups which can, for example, change the solubility of the compounds without affecting their activity. Also, the protracted profile of the compounds can be improved by attaching a lipophilic substituent to any one or more amino acid residues of a natriuretic peptide of the invention, as for example described by Huusfeldt *et al.* in International Publication WO 98/45329.

**[0212]** It is well known that amide modified analogues of natriuretic peptides are particularly potent and are thus contemplated by the present invention. For example, the carboxy-terminal residue will have a carbonyl carbon which has been substituted with an amino group to form a carboxy-terminal amido group. In general, the nitrogen atom of the amido group, covalently bound to the carbonyl carbon, will be of the general formula –NR'R", where R' and R" are substituent groups. Each substituent group can independently be hydrogen, or an organic group such as alkyl, straight chain or branched of from one to ten, usually one to six carbon atoms, including groups having substitutions of three or less nitrogen, oxygen or sulfur atoms as amido, thio or oxy, or a benzylic group (substituted or unsubstituted), and any one of which can be a nitrogen containing moiety such as hydrazide and the other can be hydrogen, or either group can be a basic or neutral dipeptide and the other can be hydrogen or an alkyl group. Representative of such amido groups are: –$NH_2$, –$NHCH_3$, –$N(CH_3)_2$ and –$NHCH_2CH_3$, among others.

**[0213]** Exemplary compounds of the present invention include sequences of any naturally-occurring natriuretic peptide, obtainable from a reptilian species, especially a species of snake and more especially a species of *Oxyuranus* or related species, provided that they include, or are modified to include, R at position 16, a hydrophobic amino acid residue, e.g., I, L or F at position 28, and/or a small amino acid residue or basic amino acid residue, e.g., P or R, at position 32, relative to the TNPc amino acid sequence. For example, naturally occurring natriuretic peptides may be isolated from

*Oxyuranus microlepidotus* (Inland Taipan), *Oxyuranus scutellatus* (Mainland or Coastal Taipan), *Oxyuranus scutellatus barringeri* subsp. Nov (Northwest Taipan) and *Oxyuranus scutellatus canni* (New Guinea Taipan).

**[0214]** Alternatively, a natriuretic peptide of the invention, or its fragments, can differ from the corresponding TNPc sequence. In one embodiment, it differs by at least one but by less than 15, 10, 8, 6, 5, 4, 3, 2 or 1 amino acid residues. In another, it differs from the corresponding TNPc sequence by at least one residue but less than 20%, 15%, 10% or 5% of the residues in it differ from the corresponding TNPc sequence. (If this comparison requires alignment the sequences should be aligned for maximum similarity. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.) The differences are, preferably, differences or changes at a non-essential residue or a conservative substitution.

**[0215]** A "*non-essential*" amino acid residue is a residue that can be altered from the wild-type sequence of an *Oxyuranus* natriuretic peptide without abolishing or substantially altering an activity of that peptide. Preferably, the alteration does not substantially alter the *Oxyuranus* natriuretic peptide activity, e.g., the activity is at least 20%, 40%, 60%, 70% or 80% of wild-type. An "*essential*" amino acid residue is a residue that, when altered from the wild-type sequence of an *Oxyuranus* natriuretic peptide, results in abolishing an *Oxyuranus* natriuretic peptide activity such that less than 20% of the wild-type activity is present. For example, conserved amino acid residues shared between the natriuretic peptides of the invention, e.g., those shown in Figure 2, are predicted to be particularly unamenable to alteration.

**[0216]** A "*conservative amino acid substitution*" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art and certain subclasses are described in Table 4. Preferred variant peptides are those having conserved amino acid substitutions. Examples of conservative substitutions include the following: aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; serine/ glycine/alanine/threonine as small amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids. Conservative amino acid substitution also includes groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the properties of

the resulting variant polypeptide. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the peptide variant. Conservative substitutions are shown in the Table 5 below under the heading of exemplary substitutions. More preferred substitutions are under the heading of preferred substitutions. Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. After the substitutions are introduced, the variants are screened for biological activity.

**[0217]** Alternatively, similar amino acids for making conservative substitutions can be grouped into three categories based on the identity of the side chains. The first group includes glutamic acid, aspartic acid, arginine, lysine, histidine, which all have charged side chains; the second group includes glycine, serine, threonine, cysteine, tyrosine, glutamine, asparagine; and the third group includes leucine, isoleucine, valine, alanine, proline, phenylalanine, tryptophan, methionine, as described in Zubay, G., *Biochemistry*, third edition, Wm.C. Brown Publishers (1993).

**[0218]** Thus, a predicted non-essential amino acid residue in an *Oxyuranus* natriuretic peptide is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of an *Oxyuranus* natriuretic peptide coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for *Oxyuranus* natriuretic peptide biological activity to identify mutants that retain activity. Following mutagenesis of such coding sequences, the encoded peptide can be expressed recombinantly and the activity of the peptide can be determined. Other methods for finding suitable NP mutants include phage display such as described, for example, in U.S. Patent No. 6,028,055.

**[0219]** Other embodiments include a peptide that contains one or more changes in amino acid sequence, e.g., a change in an amino acid residue which is not essential for activity. Such natriuretic peptides differ in amino acid sequence from TNPc, yet retain biological activity.

**[0220]** In other embodiments, the *Oxyuranus* natriuretic peptide includes an amino acid sequence having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94% 95%, 96%, 97%, 98% or more similarity to a corresponding sequence of TNPc, and has an *Oxyuranus* natriuretic peptide biological activity.

**[0221]** The natriuretic peptides of the invention contain a significant number of structural characteristics in common with each other as for example depicted in Figure 2 and Table 1. The term "family" when referring to the protein and nucleic acid molecules of the invention means two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient

amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-naturally-occurring and can be from either the same or different species. Members of a family can also have common functional characteristics.

**[0222]** Variant *Oxyuranus* natriuretic peptide sequences, which differ from a parent natriuretic peptide sequence, by the substitution, addition or deletion of at least one amino acid residue may be synthesised *de novo* using solution or solid phase peptide synthesis techniques as known in the art. Alternatively such variants, including variants of naturally-occurring *Oxyuranus* natriuretic peptide sequences may be conveniently obtained by mutagenesis of their coding sequences. Mutations in nucleotide sequences constructed for expression of variants must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridise to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed mutants screened for at least one activity of an *Oxyuranus* natriuretic peptide.

**[0223]** In one embodiment, mutations can be introduced at particular loci by synthesising oligonucleotides encoding the desired amino acid residues, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a variant having the desired amino acid insertion, substitution, or deletion. In another embodiment, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered nucleotide sequence having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder *et al.* (1986, *Gene* **42**:133); Bauer *et al.* (1985, *Gene* **37**:73); Craik (1985, *BioTechniques* Jan. 12-19, ); Smith *et al.* (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462.

**[0224]** Sources of nucleotide sequences from which nucleic acid molecules encoding the natriuretic peptides of the invention, or their nucleic acid complements, can be obtained include total or poly$A^+$ RNA from a reptilian species, e.g., snake, more preferably from a species of *Oxyuranus* from which cDNAs can be derived by methods known in the art. Other sources of the DNA molecules of the invention include genomic libraries derived from an appropriate cellular source.

**[0225]** A nucleic acid molecule encoding a native *Oxyuranus* natriuretic peptide can be identified and isolated using standard methods, as described by Sambrook *et al. Molecular Cloning – A Laboratory Manual,* Second Ed., Cold Spring Harbor Press (1989); and Ausubel *et al., Current Protocols in Molecular Biology,* John Wiley & Sons, Inc. (1998). For example, reverse-transcriptase PCR (RT-PCR) can be employed to isolate and clone natriuretic peptide cDNAs. Oligo-dT can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from isolated

RNA which contains RNA sequences of interest, e.g., total RNA isolated from human tissue. Resultant first-strand cDNAs can then be amplified by a suitable nucleic acid amplification reaction such as the Polymerase Chain Reaction (or PCR) as known in the art. Primers, which are optionally degenerate, are designed to correspond to highly conserved regions of the natriuretic peptides or nucleotide sequences which were identified and compared to generate the primers. The products of each amplification reaction are typically separated *via* agarose gel electrophoresis and all consistently amplified products are gel-purified and cloned directly into a suitable vector for subsequent sequence analysis of the cloned amplified products.

**[0226]** Another approach to identify, isolate and clone natriuretic peptide-encoding cDNAs is to screen a cDNA library. Screening for DNA fragments that encode all or a portion of a cDNA that codes for a natriuretic peptide can be accomplished by probing the library with a probe which has sequences that are highly conserved between genes believed to be related to the natriuretic peptide, e.g., the homologue of a particular natriuretic peptide from a different species, or by screening of plaques for binding to antibodies that specifically recognise a natriuretic peptide, such as antibodies that recognise the highly conserved central core of natriuretic peptides. DNA fragments that bind to a probe having sequences which are related to natriuretic peptide, or which encode amino acid sequences that are immuno-interactive with antibodies to natriuretic peptide, can be subcloned into a suitable vector and sequenced and/or used as probes to identify other cDNAs encoding all or a portion of the natriuretic peptide.

**[0227]** Thus, the present invention contemplates isolated nucleic acid molecules comprising a sequence that encodes a natriuretic peptide of the invention. In certain embodiments, an isolated nucleic acid molecule of the invention hybridises under a stringency condition described herein to a naturally-occurring nucleic acid molecule that encodes a natriuretic peptide of the invention. As used herein, the term "hybridises under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridisation and washing. Guidance for performing hybridisation reactions can be found in Ausubel *et al.*, (1998, *supra*), Sections 6.3.1-6.3.6. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridisation conditions referred to herein are as follows: 1) low stringency hybridisation conditions in 6× sodium chloride/sodium citrate (SSC) at about 45° C, followed by two washes in 0.2× SSC, 0.1% SDS at least at 50° C (the temperature of the washes can be increased to 55° C for low stringency conditions); 2) medium stringency hybridisation conditions in 6× SSC at about 45° C, followed by one or more washes in 0.2× SSC, 0.1% SDS at 60° C; 3) high stringency hybridisation conditions in 6× SSC at about 45° C, followed by one or more washes in 0.2× SSC, 0.1% SDS at 65° C; and preferably 4) very high stringency hybridisation conditions are 0.5M sodium phosphate, 7% SDS at 65° C, followed by one or more washes at 0.2× SSC, 1% SDS at 65° C. Very

high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified.

**[0228]** Other stringency conditions are well known in the art and a skilled addressee will recognise that various factors can be manipulated to optimise the specificity of the hybridisation. Optimisation of the stringency of the final washes can serve to ensure a high degree of hybridisation. For detailed examples, see Ausubel *et al.*, *supra* at pages 2.10.1 to 2.10.16 and Sambrook *et al.* (1989, *supra*) at sections 1.101 to 1.104. While stringent washes are typically carried out at temperatures from about 42° C to 68° C, one skilled in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridisation rate typically occurs at about 20° C to 25° C below the $T_m$ for formation of a DNA-DNA hybrid. It is well known in the art that the $T_m$ is the melting temperature, or temperature at which two complementary polynucleotide sequences dissociate. Methods for estimating $T_m$ are well known in the art (see Ausubel *et al.*, *supra* at page 2.10.8).

**[0229]** In general, the $T_m$ of a perfectly matched duplex of DNA may be predicted as an approximation by the formula:

**[0230]** $T_m = 81.5 + 16.6 (\log_{10} M) + 0.41 (\%G+C) - 0.63 (\%\text{ formamide}) - (600/\text{length})$,

**[0231]** wherein: M is the concentration of $Na^+$, preferably in the range of 0.01 molar to 0.4 molar; %G+C is the sum of guanosine and cytosine bases as a percentage of the total number of bases, within the range between 30% and 75% G+C; % formamide is the percent formamide concentration by volume; length is the number of base pairs in the DNA duplex.

**[0232]** The $T_m$ of a duplex DNA decreases by approximately 1° C with every increase of 1% in the number of randomly mismatched base pairs. Washing is generally carried out at $T_m - 15°$ C for high stringency, or $T_m - 30°$ C for moderate stringency.

**[0233]** The invention also contemplates derivatives of the naturally-occurring or variant natriuretic peptides disclosed herein or their biologically active portions, which have been derived from a parent sequence by modification, for example by conjugation or complexing with other chemical moieties. Such derivatives include fusions of such natriuretic peptides of the invention with other peptides or polypeptides. For example, amino acid sequence, which display at least one natriuretic peptide activity may be fused to a further protein, for example, which is not derived from the original host. The further protein may assist in the purification of the fusion protein. For instance, a polyhistidine tag or a maltose binding protein may be used in this respect as described in more detail below. Other possible fusion proteins are those which produce an immunomodulatory response. Particular examples of such proteins include Protein A or glutathione S-transferase (GST).

**[0234]** Other derivatives include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein

synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptides, portions and variants of the invention. Examples of side chain modifications include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$; reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

**[0235]** The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivatization, by way of example, to a corresponding amide.

**[0236]** The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

**[0237]** Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

**[0238]** Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides or by oxidation with N-bromosuccinimide.

**[0239]** Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

**[0240]** The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

**[0241]** Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated by the present invention is shown in Table 6.

**[0242]** The invention also contemplates chimeric natriuretic peptides that correspond to all or a portion e.g., a portion described herein of a natriuretic peptide amino acid sequence. In specific embodiments, a chimeric natriuretic peptide includes at least one (or two) biologically active

portion(s) of a natriuretic peptide of the invention. In certain embodiments, it will include at least one biologically active portion of a natriuretic peptide of the invention and at least one biologically active portion of another natriuretic peptide, e.g., from ANP, BNP, CNP or DNP or their variants. Chimeric natriuretic peptides may be prepared, for example, by using recombinant DNA based methodologies or chemical syntheses. For example, nucleic acid molecules encoding chimeric natriuretic peptides can be synthesised *de novo* using readily available machinery. Sequential synthesis of DNA is described, for example, in U.S. Patent No 4,293,652. Instead of *de novo* synthesis, recombinant techniques may be employed including use of restriction endonucleases to cleave different natriuretic peptide-encoding polynucleotides and use of ligases to ligate together in frame a plurality of cleaved polynucleotides encoding selected portions of the different natriuretic peptides. Suitable recombinant techniques are described for example in the relevant sections of Ausubel, *et al.* (*supra*) and of Sambrook, *et al.*, (*supra*). Preferably, the synthetic polynucleotide is constructed using splicing by overlapping extension (SOEing) as for example described by Horton *et al.* (1990, *Biotechniques* **8**(5): 528-535; 1995, *Mol Biotechnol.* **3**(2): 93-99; and 1997, *Methods Mol Biol.* **67**: 141-149). However, it should be noted that the present invention is not dependent on, and not directed to, any one particular technique for constructing the synthetic construct.

**[0243]** The invention therefore contemplates a method of producing a synthetic polynucleotide as broadly described above, comprising linking together in the same reading frame at least two nucleic acid sequences, wherein individual nucleic acid sequences encode a portion of a distinct natriuretic peptide to form a synthetic polynucleotide, which encodes a chimeric natriuretic peptide according to the invention.

### *3. Preparation of natriuretic peptides of the invention*

**[0244]** The subject natriuretic peptides can be prepared by a process including fractionating venom from a suitable source as described above, and testing fractions thus produced for an activity selected from natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing and/or bone growth plate size-increasing activities. Assays for testing such activities are well known to persons of skill in the art and include aortic ring contraction/relaxation assays, cell cycle GMP elevation assays (see, for instance, Examples 3 and 4, respectively) and natriuretic peptide receptor binding assays (as described, for example, in Ballermann BJ, 1988 *Am Journal of Physiology* **254**: F159-163; Burgisser *et al.*, 1985, *Biochemical and Biophysical Research Communications* **133**:1201-1209; Capper *et al.*, 1990, *Clinical Chemistry* **36**:656-658; Gutkowska *et al.*, 1988, *Analytical Biochemistry* **168**:100-106). Examples of such assays are given in anaesthetized or conscious rat and rabbit blood pressure assay, left coronary artery ligation induced heart failure in the rat, pacing induced heart failure of the sheep. An illustrative bioassay for quantification of atrial natriuretic polypeptides in rat is described by Matsui *et al.*, 1987, *Am J Physiol* **252**:R1009-14. Optionally, the fractions are purified further to provide natriuretic peptides in substantially purified form. Isolated or

chemically synthesised peptides can be tested *in vivo* for effects on blood pressure and heart rate using methods as described, for instance, in Example 5. Appropriate animal models for testing and evaluating cardiorenal properties and the effectiveness of compounds in treating heart failure are known in the art and include canine (Burnett & Lisy, WO 01/44284), rat (Arabia *et al.*, 2002, *Neuroscience* **114:** 3: 591-599) and ovine models (Byrne *et al.*, 2002, *Journal of Cardiac Failure 8.*, **2**: 108-115). Stability of the NPs can be measured against a range of enzymes including NEP24.11 and trypsin, using well established methods (see for example Lew, R. *Methods in Molecular Biology 2 51*, H P L C of Peptides and Proteins: Methods and Protocols; Aguilar, MI (Ed), Humana Press Inc., Totowa, NJ).

**[0245]** Peptides of the present invention can be synthesised by solution or solid phase synthesis methods as known in the art. For example, the widely used Merrifield solid phase synthesis method, including the experimental procedures, is described in the following references: Stewart *et al.* (1969, Solid Phase Peptide Synthesis, W. H. Freeman Co., San Francisco); Merrifield (1963, *J Am Chem Soc* **85**: 2149); Meinenhofer (1973, *Int J Pept Pro Res* **11**: 246); and Barany and Merrifield (1980, in *The Peptides*, E. Gross and F. Meinenhofer, eds., Vol. 2, Academic Press, pp. 3-285). These standard peptide synthetic methods are followed by oxidative disulfide bond formation. For example, the linear peptides may be synthesised by solid phase methodology using BOC chemistry, as described by Schnoltzer *et al.*, (1992, *Int J Pept Prot Res 40*, 180-193). Following deprotection and cleavage from the solid support the reduced peptides are purified using preparative chromatography. The purified reduced peptides are oxidised in buffered systems. The oxidised peptides are purified using preparative chromatography (e.g. Example 2 *infra*) using well documented procedures.

**[0246]** General references describing the synthesis of natriuretic peptides and derivatives thereof include Craik *et al.* (1991, *Eur. J. Biochem.* **201**: 183-190); WO89/09611 and WO90/00561.

**[0247]** Replacement of the disulfide bridges with diselenide or mixed sulfide-selenide bridges can be done using the methods of Koide *et al.* (1993, *Chem. Pharm. Bull* **41**: 502-505) or Pegoraro *et al.* (1998, *J. Mol. Biol.*, **284**: 779-792).

**[0248]** Salts of carboxyl groups of the synthesised peptides may be prepared by contacting the peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

**[0249]** Acid addition salts of the synthesised peptides may be prepared by contacting the polypeptide with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid.

**[0250]** Amides of the synthesised peptides of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A suitable method for amide formation at the C-terminal carboxyl group is to cleave the polypeptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

**[0251]** Esters of carboxyl groups of the synthesised peptides may be prepared by any of the usual means known in the art for converting a carboxylic acid or precursor to an ester. One suitable method for preparing esters of the subject peptides, when using the Merrifield synthesis technique, is to cleave the completed polypeptide from the resin in the presence of the desired alcohol either under basic or acidic conditions, depending upon the resin. Thus, the C-terminal end of the peptide when freed from the resin is directly esterified without isolation of the free acid.

**[0252]** N-acyl derivatives of an amino group of synthesised peptides may be prepared by utilising an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagent such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N-and O-acylation may be carried out together, if desired.

**[0253]** PEGylated derivatives and similar conjugates of TNPs and analogues can be prepared by methods used for other NPs (WO2004/047871).

**[0254]** The synthesis may use manual techniques or be completely automated, employing, for example, an Applied BioSystems 431A Peptide Synthesizer (Foster City, Calif.) or a Biosearch SAM II automatic peptide synthesizer (Biosearch, Inc., San Rafael, Calif.), following the instructions provided in the instruction manual and reagents supplied by the manufacturer. Disulphide bonds between Cys residues can be introduced by mild oxidation of the linear peptide by KCN as taught, for example, in U.S. Pat. No. 4,757,048 at Col. 20.

**[0255]** Alternatively, the natriuretic peptides of the invention may be prepared by a recombinant DNA technique. For example, natriuretic peptides may be prepared by a process including the steps of: (a) preparing a recombinant polynucleotide comprising a nucleotide sequence encoding a natriuretic peptide of the invention, which nucleotide sequence is operably linked to a regulatory sequence; (b) introducing the recombinant polynucleotide into a suitable host cell; (c) culturing the host cell to express recombinant peptide from the recombinant polynucleotide; and (d) isolating the recombinant peptide. In certain embodiments, the nucleotide sequence encodes at least a portion of the TNPc amino acid sequence set forth in SEQ ID NO: 90, or its fragments, variants and derivatives, illustrative examples of which are set forth in SEQ ID NO: 1-7, 15-81 *supra* and in SEQ

ID NO: 91, 94-114, 117, 118, 120, 123, 124, 126-130, 132-134, 150, 154, 160, 166-169, 173, 176 and 178-182 (see Table 2).

**[0256]** The recombinant polynucleotide is suitably in the form of an expression vector, which can be a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome. The regulatory sequences will generally need to be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, the regulatory sequences include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter.

**[0257]** In certain embodiments, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

**[0258]** The expression vector may also include a fusion partner (typically provided by the expression vector) so that the recombinant peptide of the invention is expressed as a fusion polypeptide with said fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of said fusion polypeptide. In order to express the fusion polypeptide, it is necessary to ligate a polynucleotide according to the invention into the expression vector so that the translational reading frames of the fusion partner and the polynucleotide coincide. Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc potion of human IgG, maltose binding protein (MBP) and hexahistidine ($HIS_6$), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity chromatography include, but are not restricted to, glutathione-, amylose-, and nickel- or cobalt-conjugated resins. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with ($HIS_6$) fusion partners and the Pharmacia GST purification system. In one embodiment, the recombinant polynucleotide is expressed in the commercial vector pFLAG. Another fusion partner well known in the art is green fluorescent protein (GFP). This fusion partner serves as a fluorescent "tag" which allows the fusion polypeptide of the invention to be identified by fluorescence microscopy or by flow cytometry. The GFP tag is useful when assessing subcellular localisation of the fusion polypeptide of the invention, or for isolating cells which express the fusion polypeptide of the invention. Flow cytometric methods such as fluorescence activated cell sorting (FACS) are particularly useful in this latter application. Suitably, the fusion partners also have protease cleavage sites, such as for Factor $X_a$ or Thrombin, which allow the relevant protease to partially digest the

fusion polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated polypeptide can then be isolated from the fusion partner by subsequent chromatographic separation. Fusion partners according to the invention also include within their scope "epitope tags", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-Myc, influenza virus, hemagglutinin and FLAG tags.

**[0259]** The step of introducing the recombinant polynucleotide into a host cell may be achieved by any suitable method including transfection, and transformation, the choice of which will be dependent on the host cell employed. Such methods are well known to those of skill in the art.

**[0260]** Recombinant polypeptides of the invention may be produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a natriuretic peptide the invention. The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained by one skilled in the art through routine experimentation.

**[0261]** Suitable host cells for expression may be prokaryotic or eukaryotic. One preferred host cell for expression of a polypeptide according to the invention is a bacterium. The bacterium used may be *Escherichia coli*. Alternatively, the host cell may be an insect cell such as, for example, *SF9* cells that may be utilised with a baculovirus expression system.

**[0262]** The recombinant protein may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook, *et al.*, (1989, *supra*), in particular Sections 16 and 17; Ausubel *et al.*, (1998, *supra*), in particular Chapters 10 and 16; and Coligan *et al.*, CURRENT PROTOCOLS IN PROTEIN SCIENCE (John Wiley & Sons, Inc. 1995-1997), in particular Chapters 1, 5 and 6.

**[0263]** After preparation, the recombinant peptides/polypeptides are tested for their capacity to display at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, using any suitable assay known to persons of skill in the art.

### *4. Anti-natriuretic peptide antigen-binding molecules*

**[0264]** The invention also provides an antigen-binding molecule that is specifically immuno-interactive with an *Oxyuranus* natriuretic peptide. In one embodiment, the antigen-binding molecules comprise whole polyclonal antibodies. Such antibodies may be prepared, for example, by injecting a polypeptide, fragment, variant or derivative of the invention into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are

described for example in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, (John Wiley & Sons, Inc, 1991), and Ausubel *et al.*, (1994-1998, *supra*), in particular Section III of Chapter 11.

**[0265]** In lieu of polyclonal antisera obtained in a production species, monoclonal antibodies may be produced using the standard method as described, for example, by Köhler and Milstein (1975, *Nature* **256**, 495-497), or by more recent modifications thereof as described, for example, in Coligan *et al.*, (1991, *supra*) by immortalising spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the polypeptides, fragments, variants or derivatives of the invention.

**[0266]** The invention also contemplates as antigen-binding molecules Fv, Fab, Fab' and $F(ab')_2$ immunoglobulin fragments. Alternatively, the antigen-binding molecule may comprise a synthetic stabilised Fv fragment. Exemplary fragments of this type include single chain Fv fragments (sFv, frequently termed scFv) in which a peptide linker is used to bridge the N terminus or C terminus of a $V_H$ domain with the C terminus or N-terminus, respectively, of a $V_L$ domain. ScFv lack all constant parts of whole antibodies and are not able to activate complement. Suitable peptide linkers for joining the $V_H$ and $V_L$ domains are those which allow the $V_H$ and $V_L$ domains to fold into a single polypeptide chain having an antigen binding site with a three dimensional structure similar to that of the antigen binding site of a whole antibody from which the Fv fragment is derived. Linkers having the desired properties may be obtained by the method disclosed in U.S. Patent No 4,946,778. However, in some cases a linker is absent. ScFvs may be prepared, for example, in accordance with methods outlined in Kreber *et al* (Kreber *et al.* 1997, *J. Immunol. Methods*; **201(1)**: 35-55). Alternatively, they may be prepared by methods described in U.S. Patent No 5,091,513, European Patent No 239,400 or the articles by Winter and Milstein (1991, *Nature* **349**:293) and Plückthun *et al* (1996, In *Antibody engineering: A practical approach*. 203-252).

**[0267]** In another embodiment, the synthetic stabilised Fv fragment comprises a disulphide stabilised Fv (dsFv) in which cysteine residues are introduced into the $V_H$ and $V_L$ domains such that in the fully folded Fv molecule the two residues will form a disulphide bond therebetween. Suitable methods of producing dsFv are described for example in (Glockscuther *et al. Biochem.* **29**: 1363-1367; Reiter *et al.* 1994, *J. Biol. Chem.* **269**: 18327-18331; Reiter *et al.* 1994, *Biochem. 33*: 5451-5459; Reiter *et al.* 1994. *Cancer Res.* **54**: 2714-2718; Webber *et al.* 1995, *Mol. Immunol.* **32**: 249-258).

**[0268]** Also contemplated as antigen-binding molecules are single variable region domains (termed dAbs) as for example disclosed in Ward *et al.* (1989, *Nature* **341**: 544-546); Hamers-Casterman *et al.* (1993, *Nature*. **363**: 446-448); Davies & Riechmann, (1994, *FEBS Lett.* **339**: 285-290). Alternatively, the antigen-binding molecule may comprise a "minibody". In this regard, minibodies are small versions of whole antibodies, which encode in a single chain the essential elements of a whole antibody. Suitably, the minibody is comprised of the $V_H$ and $V_L$ domains of a

native antibody fused to the hinge region and CH3 domain of the immunoglobulin molecule as, for example, disclosed in U.S. Patent No 5,837,821.

**[0269]** In an alternate embodiment, the antigen binding molecule may comprise non-immunoglobulin derived, protein frameworks. For example, reference may be made to Ku & Schultz, (1995, *Proc. Natl. Acad. Sci. USA*, **92**: 652-6556) which discloses a four-helix bundle protein cytochrome b562 having two loops randomised to create complementarity determining regions (CDRs), which have been selected for antigen binding.

**[0270]** The antigen-binding molecule may be multivalent (*i.e.*, having more than one antigen binding site). Such multivalent molecules may be specific for one or more antigens. Multivalent molecules of this type may be prepared by dimerisation of two antibody fragments through a cysteinyl-containing peptide as, for example disclosed by Adams *et al.*, (1993, *Cancer Res.* **53**: 4026-4034) and Cumber *et al.* (1992, *J. Immunol.* **149**: 120-126). Alternatively, dimerisation may be facilitated by fusion of the antibody fragments to amphiphilic helices that naturally dimerise (Pack P. Plünckthun, 1992, *Biochem.* **31**: 1579-1584), or by use of domains (such as the leucine zippers jun and fos) that preferentially heterodimerise (Kostelny *et al.*, 1992, *J. Immunol.* **148**: 1547-1553). In an alternate embodiment, the multivalent molecule may comprise a multivalent single chain antibody (multi-scFv) comprising at least two scFvs linked together by a peptide linker. In this regard, non-covalently or covalently linked scFv dimers termed "diabodies" may be used. Multi-scFvs may be bispecific or greater depending on the number of scFvs employed having different antigen binding specificities. Multi-scFvs may be prepared for example by methods disclosed in U.S. Patent No. 5,892,020.

**[0271]** Phage display and combinatorial methods for generating natriuretic peptide antigen-binding molecules are known in the art (as described in, e.g., Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.*, 1992, *Hum Antibod Hybridomas* 3:81-85; Huse *et al.*, 1989, *Science* **246**:1275-1281; Griffths *et al.*, 1993, *EMBO J* **12**:725-734; Hawkins *et al.*, 1992, *J Mol Biol* **226**:889-896; Clackson *et al.*, 1991, *Nature* **352**:624-628; Gram *et al.*, 1992, *Proc.Natl. Acad. Sci USA* **89**:3576-3580; Garrad *et al.*, 1991, *Bio/Technology* **9**:1373-1377; Hoogenboom *et al.*, 1991, *Nucleic Acid Res* **19**:4133-4137; and Barbas *et al.*, 1991, *Proc.Natl. Acad. Sci USA* **88**:7978-7982).

**[0272]** The antigen-binding molecules can be used to screen expression libraries for variant natriuretic peptides of the invention as described herein. They can also be used to detect and/or isolate the natriuretic peptides of the invention. Thus, the invention also contemplates the use of

antigen-binding molecules to isolate *Oxyuranus* natriuretic peptides using, for example, any suitable immunoaffinity based method including, but not limited to, immunochromatography and immunoprecipitation. A preferred method utilises solid phase adsorption in which anti-natriuretic peptide antigen-binding molecules are attached to a suitable resin, the resin is contacted with a sample suspected of containing a natriuretic peptides, and the natriuretic peptides, if any, are subsequently eluted from the resin. Preferred resins include: Sepharose® (Pharmacia), Poros® resins (Roche Molecular Biochemicals, Indianapolis), Actigel Superflow™ resins (Sterogene Bioseparations Inc., Carlsbad Calif.), and Dynabeads™ (Dynal Inc., Lake Success, N.Y.).

**[0273]** Preferred epitopes encompassed by the antigenic peptide are regions of *Oxyuranus* natriuretic peptides which are located in the N-terminal, central core and especially C-terminal portions, as well as regions with high antigenicity.

**[0274]** The antigen-binding molecule can be coupled to a compound, e.g., a label such as a radioactive nucleus, or imaging agent, e.g. a radioactive, enzymatic, or other, e.g., imaging agent, e.g., a NMR contrast agent. Labels which produce detectable radioactive emissions or fluorescence are preferred.

**[0275]** An anti-natriuretic peptide antigen-binding molecule (e.g., monoclonal antibody) can be used to isolate a natriuretic peptide by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, an anti-natriuretic peptide antigen-binding molecule can be used to detect natriuretic peptides (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the protein. Anti-natriuretic peptide antigen-binding molecules can be used diagnostically to monitor natriuretic peptide levels in tissue as part of a clinical testing procedure (e.g., for the diagnosis, prognosis or monitoring of congestive heart failure). Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance (i.e., antibody labelling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include $^{125}I$, $^{131}I$, $^{35}S$ or $^{3}H$.

**[0276]** The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium ($Eu^{34}$), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic

polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

**[0277]** A large number of enzymes useful as labels is disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338. Enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme in solution.

### *5. Pharmaceutical Compositions*

**[0278]** The invention also provides pharmaceutical compositions that include a natriuretic peptide of the invention and a pharmaceutically acceptable carrier, excipient and/or diluent. The pharmaceutical compositions of the invention can be used to promote or otherwise facilitate at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals. Accordingly, the compositions are considered useful for treating or preventing a variety of conditions including oedematous states such as heart failure, nephrotic syndrome, cirrhosis of the liver, hypertension, kidney failure and the like, bacterial infections, asthma, weight loss, inflammatory-related diseases, erectile dysfunction, hypercholesterolemia, skeletal dysplasias and as a protectant for toxicity of anti-cancer drugs.

**[0279]** The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable non-toxic salts include the acid addition salts (formed with the free amino groups) and which are formed by reaction with inorganic acids such as, for example, hydrochloric, sulfuric or phosphoric acids, or organic acids such as, for example, acetic, oxalic, tartaric, mandelic, citric, malic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases such as amines, i.e., isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

**[0280]** The natriuretic peptides of the invention are suitably administered to a subject, e.g., a human or a non-human mammal, such as a domestic animal, at dosages of at least about 0.01 to about 100 mg/kg, more preferably about 0.05 to about 50 mg/kg, and even more preferably about 0.1 to about 30 mg/kg, of body weight (e.g., about 10 to about 50 ng/kg in dogs), although other dosages may provide beneficial results. The amount administered will vary depending on various factors including, but not limited to, the agent chosen, the disease, and whether prevention or treatment is to be achieved. Both local and systemic administration are envisioned. Systemic administration is preferred. Thus, administration of the therapeutic agents in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition,

whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses.

**[0281]** One or more suitable unit dosage forms comprising a natriuretic peptide of the invention, which may optionally be formulated for sustained release, can be administered by a variety of routes including oral, or parenteral, including by rectal, buccal, vaginal and sublingual, transdermal, subcutaneous, intravenous, intramuscular, intraperitoneal, intrathoracic, intracoronary, intrapulmonary and intranasal routes. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

**[0282]** When the natriuretic peptide of the invention is prepared for oral administration, it is preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations comprise from 0.1 to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipient, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for oral administration may be present as a powder or as granules; as a solution, a suspension or an emulsion; or in achievable base such as a synthetic resin for ingestion of the active ingredients from a chewing gum. The active ingredient may also be presented as a bolus, electuary or paste. A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference. Supplementary active compounds can also be incorporated into the compositions.

**[0283]** Pharmaceutical formulations containing the natriuretic peptide of the invention can be prepared by procedures known in the art using well known and readily available ingredients. For example, the natriuretic peptide can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose, HPMC and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturising agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

[0284] For example, tablets or caplets containing the nucleic acid molecule or peptide of the invention can include buffering agents such as calcium carbonate, magnesium oxide and magnesium carbonate. Caplets and tablets can also include inactive ingredients such as cellulose, pregelatinized starch, silicon dioxide, hydroxy propyl methyl cellulose, magnesium stearate, microcrystalline cellulose, starch, talc, titanium dioxide, benzoic acid, citric acid, corn starch, mineral oil, polypropylene glycol, sodium phosphate, and zinc stearate, and the like. Hard or soft gelatin capsules containing the nucleic acid molecule or peptide of the invention can contain inactive ingredients such as gelatin, microcrystalline cellulose, sodium lauryl sulfate, starch, talc, and titanium dioxide, and the like, as well as liquid vehicles such as polyethylene glycols (PEGs) and vegetable oil. Moreover, enteric coated caplets or tablets of the nucleic acid molecule or peptide of the invention are designed to resist disintegration in the stomach and dissolve in the more neutral to alkaline environment of the duodenum.

[0285] In some embodiments, a natriuretic peptide of the invention is the only component present in the pharmaceutical composition. In some embodiments, the pharmaceutical composition can include an additional component or adjuvant. For example, the composition can include one or more of: an anti-microbial, e.g., an antibiotic, an antiviral, an antifungal, an antiparasitic agent, an anti-inflammatory agent, an antihistamine, an anti-fibrolytic agent, and a growth factor. Examples of antibiotics include tetracycline, ciprofloxacin, gentamycin, cyclosporin cefotaxim, and the like. Examples of antivirals include gangcyclovir, zidovudine, amantidine, vidarabine, ribaravin, trifluridine, acyclovir, dideoxyuridine, and the like. Antifungals include, but are not limited to, diflucan, ketaconizole, nystatin, and the like. Antiparasitic agents such as pentamidine can be included. The composition may further include an anti-inflammatory agent such as I-1-anti-trypsin, I-1-antichymotrypsin, and the like. Other compounds that can be included in the composition include anaesthetics, e.g., local anaesthetics.

[0286] The pharmaceutical composition can be formulated to promote stability of a natriuretic peptide of the invention, e.g., to reduce digestion, e.g., autodigestion, of the natriuretic peptide. The stability of the natriuretic peptide can be promoted, for example, by preparing the natriuretic peptide in a pharmaceutical composition having a pH of about 5 to 9, preferably about 6.5 to 7. The stability of the natriuretic peptide can also be stabilised by providing the natriuretic peptide in a pharmaceutical composition, which further includes, e.g., a stabiliser, such as a polyol. In such embodiments, the pharmaceutical composition can include about 5%, 10%, 20% or more of a polyol (or polyols). An example of a polyol which can be used in the pharmaceutical composition is glycerol. In other aspects, the stability of the natriuretic peptide can be increased by providing the natriuretic peptide in a crystallised, freeze-dried or lyophilised form. If the composition is frozen, the composition should be thawed prior to the time of use. In another embodiment, the invention features a composition which includes a natriuretic peptide, e.g., a natriuretic peptide described herein, and

which has a pH of about 5 to 9, preferably about 6.5 to 7. The invention also features a composition which includes a natriuretic peptide, e.g., a natriuretic peptide described herein, and a stabilising agent, e.g., a polyol, e.g., glycerol. The polyol can be present at about 5%, 10% or 20%.

**[0287]** The dosage of the composition comprising the natriuretic peptide depends upon the particular use of the natriuretic peptide, but the dosage should be an effective amount for the composition to perform its intended use. Data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. Generally, for a composition comprising a natriuretic peptide that is an aqueous solution, it is believed that from about 1 mL to about 50 mL of such composition is sufficient to promote a natriuretic activity as described herein. However, depending on the use of the composition, the dosage can range from about 1 mL to about 200 mL.

**[0288]** The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

**[0289]** Also within the scope of the invention are kits comprising a natriuretic peptide described herein. The kit can include one or more other elements including: instructions for use; other reagents, e.g., other therapeutic agents (e.g., one or more of: an anti-microbial, e.g., an antibiotic, an antiviral, an antifungal, an antiparasitic agent, an anti-inflammatory agent, an antihistamine, an anti-fibrolytic agent and an analgesic); a diluent; devices, e.g., containers, e.g., sterile containers, or other materials for preparing the natriuretic peptide for administration; pharmaceutically acceptable carriers (e.g., a stabiliser); and devices or other materials for administration to a subject (e.g., syringes, applicators, bandages, spray or aerosol devices). The instructions can include instructions for therapeutic application including suggested dosages and/or modes of administration, e.g., in a patient with congestive heart failure. In some applications, the natriuretic peptide can be administered in combination with other components and the kit can include instructions on the amount, dosage, and timing of administration of the natriuretic peptide and the other components.

**[0290]** In some embodiments, the natriuretic peptide may be supplied in lyophilised or freeze dried form. In such embodiments, the kit can include one or more of: instructions for thawing and/or hydrolysing, and a pharmaceutically acceptable carrier or diluent. In some embodiments, the kit can include instructions for a diluent or a premeasured amount of a diluent.

**[0291]** The present invention also contemplates methods for specifically detecting or quantifying different natriuretic peptides, including the TNPs of the present invention, in a biological sample of interest. These methods generally comprise: (a) separating peptides present in the biological sample as a function of their molecular mass (e.g., by a mass spectroscopic technique such as differential time of flight), wherein each peptide correlates with a detectable signal; (b) detecting the detectable signals during, or at the completion of, the separation; and (c) processing the detectable

signals to deduce which, if any, natriuretic peptide is present in the biological sample. In some embodiments, the detectable signals are processed by comparing them to a set of reference detectable signals, each of which correlate(s) with a distinct natriuretic peptide, to thereby determine whether any of the sample detectable signals match the reference detectable signals. Advantageously, these methods permit discrimination and quantification of different natriuretic peptides and can aid in the detection of major metabolites (e.g., enzyme cleaved products) that may result in a NP associated condition (e.g., congestive heart failure).

**[0292]** In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

## EXAMPLES

### EXAMPLE 1

### ISOLATION, PURIFICATION AND IDENTIFICATION OF PEPTIDES FROM SNAKE VENOM

#### *Materials And Methods*

Venom Collection

**[0293]** Venom was collected by from specimens of *Oxyuranus microlepidotus* (Inland taipan) from Goydnor's Lagoon, South Australia, Australia and pooled to minimise the effects of individual variations.

Centrifuge Filtration

**[0294]** Venoms were diluted to 10 mg/mL with buffered saline solution, centrifuged at 1,500×g for two hours in Centricon 50 centrifuge filters (Millipore, 50 kDa cut-off), and then the filtrate centrifuge filtered again at 3,000 G using Centricon 10 tubes (Millipore, 10 kDa cut-off). The filtrates from this step were then lyophilised and stored until needed for LC-MS analysis and RP-HPLC analysis.

LC-MS analysis of centrifuge fractionated venom

**[0295]** Venom fractions <10 kDa were dissolved in 0.1% TFA to a concentration of 1 mg/mL. On-line LC-MS of venoms was performed on a Vydac C18 analytical column (2.1 x 35 mm, 5μ particle size, 300 Å) with Solvent A (0.05% TFA, $H_2O$) and Solvent B (90% acetonitrile in 0.045% TFA) at a turbospray flow rate of 130 μL/min. The solvent delivery and gradient formation was achieved using an Applied Biosystems 140 B solvent delivery system. The variable gradient was 0-20% in the first two minutes and then 20-45% over the next 12 minutes followed by 45-80% over the next minute. Electrospray mass spectra were acquired on a PE-SCIEX API III triple quadrupole mass spectrometer that was equipped with an Ionspray atmospheric pressure ionisation source. Samples (10 μL) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data was acquired at an orifice potential of 80V over the mass range 400-2100 Da with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

Preparative RP-HPLC

**[0296]** A Vydac Preparative C18 column (20x250mm, 10μ, 300 Å) was used on a Waters 600 HPLC system and UV absorbance was monitored at 214 nm by a Waters 486 tuneable absorbance detector. Samples were dissolved in two mL of buffer A (0.1% trifluroacetic acid), manually injected and then the column run at 100% A to wash off the concentrated salts present in the >10 kDa fraction.

The following gradient conditions of buffer B (90% acetonitrile, 0.09% trifluroacetic acid) were then used: 0 to 20% in five minutes, (4% gradient) 20% to 60% over forty minutes (1% gradient) and then 60% to 80% in five minutes (4% gradient).

Edman Degradation Sequencing

**[0297]** 0.1 mg of pure peptide was dissolved in 100 µL of 0.1 M ammonium bicarbonate at pH 7.0. 10 µL of 0.1 TCEP (pH 7) was added and incubated at 50° C for 20–30 minutes. 30 µL of 0.1M maleimide was then added and incubated at 50° C for a further 20–30 minutes. Maleimide added 97 amu per cysteine and mass spectroscopy showed an increase of 194 Daltons per disulphide bond. The reduced/alkylated peptides were N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 477A Protein Sequencer.

***Results and Discussion***

**[0298]** LC-MS analysis of the fractionated venom revealed the presence of components in the 3-4 kDa range (Figure 1). Three components, with molecular weights of 3651, 3661 and 4112 Daltons, respectively, were purified by preparative RP-HPLC. Edman degradation sequencing provided the full sequences, revealing a significant degree of homology between the venom components and circulating isoforms of ANP/BNP (Figure 2). As such these components were designated TNPa, TNPb and TNPc, respectively. The nomenclature chosen reflects the common name source (*Taipan*), structural homology to natriuretic peptides and chronological order of isolation.

EXAMPLE 2

**SYNTHESIS OF NATRIURETIC PEPTIDES**

***Synthesis and oxidation***

**[0299]** Analogues of the naturally occurring natriuretic peptides were manually synthesised, deprotected, and cleaved from resin in accordance with known methods previously described for other disulfide bearing peptides (Nielsen *et al.*, (1999) J. Mol. Biol. 289, 1405–1421). *In brief,* the peptides, including ANP, BNP, and TNP toxins and analogues (see sequences in Tables 1 and 2) were manually assembled using the stepwise in situ neutralization protocol for Boc chemistry. The amino acid side-chain protection was as follows: Cys(MeBzl), Tyr (BrZ), Asn (Xan), Asp (OcHex), selenocys(MeBzl) and Ser (Bzl). All the syntheses were performed on either Boc-E-(OBzl)-$OCH_2$-Pam, Boc-E-(OcHx)-$OCH_2$-Pam or Boc-A-$OCH_2$-Pam resins. Deprotection and cleavage were carried out in HF:p-cresol:p-thiocresol (9:0.5:0.5) for 1.5 h. The pure, reduced peptides (0.02-0.05 mM) were oxidised in aqueous 0.1M $NH_4HCO_3$ (pH 8.0, adjusted with 0.1 M $NH_4OH$). The peptide solutions were then stirred for 2-4 days at room temperature to achieve oxidation. Oxidised peptides were purified by preparative RP-HPLC on C18 semi-preparative or preparative HPLC columns. HPLC

studies were performed on Waters 600E and Shimadzu LC-2010 instruments. All the preparative HPLC was carried out using a Phenomenex $C_{18}$ column (250x21.20mm, 10μm). All of the analytical HPLC were carried out using either a Zorbax 300SB-$C_8$ (150x2.1mm, 5μm) column or a Phenomenex $C_{18}$ (250x4.6mm, 10μm) with a 90% Acetonitrile and 0.043% Trifluroacetic acid as the eluting buffer B and 1%B per minute as the gradient. Electrospray mass spectra were acquired on a Micromass LCT (UK) which is a liquid chromatography-orthogonal acceleration reflecting TOF mass spectrometer, coupled to an agilent HPLC system.

***Analysis***

**[0300]** Peptides were quantified initially by triplicate amino acid analysis and then by RP-HPLC (HP 1100) using an external reference standard for each peptide. Mass spectra were acquired on a PE-Sciex API III triple quadrupole electrospray mass spectrometer (MS) in positive ion mode (m/z 500-2000, at 0.1-0.2-Da steps, declustering potentials of 10-90 V, and dwell times of 0.4-1.0 s). Data were deconvoluted (MacSpec 3.2, Sciex, Canada) to obtain the molecular weight from the multiply charged species. MS was used to confirm purity and to monitor peptide oxidation.

***Reduction and alkylation***

**[0301]** Purified peptides (~ 100 pmol) were fully reduced in the presence of TCEP and 50 mM ammonium acetate at pH 8 (37°C for 1 h) and then alkylated in the presence of maleimide (37°C, 1 h). The alkylated peptides were purified by RP-HPLC, applied to a Biobrene treated glass fibre filter, and analysed by Edman chemistry using an ABI model 470A protein sequencer. Alkylation of peptides with maleimide allowed their cysteine residues to be observed as PTH-cys-maleimide doublets (diastereomers)

Results

**[0302]** Synthetic procedures based on Boc *in situ* neutralisation chemistry for TNP toxins and their analogues has been successfully established. Chain assembly proceeded efficiently with every coupling yield above 99.6% according to the quantitative ninhydrin test. Illustrative examples of the results are provided in Figure 3.

***Discussion***

**[0303]** Examination of the sequences of the peptides showed that despite the strong homology with both ANP and BNP the *O. microlepidotus* venom peptides differ at several key points. The natriuretic peptides can be considered to consist of three distinct regions: the ring structure, the N-terminal head and the C-terminal tail. Within the ring structure, the following observations can be made. TNPa and TNPb have the invariant R (ie., present in the NP signature) at position eight of the loop replaced by an H. Although, this would normally be considered a conservative substitution, R → H in TNPc resulted in reduced effects on lowering blood pressure compared to TNPc. This provides

support for the notion that the NP signature requires an R at this position. Previous examinations within the ring structure of ANP have shed some light on functionality (Watanabe *et al.*, 1988, *Eur. J. Pharmacol.* **147**:49-57; Thibault *et al.*, 1995, *Mol. Pharm.* **48**: 1046-1053]. Replacement of Phe at position 2 within the loop of ANP with the D-amino form of the same amino acid almost completely abolished activity. However, replacement of Gly at position nine within the ring with D-Ala increased the activity. A similar effect to Phe occurred when Asp at position 6 within the ring was replaced with the D-Asp, activity being reduced to only negligible amounts. The presence of Pro in the venom peptides at position five cannot explain any differences in activity as all three *O. microlepidotus* peptides, whether equipotent to ANP or not, contain the residue at this location. These venom peptides are novel in containing prolines at this location.

**[0304]** Strikingly, the C-terminal tails of the TNP molecules shared no homology with this region in ANP or other NPs. The C-terminal tails in TNPs are as long (TNPa and TNPb) or longer than (TNPc) the length previously shown to be the minimum for full activity [Watanabe *et al.*, 1988; Wakitani *et al.*, 1985, *Circ. Res.* **56**: 621-627]. TNPc has a C-terminal tail, that differs in length and sequence to that of the other TNPs and each of the TNPs contain two prolines within the C-terminal tail. Due to its restrained conformation, prolines are contained within turns in secondary structure. Yet the positions of the prolines differ amongst TNPa/b and TNPb, suggesting that the tails may adopt different conformations. It is notable that the two peptides (TNPa and TNPb) that are inactive in assays specific for ANP/BNP activity have a Pro at a location different than TNPc which is equipotent to ANP. Interestingly, substitution of the C-terminal Ile in TNPc by the equivalent Pro in TNPa/b reduces the activity significantly (see Example 8). Considering that all other active NPs have a hydrophobic amino acid (ie., Val, Ile) in that position, it is not unreasonable to speculate that the NP signature should be considered to extend beyond the loop consensus structure to additionally consider elements of tail structure. The C-terminal region, which is associated with NP stability *in vivo*, may play a role in protecting the core ring structure *via* tertiary interactions. The reduced activity of TNPa and TNPb can be explained, at least in part, by the departure (despite its apparent conservative nature) from the consensus NP sequence and the presence of a tail Pro where a hydrophobic residue is found consistently in other NPs. TNPc and its derivatives have superior activity because the NP signature is conserved. Other features present in the TNP family, particularly TNPc, contribute to enhanced stability over ANP (consistent with C-terminal tail theory).

## EXAMPLE 3

### AORTIC RING EXPERIMENTS

### ***Materials And Methods***

**[0305]** Male Sprague Dawley rats were killed and the descending thoracic aorta rapidly removed and flushed with physiological solution. After being cleared of adhering fat and connective

tissue, 5 mm rings were mounted under 10 g resting tension, between two stainless steel hooks, in organ baths (37°C) containing carbogenated (95% $O_2$ and 5% $CO_2$) physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2 and $CaCl_2$, 2.5. The lower hook was attached to a tissue holder and the upper hook to a force displacement transducer (Grass FTO3) Where indicated endothelial cells were removed by gentle rubbing of the intimal surface with a wire. To confirm the presence or absence of endothelial cells, tissues were precontracted with a sub-maximal concentration of phenylephrine (0.3 μM) and the response to acetylcholine (ACh, 10 μM) observed. Ach-induced relaxation greater than 80% of maximum indicated the presence of endothelium; whereas Ach-induced relaxation less than 10% indicated the absence of endothelium. Ring preparations under resting tension were conditioned by two contraction/relaxation cycles with 40 mM KCl, contracted by a third application of 40 mM KCl and then relaxed with different doses of the venom peptides. Responses were expressed as a percentage relaxation of the precontraction by KCl.

**[0306]** Pharmacological analysis of the peptides showed that the activity profile differs amongst the suite of peptides (Figure 4) despite the highly conserved sequences. For example, TNPc was equipotent to ANP and DNP in producing a near 100% relaxation in pre-contracted aortae. These effects were consistent between endothelium-intact (NPR-C mediated) and -denuded rings (GC-A mediated). However, TNPa and TNPb produced only minor (approximately 20% maximum) relaxation effects in the endothelium-intact aortic rings and no relaxation in denuded rings.

**[0307]** In addition, neither TNPa or TNPb were able to competitively inhibit the binding of TNPc to either the endothelium intact or endothelium denuded aortic rings (Figure 5, TNPb results shown). Taken together, these results suggest a variation of selectivity amongst the peptides which is dictated by only relatively minor alterations in peptide sequence. The high potency of TNPc provides evidence that this peptide will have an activity profile similar to hANP and BNP.

## EXAMPLE 4

### DETERMINATION OF CELL CYCLIC GMP ELEVATIONS

### *Materials And Methods*

**[0308]** The human embryonic kidney 293 (HEK-293) cell line was used for the stable expression of rat GC-A (GC-A/293). Cells expressing rat GC-A (GC-A/293) in the HEK-293 cell line were as previously described (Potter L.R., and Garbers D.L.(1992) J Biol Chem. 26, 14531-4) and determination of cGMP elevations was undertaken as previously described (Chrisman *et al.*T.D., Schulz, S., Potter, L.R. and Garbers, D.L. (1993) J. Biol. Chem. (1993) 268, 3698-3703). Responses were expressed as a percentage of the effect produced by ANP.

***Results and Discussion***

**[0309]** The peptides TNPa, TNPb and TNPc were tested on the GC-A receptor overexpressed in HEK-293 cells and cGMP elevations were determined and compared to those of other natriuretic peptides ANP and DNP. These results (Figure 4) showed that the *O. microlepidotus* peptides display a range of activities. For example, TNPc is equipotent to ANP and DNP, whereas TNPa and TNPb displayed lesser effects on cGMP elevations. This result is consistent with those obtained using the aortic ring experiments, suggesting that TNPc is a natriuretic peptide with similar potency to ANP, acting at both the NPR-A and NPR-C receptors.

**[0310]** In summary, in specific tissue and cell-based assays, TNPc is equipotent to DNP and ANP whereas TNPa and TNPb were the first ANP/BNP-like natriuretic peptides to be inactive or only weakly active upon the GC-A cell based assays as well as the endothelium-denuded aortic rings. In addition, these peptides were only weakly active on the NPR-C mediated relaxation in endothelium-intact aortic rings. As demonstrated by their inability to inhibit the binding of TNPc, these peptides do not bind at all, even in an antagonistic manner, to GC-A and have a low affinity for NPR-C. Within the ring structure these peptides have a histidine substituted for the invariant arginine at position eight and contain a proline a ring position five. Uniquely, these peptides contain a proline at residue three of the C-terminal tail, a location previously shown to be crucial for binding. TNPa and TNPb are the first natriuretic peptides with this change in structure of the C-terminal tail and also the first with replacement of a crucial invariant residue within the ring structure. Due to sequence divergence, the snake venom natriuretic peptides may represent a distinct group within the natriuretic peptide family, which possess activity divergent from the rest of the family.

EXAMPLE 5

**SOLUTION STABILITY OF NPS**

***Methods***

**[0311]** Peptides are dissolved at 1 mg/mL in 5 mM sodium acetate buffer/0.9% saline. The samples are stored at 37° C and aliquots taken at intervals over a 31-day period. For comparison studies, a fresh sample of each peptide is made up from dry lyophilised powder stored at –20° C at the same concentration in water just prior to evaluation. Samples are evaluated by RP-HPLC/MS using an optimised chromatography program over a mass range of 300-1800amu.

EXAMPLE 6

PLASMA STABILITY OF NATRIURETIC PEPTIDES

***Material and Methods***

**[0312]** *Plasma preparation:* Blood samples (from healthy human volunteers) were drawn into a sealed tube containing ethylene diamine tetra-acetic acid (EDTA). After sampling, the plasma was separated by centrifugation at 14,000 rpm for 30 min. Plasma was taken out and stored in separated eppendorf tubes at –20 °C.

**[0313]** *Stability assay in human and rat plasma:* Human or rat plasma (sample size 200µl) was incubated at 37°C for 5 min. 50 µl peptide stock solution (5 mg/ml) was added to the plasma. The vortexed reaction mixture was incubated at 37°C. Sample aliquots of 30 µl were taken from the reaction mixture at sequential intervals of 0 h, 1 h, 2 h, 4 h and 24 h and the sample reaction quenched immediately with 30 µl extraction buffer containing 50% Acetonitrile, 50% 0.1 M NaCl and 1% TFA. The quenched aliquots were cooled in ice for 5 min prior to centrifugation at 14,000 rpm for 15 min. 20 µl of the supernatant from each of the centrifuged aliquots was injected into RP-HPLC for analysis.

**Results**

**[0314]** These studies were undertaken to evaluate the plasma stability of TNPc at normal body temperature. The results for both rat and human plasma are summarised diagrammatically in Figure 6A. This shows clearly that TNPc is stable in plasma for at least 24 h at 37°C (with 72% remaining in human plasma and 76% remaining in rat plasma) The results demonstrate unambiguously that TNPc has a much longer half-life in plasma compared to WT-hANP (55 min in human plasma and 58 min in rat plasma).

EXAMPLE 7

STABILITY OF NATRIURETIC PEPTIDES TO ENZYMATIC DEGRADATION

***Material and Methods***

**[0315]** The incubation buffer contained 100 mM Tris/HCl and 150 mM NaCl and the pH was adjusted to 7.2-7.4. In each assay, 100 µl of peptide [500 µM] was incubated with 300 µl buffer (with 500 ng Endopeptidase (NEP) or 10µg Plasmin) at 37°C. 50 µl aliquots were taken at times 5 min, 30 min, 60 min, 90 min, 180 min and 24 h. The reaction was quenched by heated to 100°C for 4 min and the remaining peptide concentrations measured by RP-HPLC. Note that the levels of Plasmin required to degrade these peptides was several thousands time the levels present in human plasma.

## Results

**[0316]** Endopeptidase (EC 3.4.24.11) is an ectoenzyme which involves with the clearance of NPs *in vivo*. It inactivates NPs by opening their 17-member ring structure. Plasmin, a serine proteinase, is able to degrade most extracellular proteins. It possesses tryptic-like specificity and hydrolyses protein and peptides at lysyl- and arginyl-bonds. It also has esterolytic activity toward basic amino acid esters and amidolytic activity toward basic amino acid amides. Results from these two assays confirmed the results of the plasma assay: TNPc is more stable than either ANP and BNP, supporting its candidacy as a therapeutic for heart failure (Results are shown in Figures 6b-c). The result against NEP is interesting. TNPc did not show any degradation over the 180 min measurement period (by comparison ANP had been completely degraded by that time), while BNP had been significantly degraded. As NEP presents a known degradation pathway for NPs, this result represents a significant advantage for TNPc. It is also evident that the increased NEP stability cannot be explained by the length of the tail region alone as the NEP cleavage site residues at the N-terminal end of the loop region. These results demonstrate that TNPc is more stable than either ANP and BNP, supporting its candidacy as a therapeutic for heart failure.

## EXAMPLE 8

### EFFECT OF NATRIURETIC PEPTIDES ON BLOOD PRESSURE AND HEART RATE IN RABBITS

### *Material and Methods*

#### Animals

**[0317]** Experiments were conducted using 12 naïve male New Zealand white rabbits, bred from stock and housed at the Baker Heart Research Institute and whose weight ranged from 2.5 to 3.3 kg. Prior to and during experimentation each animal was housed in individual standard rabbit cages, under conditions of constant ambient temperature, constant humidity and normal light/dark cycle (with the lights on from 06:00 to 18:00). Food and water were accessible ad libitum for the duration of the study. All procedures were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government Publishing Service, Canberra, Australia, 1990) and were approved by the Animal Experimentation Committee of the Alfred Hospital/Baker Heart Research Institute.

#### Experimental Procedures

**[0318]** On the day of the experiment, the rabbit was placed in a standard single rabbit holding box (15 cm high and wide and 35 cm long) with wire top and raised wire grid floor. Under local anaesthesia (Lignocaine HCl, 1%), the central ear artery and marginal ear vein were catheterised. A one-hour recovery period was allowed before commencing the experiment.

[0319] Pulsatile arterial blood pressure was measured with a Statham P23ID strain gauge pressure transducer (Statham, Hato Rey, Puerto Rico) and HR was derived by a rate-meter triggered from the arterial pulse. Mean arterial pressure (MAP), HR and respiration rate were digitised and averaged over two-second periods by computer using the LabVIEW programming language (National Instruments, Austin, TX, USA). Throughout the experiment, cardiovascular parameters were recorded on a six-channel polygraph (Grass Instruments, model 7D, Quincy, Massachusetts, USA).

Experimental Design

*Protocol*

[0320] Each rabbit received up to eight experiments separated by a 1 week recovery. During each experiment, a dose response curve to TNP (a, b, c, c I28P, c R16H, c N7D, c P32R, c Cha-10, c R-18, c A7, c K7, c BTT), DNP, human ANP (WT-hANP), human ANP methionion oxide (hANP m/o) or a control experiment was performed with the drug administered via the marginal ear vein catheter (Table 8). For each experiment there was an initial 60-minute acclimatization period and a 45-minute period during which baseline cardiovascular parameters were obtained in duplicate. Dose-response curves were performed using 3 increasing doses of the peptides administered using a variable rate infusion pump (Harvard Apparatus, model 22 I/w, South Natick, Massachusetts, USA) at 3, 6, and 12 ml/hr to give doses of 1, 2, and 4 μg/ kg/ minute. These were the same doses as those used for ANP to give a maximum 14% reduction in blood pressure (Woods *et al.*, 1989, *J Cardiovasc* **13**(2): p. 177-185). For the time control experiment, the same volumes of vehicle (0.9% saline) were administered at the same infusion rates. Each dose was infused for 45 minutes. The order of experiments was randomised and only one dose-response curve was performed in each animal per experiment. The doses of the TNP peptides and analogues were adjusted by molecular weight to give equal molar equivalents of the 1, 2 and 4 mg/kg dose of hANP.

Data Analysis

*Haemodynamic data*

[0321] Two-second averages of all parameters were displayed on the computer and movement artefacts were excluded from the measurements. Data was averaged over 20 minutes for each of the control periods, and over the last 15 minutes of the treatments at each dose. The 3 values for each dose were also averaged to give an "average response" value.

Statistical Analysis

[0322] Values were expressed as mean difference from control ± standard error of the difference (SED). The analysis of data was performed by a multi factor split plot (nested) ANOVA which combined a repeated measure within animal design and also allowed for comparisons between groups (treatments as described in Table 8). The total sums of squares (SS) was divided into between-

groups and within- groups SS. The latter contained the between treatments SS, between animals SS and animal x treatment interaction for each of the groups (Snedecor and Cochran, 1980, Statistical methods. 7th ed. The Iowa State Univ. Press, Ames, IA). Comparisons within each group were made using a set of orthogonal contrasts. The F ratio for each contrast was calculated as the mean square for the contrast (MS) divided by the total residual MS of the groups. Contrasts within groups were made by using a set of comparisons in which the between column SS and the between animal SS were subtracted from the total SS to give the residual SS. The latter were used to calculate the average treatment standard error of the mean (indicating variation within animals). Thus, the estimate of the within group variance was made with a contribution from all the groups.

Compounds used

**[0323]** The compounds tested are listed in Table 8 and include the Taipan natriuretic peptides- (a, b, c, c I28P, c R16H, c N7D, c P32R, c Cha-10, c R-18, c A7, c K7, c BTT), DNP, human ANP and human ANP methionion oxide. The doses of drugs were expressed in μg of the base and were dissolved in 0.9% saline.

***Results and Discussion***

Blood pressure and heart rate

**[0324]** In a randomised design, several different natriuretic peptides were examined for cardiovascular effects in separate experiments, over a period of several weeks using conscious rabbits. Over the course of the experimental period, basal values of BP and HR remained constant. During each experiment, 45 minutes infusions of 1, 2 and 4 μg/ kg/min (of ANP or the molar equivalent of the peptide to be tested) were administered intravenously. The results are summarised in Table 9a-b.

**[0325]** The human form of ANP (WT-hANP) markedly lowered MAP (-15.8 ± 0.9 mmHg, $P < 0.001$, Table 9). It produced a small increase in HR of 15± 3 b/min ($P < 0.002$). Of the 4 natriuretic-like peptides, only TNPc and DNP reduced MAP, with similar average falls of 14.9 ± 0.7 mmHg and 14.2 ± 0.8 mmHg, respectively ($P < 0.001$, Table 9A). There was no difference between these responses and the MAP reduction by WT-hANP and none of the natriuretic-like peptides had a significant effect on HR. Systolic BP and diastolic BP followed the same trend as MAP. Consistent with the experiments described above, TNPa and TNPb had little or insignificant effects on blood pressure or heart rate.

**[0326]** Cardiovascular responses to a variant of human ANP, hANP methionion oxide, were compared with those to WT-hANP (Table 9B). WT-hANP m/o lowered MAP by an average – 10.6 ± 0.9 mmHg ($P < 0.05$ compared to vehicle) which was less than the response to WT-hANP (-15.8 mmHg, $P < 0.01$). Tachycardia of 28 ± 7 bpm ($P < 0.05$ compared to vehicle) accompanied the fall in MAP but the variance in HR was also increased, resulting in no difference between the HR

effects of the two human forms of ANP. The major findings were that TNPc when compared to vehicle administration produced a marked decrease in systolic and diastolic blood pressure that was similar to the human form of ANP, WT-hANP. TNPc was the most hypotensive taipan natriuretic-like peptide examined whereas the effects of TNPa and TNPb and hANP-M.O. on blood pressure were similar to those produced by vehicle in conscious normotensive rabbits.

**[0327]** Table 10A-B summarises the results of the changes to mean arterial blood pressure (MAP) during the three dose infusions and during a one hour recovery period for the range of TNP analogues described in Table 8 as belonging to group 2a and 2b. Compared to vehicle, ANP, DNP, TNPc and the majority of TNPc analogues produce reductions in MAP that are significant. In contrast, effects of TNPa, TNPb and those analogues of TNPc that substitute residues of TNPa into TNPc (I28P and R16H), do not display significant reductions in MAP compared to vehicles. These results reflect the observation made in Examples 3 and 4, that indicate the parity of effectiveness between ANP and TNPc compared to the lesser active peptides, TNPa and TNPb. Without exception, the remaining analogues of TNPc (N7D, P32R, Cha-10, R18, A7, K7, BTT) all produce reductions in MAP that are significant compared to vehicle. These results support the conception that TNPc and related analogues are effective alternatives for NP therapy.

**[0328]** The observations of the recovery period are further illuminating. The halt of WT-hANP and hANP-M.O. infusion produced an increase in mean arterial pressure (Table 10A), an observation consistent with their short half lives. Similarly, halting infusion of TNPa gave rise to an increase in blood pressure. In contrast, upon the end of TNPc infusion the MAP did not alter, suggesting that TNPc has an increased half life compared to ANP. Consistent with this, the majority of TNPc analogues also had a reduced recovery compared to ANP, Cha10 and N7D being the only exceptions. Indeed, the data imply that TNPc is even more stable than DNP, a NP that has heretofore been noted for its stability.

**[0329]** From these data it can be inferred that TNPc and related analogues are potent NPs and as such are effective at lowering blood pressure in mammals. Given that a significant problem in previous NPs is the lack of stability, possessing the characteristic of a longer half-life is a distinct advantage of TNPc over other NPs. Their exceptional stability as demonstrated here provides them with advantages that enable their usefulness as the CHF therapeutics, particularly in the chronic CHF market, which has heretofore been denied to the less robust ANP and BNP. TNPc, or related analogues, may provide a more practical long-term treatment for CHF as its half-life is longer than WT-hANP.

EXAMPLE 9

**EFFECT OF NATRIURETIC PEPTIDES ON AN OVINE MODEL OF CONGESTIVE HEART FAILURE**

**[0330]** The aim of these experiments were to compare the efficacy of TNPc with ANP for the acute treatment of moderate CHF. The animal model used was the Ovine model of tachycardia induced CHF.

***Experimental Protocol***

**[0331]** Number of Animals: 6

**[0332]** 1. Implant pacemakers in 6 adult sheep (BW 50-55 kg)

**[0333]** 2. Pace for 4 weeks at 180 bpm to create CHF (NYHC III)

**[0334]** 3. Anaesthetise and perform an echocardiographic examination. Perform a conductance catheter evaluation.

**[0335]** 4. Day 1. Commence drug infusion (randomise either TNPc or ANP to days 1 or 2) at 18 (low) and 84 ng/min/kg (high), each for 30 mins. Perform an echocardiographic and conductance catheter evaluation after each (ie at 30 mins) after each drug level. Recover animal.

**[0336]** 5. Day 2. Commence drug infusion (either TNPc or ANP according to day 1 treatment) at 18 (low) and 84 ng/min/kg (high), each for 30 mins. Perform an echocardiographic and conductance catheter evaluation after each (ie at 30 mins) after each drug level. Euthanse animal.

***Parameters Measured***

**[0337]** a) Echocardiography

**[0338]** 1. Left Ventricular Ejection Fraction

**[0339]** b) Conductance Catheter

**[0340]** 1. Left Ventricular dP/dt

**[0341]** 2. Left Ventricular End Diastolic Pressure

**[0342]** 3. Left Ventricular ESPVR

**[0343]** 4. Heart Rate

**[0344]** 5. Mean Arterial Pressure (MAP)

**[0345]** Six adult sheep were used, details are given below in Table 11a. Animals were randomised to either TNPc or ANP on two consecutive days, according to the schedule given in Table 11b. Studies were conducted over a period of 4 days with two study groups. Data is expressed as means+/- SEM. Data was analysed by Students t-test. A value of $p<0.05$ is considered significant. Stock solutions of each peptide were made up on under sterile conditions and using 600 ml of sterile

saline. The stock solutions were protected from light with aluminium foil and kept refrigerated. Animals were infused at the rates below *via* a fore-leg vein using new calibrated Terumo syringe pumps.

***Results and Discussion***

**[0346]** ANP at the high dose rate significantly increased LV Ejection Fraction (EF) from baseline and from the low dose. This was not maintained by the end of the of the recovery period (30 min). ANP significantly decreased LV End Diastolic Pressure (EDP) at the high dose rate. Once again this was not maintained during the recovery period. A decrease in EDP and an increase in ejection fraction is expected for effective treatment of CHF as it indicates that the heart is performing much more effectively as a pump. Notably, no significant increase in Heart Rate was observed for ANP, consistent with the notion that NPs do not induce tachycardia. The results from the TNPc infusions were remarkably similar to ANP. Once again there were improvements in the LVEF and decreases in the LVEDP at the high dose rates supporting that this peptide is effective in treating CHF.

**[0347]** There were no significant changes in any other parameters associated with either substance. Acute improvements in LVEF and LVEDP in heart failure subjects are a very positive finding for both substances.

**[0348]** The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

**[0349]** The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

**[0350]** Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appended claims.

## TABLE 1

### SEQUENCES OF KNOWN NATRIURETIC PEPTIDES IN HUMAN AND SNAKE

| Natriuretic Peptide | Sequence | SEQ ID |
|---|---|---|
| hANP | SLRRSSCFGGRMDRIGAQSGLGCNSFRY | SEQ ID NO:82 |
| hBNP | SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH | SEQ ID NO:83 |
| hCNP | YKGANKKGLSKGCFGLKLDRIGSMSGLGC | SEQ ID NO:84 |
| DNP | EVKYDPCFGHKIDRINHVSNLGCPSLRDPRPNAPSTSA | SEQ ID NO:85 |
| Lebetin 2 isoform α | GDNKPPKKGPPNGCFGHKIDRIGSHSGLGCNKVDDNKG | SEQ ID NO:86 |
| PNP | GENEPPKKKAPDGCFGHKIDRIGSHSGLGCNKFKPGH | SEQ ID NO:87 |
| **NP consensus** | **CFGXXXDRIXXXSXLGC** | SEQ ID NO:88 |

## TABLE 2

### SEQUENCES OF NOVEL TNPS AND THEIR SYNTHETIC ANALOGUES.

U = Selenocysteine; O = hydroxyproline, Z = pyroglutamate

| NAME | SEQUENCE | SEQUENCE ID |
|---|---|---|
| TNPa | SDSKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK | SEQ ID NO:89 |
| TNPb | SDPKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK | SEQ ID NO:90 |
| TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:91 |
| R32-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNRPKKFSGE | SEQ ID NO:92 |
| H16-TNPc | SDSKIGNGCFGFPLDHIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:93 |
| P28-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRPMQNPPKKFSGE | SEQ ID NO:94 |
| V29-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIVQNPPKKFSGE | SEQ ID NO:95 |
| R29-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIRQNPPKKFSGE | SEQ ID NO:96 |
| K26-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCKRIMQNRPKKFSGE | SEQ ID NO:97 |
| U9,U25-TNPc | SDSKIGNGUFGFPLDRIGSVSGLGUNRIMQNPPKKFSGE | SEQ ID NO:98 |
| U25-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGUNRIMQNPPKKFSGE | SEQ ID NO:99 |
| U9-TNPc | SDSKIGNGUFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:100 |
| Q7-TNPc | SDSKIGQGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:101 |
| A7-TNPc | SDSKIGAGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:102 |
| K7-TNPc | SDSKIGKGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:103 |
| S6,K7-TNPc | SDSKISKGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:104 |
| S7-TNPc | SDSKIGSGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:105 |
| R6,S7-TNPc | SDSKIRSGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:106 |
| Nle29-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRInQNPPKKFSGE | SEQ ID NO:107 |
| A31-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQAPPKKFSGE | SEQ ID NO:108 |
| Q31-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQQPPKKFSGE | SEQ ID NO:109 |
| A31,O32-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQAOPKKFSGE | SEQ ID NO:110 |
| Q31,O32-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQQOPKKFSGE | SEQ ID NO:111 |
| Z0-TNPc | ZSDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:112 |
| A7,U9,U25,Nle29,A31-TNPc | SDSKIGAGUFGFPLDRIGSVSGLGUNRInQAPPKKFSGE | SEQ ID NO:113 |
| Z0,A7,U9,U25,Nle29,A31-TNPc | ZSDSKIGAGUFGFPLDRIGSVSGLGUNRInQAPPKKFSGE | SEQ ID NO:114 |

| NAME | SEQUENCE | SEQUENCE ID |
|---|---|---|
| BBT | SPKMVQGSGCFGRKMDRISSSSGLGCNRIMQNPPKKFSGE | SEQ ID NO:115 |
| BTB | SPKMVQGSGCFGFPLDRIGSVSGLGCKVLRRH | SEQ ID NO:116 |
| TBB | SDSKIGNGCFGRKMDRISSSSGLGCKVLRRH | SEQ ID NO:117 |
| BTT | SPKMVQGSGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:118 |
| TBT | SDSKIGNGCFGRKMDRISSSSGLGCNRIMQNPPKKFSGE | SEQ ID NO:119 |
| TTB | SDSKIGNGCFGFPLDRIGSVSGLGCKVLRRH | SEQ ID NO:120 |
| AAT | SLRRSSCFGGRMDRIGAQSGLGCNRIMQNPPKKFSGE | SEQ ID NO:121 |
| ATA | SLRRSSCFGFPLDRIGSVSGLGCNSFRY | SEQ ID NO:122 |
| TAA | SDSKIGNGCFGGRMDRIGAQSGLGCNSFRY | SEQ ID NO:123 |
| ATT | SLRRSSCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:124 |
| TAT | SDSKIGNGCFGGRMDRIGAQSGLGCNRIMQNPPKKFSGE | SEQ ID NO:125 |
| TTA | SDSKIGNGCFGFPLDRIGSVSGLGCNSFRY | SEQ ID NO:126 |
| DDT | EVKYDPCFGHKIDRINHVSNLGCNRIMQNPPKKFSGE | SEQ ID NO:127 |
| DTD | EVKYDPCFGFPLDRIGSVSGLGCPSLRDPRPNAPSTSA | SEQ ID NO:128 |
| TDD | SDSKIGNGCFGHKIDRINHVSNLGCPSLRDPRPNAPSTSA | SEQ ID NO:129 |
| DTT | EVKYDPCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:130 |
| TDT | SDSKIGNGCFGHKIDRINHVSNLGCNRIMQNPPKKFSGE | SEQ ID NO:131 |
| TTD | EVKYDPCFGFPLDRIGSVSGLGCPSLRDPRPNAPSTSA | SEQ ID NO:132 |
| TLT | SDSKIGNGCFGHKIDRIGSHSGLGCNRIMQNPPKKFSGE | SEQ ID NO:133 |
| DLT | EVKYDPCFGHKIDRIGSHSGLGCNRIMQNPPKKFSGE | SEQ ID NO:134 |
| Z1-TNPc | ZDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:135 |
| A4-TNPc | SDSAIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:136 |
| A5-TNPc | SDSKAGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:137 |
| A10-TNPc | SDSKIGNGCAGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:138 |
| A12-TNPc | SDSKIGNGCFGAPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:139 |
| A14-TNPc | SDSKIGNGCFGFPADRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:140 |
| A15-TNPc | SDSKIGNGCFGFPLARIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:141 |
| A16-TNPc | SDSKIGNGCFGFPLDAIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:142 |
| A17-TNPc | SDSKIGNGCFGFPLDRAGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:143 |
| A20-TNPc | SDSKIGNGCFGFPLDRIGSASGLGCNRIMQNPPKKFSGE | SEQ ID NO:144 |

| NAME | SEQUENCE | SEQUENCE ID |
|---|---|---|
| A23-TNPc | SDSKIGNGCFGFPLDRIGSVSGAGCNRIMQNPPKKFSGE | SEQ ID NO:145 |
| A26-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCARIMQNPPKKFSGE | SEQ ID NO:146 |
| A27-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNAIMQNPPKKFSGE | SEQ ID NO:147 |
| A28-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRAMQNPPKKFSGE | SEQ ID NO:148 |
| A29-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIAQNPPKKFSGE | SEQ ID NO:149 |
| A30-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMANPPKKFSGE | SEQ ID NO:150 |
| A31-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQAPPKKFSGE | SEQ ID NO:151 |
| A34-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPAKFSGE | SEQ ID NO:152 |
| A35-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKAFSGE | SEQ ID NO:153 |
| A36-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKASGE | SEQ ID NO:154 |
| A39-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGA | SEQ ID NO:155 |
| Q4-TNPc | SDSQIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:156 |
| Q16-TNPc | SDSKIGNGCFGFPLDQIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:157 |
| Q27-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNQIMQNPPKKFSGE | SEQ ID NO:158 |
| Q34-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPQKFSGE | SEQ ID NO:159 |
| Q35-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKQFSGE | SEQ ID NO:160 |
| P31,R32-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQPRPKKFSGE | SEQ ID NO:161 |
| E4-TNPc | SDSEIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:162 |
| E16-TNPc | SDSKIGNGCFGFPLDEIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:163 |
| E27-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNEIMQNPPKKFSGE | SEQ ID NO:164 |
| E34-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPEKFSGE | SEQ ID NO:165 |
| E35-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKEFSGE | SEQ ID NO:166 |
| K26,V27,L28,R29,R30,H31-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCKVLRRHPPKKFSGE | SEQ ID NO:167 |
| Y10-TNPc | SDSKIGNGCFGYPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:168 |
| TNPc-NH2 | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGENH2 | SEQ ID NO:169 |
| Z1-TNPc-NH2 | ZDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGENH2 | SEQ ID NO:170 |
| L5-TNPc | SDSKLGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:171 |
| L10-TNPc | SDSKIGNGCLGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:172 |
| L12-TNPc | SDSKIGNGCFGLPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:173 |
| L20-TNPc | SDSKIGNGCFGFPLDRIGSLSGLGCNRIMQNPPKKFSGE | SEQ ID NO:174 |

| NAME | SEQUENCE | SEQUENCE ID |
|---|---|---|
| L27-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNLIMQNPPKKFSGE | SEQ ID NO:175 |
| L28-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRLMQNPPKKFSGE | SEQ ID NO:176 |
| L29-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRILQNPPKKFSGE | SEQ ID NO:177 |
| L30-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMLNPPKKFSGE | SEQ ID NO:178 |
| L36-TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKLSGE | SEQ ID NO:179 |
| L17-TNPc | SDSKIGNGCFGFPLDRLGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:180 |
| R12-TNPc | SDSKIGNGCFGRPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:181 |
| K13-TNPc | SDSKIGNGCFGFKLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:182 |
| R13-TNPc | SDSKIGNGCFGFRLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:183 |
| R18-TNPc | SDSKIGNGCFGFPLDRIRSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO:184 |
| A7,R18,U9,U25,Nle29-TNPc | SDSKIGAGUFGFPLDRIRSVSGLGUNRInQNPPKKFSGE | SEQ ID NO:185 |
| A7,R18,U9,U25,Nle29, A31-TNPc | SDSKIGAGUFGFPLDRIRSVSGLGUNRInQAPPKKFSGE | SEQ ID NO:186 |

## TABLE 3

AMINO ACID SUB-CLASSIFICATION

| Sub-classes | Amino acids |
|---|---|
| Acidic | Aspartic acid, Glutamic acid |
| Basic | Noncyclic: Arginine, Lysine; Cyclic: Histidine |
| Charged | Aspartic acid, Glutamic acid, Arginine, Lysine, Histidine |
| Small | Glycine, Serine, Alanine, Threonine, Proline |
| Polar/neutral | Asparagine, Histidine, Glutamine, Cysteine, Serine, Threonine |
| Polar/large | Asparagine, Glutamine |
| Hydrophobic | Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan |
| Aromatic | Tryptophan, Tyrosine, Phenylalanine |
| Residues that influence chain orientation | Glycine and Proline |

## TABLE 4

### ABBREVIATIONS FOR AMINO ACIDS

| Amino Acid | 1-Letter Symbol | 3-Letter Symbol | Amino Acid | 1-Letter Symbol | 3-Letter Symbol |
|---|---|---|---|---|---|
| Alanine | A | Ala | Leucine | L | Leu |
| Arginine | R | Arg | Lysine | K | Lys |
| Asparagine | N | Asn | Methionine | M | Met |
| Aspartic acid | D | Asp | Phenylalanine | F | Phe |
| Cysteine | C | Cys | Proline | P | Pro |
| Glutamine | Q | Gln | Serine | S | Ser |
| Glutamic acid | E | Glu | Threonine | T | Thr |
| Glycine | G | Gly | Tryptophan | W | Trp |
| Histidine | H | His | Tyrosine | Y | Tyr |
| Isoleucine | I | Ile | Valine | V | Val |
| Pyroglutamate | Z | Pyr | Norleucine | n | Nle |

## TABLE 5

### EXEMPLARY AND PREFERRED AMINO ACID SUBSTITUTIONS

| Original Residue | Exemplary Substitutions | Preferred Substitutions |
|---|---|---|
| Ala | Val, Leu, Ile | Val |
| Arg | Lys, Gln, Asn | Lys |
| Asn | Gln, His, Lys, Arg, Asp | Gln |
| Asp | Glu, Asn | Glu |
| Cys | Ser | Ser |
| Gln | Asn, His, Lys, | Asn |
| Glu | Asp, Lys, Gla | Asp |
| Gly | Pro | Pro |
| His | Asn, Gln, Lys, Arg | Arg |
| Ile | Leu, Val, Met, Ala, Phe, Norleu | Leu |
| Leu | Norleu, Ile, Val, Met, Ala, Phe | Ile |
| Lys | Arg, Gln, Asn, Orn | Arg |
| Met | Leu, Ile, Phe, Nle | Leu |
| Phe | Leu, Val, Ile, Ala | Leu |
| Pro | Gly, Hyp, Ser, Thr | Gly |
| Ser | Thr | Thr |
| Thr | Ser | Ser |
| Trp | Tyr | Tyr |
| Tyr | Trp, Phe, Thr, Ser | Phe |
| Val | Ile, Leu, Met, Phe, Ala, Nle | Leu |

**TABLE 6**

| Non-Conventional Amino Acids | |
|---|---|
| α-aminobutyric acid | L-N-methylalanine |
| α-amino-α-methylbutyrate | L-N-methylarginine |
| aminocyclopropane-carboxylate | L-N-methylasparagine |
| aminoisobutyric acid | L-N-methylaspartic acid |
| aminonorbornyl-carboxylate | L-N-methylcysteine |
| cyclohexylalanine | L-N-methylglutamine |
| cyclopentylalanine | L-N-methylglutamic acid |
| L-N-methylisoleucine | L-N-methylhistidine |
| D-alanine | L-N-methylleucine |
| D-arginine | L-N-methyllysine |
| D-aspartic acid | L-N-methylmethionine |
| D-cysteine | L-N-methylnorleucine |
| D-glutamate | L-N-methylnorvaline |
| D-glutamic acid | L-N-methylornithine |
| D-histidine | L-N-methylphenylalanine |
| D-isoleucine | L-N-methylproline |
| D-leucine | L-N-medlylserine |
| D-lysine | L-N-methylthreonine |
| D-methionine | L-N-methyltryptophan |
| D-ornithine | L-N-methyltyrosine |
| D-phenylalanine | L-N-methylvaline |
| D-proline | L-N-methylethylglycine |
| D-serine | L-N-methyl-t-butylglycine |
| D-threonine | L-norleucine |
| D-tryptophan | L-norvaline |
| D-tyrosine | α-methyl-aminoisobutyrate |
| D-valine | α-methyl-γ-aminobutyrate |
| D-α-methylalanine | α-methylcyclohexylalanine |
| D-α-methylarginine | α-methylcylcopentylalanine |
| D-α-methylasparagine | α-methyl-α-napthylalanine |
| D-α-methylaspartate | α-methylpenicillamine |
| D-α-methylcysteine | N-(4-aminobutyl)glycine |
| D-α-methylglutamine | N-(2-aminoethyl)glycine |
| D-α-methylhistidine | N-(3-aminopropyl)glycine |

| Non-Conventional Amino Acids | |
|---|---|
| D-α-methylisoleucine | N-amino-α-methylbutyrate |
| D-α-methylleucine | α-napthylalanine |
| D-α-methyllysine | N-benzylglycine |
| D-α-methylmethionine | N-(2-carbamylediyl)glycine |
| D-α-methylornithiine | N-(carbamylmethyl)glycine |
| D-α-methylphenylalanine | N-(2-carboxyethyl)glycine |
| D-α-methylproline | N-(carboxymethyl)glycine |
| D-α-methylserine | N-cyclobutylglycine |
| D-α-methylthreonine | N-cycloheptylglycine |
| D-α-methyltryptophan | N-cyclohexylglycine |
| D-α-methyltyrosine | N-cyclodecylglycine |
| L-α-methylleucine | L-α-methyllysine |
| L-α-methylmethionine | L-α-methylnorleucine |
| L-α-methylnorvatine | L-α-methylornithine |
| L-α-methylphenylalanine | L-α-methylproline |
| L-α-methylserine | L-α-methylthreonine |
| L-α-methyltryptophan | L-α-methyltyrosine |
| L-α-methylvaline | L-N-methylhomophenylalanine |
| N-(N-(2,2-diphenylethyl carbamylmethyl)glycine | N-(N-(3,3-diphenylpropyl carbamylmethyl)glycine |
| 1-carboxy-1-(2,2-diphenyl-ethyl amino)cyclopropane | |

## TABLE 7

### PEPTIDE HALF LIFE IN PLASMIN

***One phase exponential decay; best fit values***

| | TNPc | DNP | BNP | ANP |
|---|---|---|---|---|
| SPAN | 0.8454 | 0.7306 | 0.9926 | 0.9579 |
| K | 0.02935 | 0.02381 | 0.053 | 0.3413 |
| PLATEAU | 0.1128 | 0.1675 | 0.03403 | 0.04207 |
| HalfLife (min) | 23.61 | 29.11 | 13.08 | 2.031 |

**TABLE 8**

**PEPTIDES TESTED AND THEIR GROUPINGS**

| Group 1 | Group 2a | Group 2b | Group 3 |
|---|---|---|---|
| TNP variants | TNPc variants | TNPc variants | Human ANP |
| vehicle | vehicle | vehicle | vehicle |
| WT-hANP | TNPc | TNPc | WT-hANP |
| TNPa | TNPc 128P | TNPc Cha-10 | hANP m/o |
| TNPb | TNPc R16H | TNPc R-18 | |
| TNPc | TNPc N7D | TNPc A7 | |
| DNP | TNPc P32R | TNPc K7 | |
| | | TNPc BTT | |

Group 1: TNP peptides are compared with human ANP

Group 2: TNPc variants are compared with TNPc

Group 3: Human ANP variants are compared with human ANP

## TABLE 9A

## EFFECT OF INTRAVENOUS VEH, WT-HANP, TNPA, TNPB, TNPC AND DNP IN CONSCIOUS RABBITS

| | | | | | | | | | | Significance | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Dose (μg/kg/min) | | | | Linear | Non | Average | | Veh vs | WT-hANP vs |
| | n | 1.0 | 2.0 | 4.0 | SED | Trend | Linear | Response | SED | treatment | treatment |
| **Veh** | 10 | | | | | | | | | | |
| Systolic BP (mmHg) | | -3.0 | -5.9 | -7.5 | 1.6 | * | NS | -5.5 ± 0.9 | | | |
| Diastolic BP (mmHg) | | -5.8 | -6.7 | -5.4 | 1.1 | NS | NS | -5.9 ± 0.6 | | | |
| Mean BP (mmHg) | | -5.9 | -7.3 | -6.8 | 1.1 | NS | NS | -6.7 ± 0.7 | | | |
| HR (bpm) | | -2.8 | 3.6 | 12.9 | 7.3 | NS | NS | 4.6 ± 4.2 | | | |
| **WT-hANP** | 8 | | | | | | | | | | |
| Systolic BP (mmHg) | | -10.6 | -13.0 | -14.5 | 1.6 | NS | NS | -12.7 ± 0.9 | | *** | |
| Diastolic BP (mmHg) | | -9.7 | -12.3 | -14.4 | 1.4 | * | NS | -12.1 ± 0.8 | | *** | |
| Mean BP (mmHg) | | -13.1 | -15.7 | -18.5 | 1.3 | ** | NS | -15.8 ± 0.7 | | *** | |
| HR (bpm) | | 17.1 | 19.0 | 9.0 | 5.5 | NS | NS | 15.1 ± 3.2 | | NS | |
| **TNPa** | 5 | | | | | | | | | | |
| Systolic BP (mmHg) | | -2.8 | -4.9 | -9.4 | 2.3 | * | NS | -5.7 ± 1.3 | | NS | *** |
| Diastolic BP (mmHg) | | -6.0 | -7.7 | -9.9 | 1.6 | NS | NS | -7.9 ± 0.9 | | NS | * |
| Mean BP (mmHg) | | -6.4 | -8.0 | -11.6 | 1.8 | NS | NS | -8.7 ± 1.1 | | NS | *** |
| HR (bpm) | | -16.1 | -15.9 | -9.8 | 6.6 | NS | NS | -13.9 ± 3.8 | | * | *** |
| **TNPb** | 5 | | | | | | | | | | |
| Systolic BP (mmHg) | | -7.9 | -8.8 | -10.0 | 2.4 | NS | NS | -8.9 ± 1.4 | | NS | NS |
| Diastolic BP (mmHg) | | -7.0 | -7.9 | -8.6 | 1.6 | NS | NS | -7.8 ± 0.9 | | NS | * |
| Mean BP (mmHg) | | -8.0 | -9.8 | -11.0 | 1.8 | NS | NS | -9.6 ± 1.1 | | NS | *** |
| HR (bpm) | | -3.9 | -5.1 | 4.7 | 6.0 | NS | NS | -1.4 ± 3.5 | | NS | NS |
| **TNPc** | 5 | | | | | | | | | | |
| Systolic BP (mmHg) | | -9.1 | -15.6 | -14.9 | 1.3 | NS | NS | -13.2 ± 0.8 | | *** | NS |
| Diastolic BP (mmHg) | | -10.0 | -14.7 | -12.8 | 1.1 | NS | NS | -12.5 ± 0.6 | | *** | NS |
| Mean BP (mmHg) | | -11.0 | -17.7 | -15.9 | 1.2 | NS | NS | -14.9 ± 0.7 | | *** | NS |
| HR (bpm) | | -3.5 | 3.7 | 13.8 | 6.6 | NS | NS | 4.7 ± 3.8 | | NS | NS |
| **DNP** | 5 | | | | | | | | | | |
| Systolic BP (mmHg) | | -11.7 | -11.9 | -16.4 | 1.4 | NS | NS | -13.3 ± 0.8 | | *** | NS |
| Diastolic BP (mmHg) | | -8.5 | -6.9 | -11.7 | 1.6 | NS | NS | -9.0 ± 0.9 | | NS | NS |
| Mean BP (mmHg) | | -13.0 | -12.6 | -17.2 | 1.4 | NS | NS | -14.2 ± 0.8 | | *** | NS |
| HR (bpm) | | 17.5 | 25.9 | 8.5 | 11.1 | NS | NS | 17.3 ± 6.4 | | NS | NS |

Values are mean difference from control and SED indicating group variance
Significance :* P <0.05, ** P<0.01 , *** P<0.001, NS P>0.05

## TABLE 9B

### VEHICLE VS WT- hANP AND hANP M/O IN CONSCIOUS RABBITS

| | n | Dose (μg/kg/min) 1.0 | 2.0 | 4.0 | SED | Linear Trend | Non Linear | Average Response | SED | Significance Vehicle vs treatment | WT- hANP vs hANP m/o |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Vehicle** | 10 | | | | | | | | | | |
| Systolic BP (mmHg) | | **-3.0** | **-5.9** | **-7.5** | 1.6 | NS | NS | -5.5 ± 0.9 | | | |
| Diastolic BP (mmHg) | | **-5.8** | **-6.7** | **-5.4** | 1.1 | NS | NS | -5.9 ± 0.6 | | | |
| Mean BP (mmHg) | | **-5.9** | **-7.3** | **-6.8** | 1.1 | NS | NS | -6.7 ± 0.7 | | | |
| HR (bpm) | | **-2.8** | **3.6** | **12.9** | 7.3 | NS | NS | 4.6 ± 4.2 | | | |
| **WT- hANP** | 8 | | | | | | | | | | |
| Systolic BP (mmHg) | | **-10.6** | **-13.0** | **-14.5** | 1.6 | NS | NS | -12.7 ± 0.9 | | *** | |
| Diastolic BP (mmHg) | | **-9.7** | **-12.3** | **-14.4** | 1.4 | * | NS | -12.1 ± 0.8 | | *** | |
| Mean BP (mmHg) | | **-13.1** | **-15.7** | **-18.5** | 1.3 | * | NS | -15.8 ± 0.7 | | *** | |
| HR (bpm) | | **17.1** | **19.0** | **9.0** | 5.5 | NS | NS | 15.1 ± 3.2 | | NS | |
| **hANP m/o** | 8 | | | | | | | | | | |
| Systolic BP (mmHg) | | **-3.1** | **-11.8** | **-10.5** | 1.5 | ** | * | -8.5 ± 0.8 | | NS | * |
| Diastolic BP (mmHg) | | **-7.6** | **-10.0** | **-8.1** | 1.7 | NS | NS | -8.6 ± 1.0 | | NS | NS |
| Mean BP (mmHg) | | **-8.2** | **-12.3** | **-11.2** | 1.5 | NS | NS | -10.6 ± 0.9 | | * | ** |
| HR (bpm) | | **7.5** | **27.1** | **47.7** | 11.4 | * | NS | 27.5 ± 6.6 | | * | NS |

Values are mean difference from control and SED indicating group variance
Significance :* P <0.05, ** P<0.01 , *** P<0.001, NS P>0.05

## TABLE 10A

### CHANGE OF MEAN BLOOD PRESSURE DURING THE 1 HOUR RECOVERY PERIOD FOR GROUP 2A PEPTIDES

| | | | | | Recovery | | |
|---|---|---|---|---|---|---|---|
| Treatment | Treatment Effect | Significance | MAP at Final dose | Recovery | Delta | SEM | Significance |
| Veh | -6.6 | | 67.5 | 67.2 | -0.3 ± | 0.9 | NS |
| WT-hANP | -18.5 | *** | 53.8 | 59.2 | 5.8 ± | 0.9 | *** |
| hANP- M.O. | -11.2 | NS | 60.5 | 62.5 | 2.0 ± | 2.4 | NS |
| TNPa | -11.6 | NS | 62.6 | 65.5 | 2.9 ± | 3.3 | NS |
| TNPb | -11.0 | NS | 66.5 | 65.6 | -0.9 ± | 0.6 | NS |
| TNPc | -15.9 | *** | 56.3 | 56.5 | 0.1 ± | 2.8 | NS |
| TNPc I28P | -7.5 | NS | 62.6 | 64.8 | 2.1 ± | 2.3 | NS |
| TNPc R16H | -5.9 | NS | 67.3 | 68.6 | 1.3 ± | 4.5 | NS |
| TNPc N7D | -19.8 | *** | 57.5 | 67.5 | 10.0 ± | 5.3 | NS |
| TNPc P32R | -19.9 | *** | 56.9 | 57.8 | 0.9 ± | 1.1 | NS |
| DNP | -17.2 | *** | 58.9 | 61.4 | 2.5 ± | 1.1 | NS |

Values are mean and SEM indicating group variance
Significance :* P <0.05, ** P<0.01 , *** P<0.001, NS P>0.05 compared to vehicle

## TABLE 10B

### CHANGE OF MEAN BLOOD PRESSURE DURING THE 1 HOUR RECOVERY PERIOD FOR GROUP 2B PEPTIDES

| | | | | Recovery | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Treatment Effect | Significance | MAP at Final dose | Recovery | Delta | SEM | Significance |
| **Veh** | -4.2 | | 67.5 | 67.2 | **-0.3** | **0.9** | **NS** |
| **WT-hANP** | -18.5 | *** | 53.8 | 59.2 | **5.8** | **0.9** | *** |
| **TNPc** | -15.9 | *** | 56.3 | 56.5 | **0.1** | **2.8** | **NS** |
| **cha-10** | -15.9 | ** | 64.1 | 72.7 | **8.6** | **2.2** | **NS** |
| **R-18** | -13.2 | * | 62.0 | 65.0 | **3.0** | **1.1** | **NS** |
| **A7** | -14.9 | ** | 62.6 | 66.4 | **3.9** | **6.4** | **NS** |
| **K7** | -12.4 | * | 59.9 | 64.4 | **4.4** | **3.1** | **NS** |
| **BTT** | -17.0 | *** | 58.8 | 62.3 | **3.5** | **2.2** | **NS** |

Values are mean and SEM indicating group variance
Significance :* P <0.05, ** P<0.01 , *** P<0.001, NS P>0.05 compared to vehicle

## TABLE 11A

### DRUG CONCENTRATIONS AND INFUSION LEVELS

| Animal ID | Drug Conc ng/mL | Low ng/min | Low Total | Low Inf ml/min | Hi ng/min | Hi Total | Hi Inf ml/min |
|---|---|---|---|---|---|---|---|
| 100 | 2000 | 945 | 28350 | 0.47 | 4536 | 136080 | 2.27 |
| 226 | | 1120 | 33600 | 0.56 | 5376 | 161280 | 2.69 |
| 81 | | 910 | 27300 | 0.46 | 4368 | 131040 | 2.18 |
| 229 | | 910 | 27300 | 0.46 | 4368 | 131040 | 2.18 |
| 236 | | 1207.5 | 36225 | 0.60 | 5796 | 173880 | 2.90 |
| 207 | | 910 | 27300 | 0.46 | 4368 | 131040 | 2.18 |

## TABLE 11B

### DOSAGE SCHEDULE

| Animal ID | Weight kg | Day 1 | Day2 | Start Date |
|---|---|---|---|---|
| 100 | 54 | ANP | TNP | 19/05/2005 |
| 226 | 64 | TNP | ANP | 19/05/2005 |
| 81 | 52 | ANP | TNP | 19/05/2005 |
| 229 | 52 | TNP | ANP | 23/05/2005 |
| 236 | 69 | ANP | TNP | 23/05/2005 |
| 207 | 52 | TNP | ANP | 23/05/2005 |

**TABLE 12A**

**EFFECTS OF ANP ON HEMODYNAMIC PARAMETERS IN CHF-INDUCED SHEEP**

| | ejection fraction | EDP | +dP/dt | -dP/dt | ESPR slope | heart rate |
|---|---|---|---|---|---|---|
| baseline | 37.38 ± 2.60 | 7.67 ± 2.06 | 1292.50 ± 191.39 | 1535.33 ± 160.82 | 2.97 ± 0.69 | 98.83 ± 8.39 |
| low | 42.17 ± 2.57 | 5.83 ± 2.82 | 949.33 ± 45.10 | 1336.50 ± 189.70 | 2.85 ± 0.44 | 91.00 ± 8.49 |
| high | 54.50 ± 3.94 *#§ | 3.33 ± 3.08 *# | 1047.00 ± 39.92 | 1332.17 ±164.33 | 3.04 ± 0.42 | 96.33 ± 7.77 |
| recovery | 41.00 ± 2.99 | 6.33 ± 3.20 | 1035.83 ±58.66 | 1232.17 ± 176.54 | 3.44 ± 0.59 | 91.00 ± 4.09 |

* $p<0.05$ vs baseline; # $p<0.05$ vs low; § $p<0.05$ vs recovery

**TABLE 12B**

**EFFECTS OF TNPC ON HEMODYNAMIC PARAMETERS IN CHF-INDUCED SHEEP**

| | ejection fraction | EDP | +dP/dt | -dP/dt | ESPR slope | heart rate |
|---|---|---|---|---|---|---|
| baseline | 41.00 ± 3.16 | 8.50 ± 1.65 | 1271.83 ± 203.40 | 1774.67 ± 168.58 | 2.53 ± 0.24 | 103.83 ± 7.27 |
| low | 48.17 ± 4.66 | 5.83 ± 1.51 | 1079.67 ± 182.62 | 1469.50 ± 252.11 * | 2.72 ± 0.56 | 98.00 ± 9.71 |
| high | 61.83 ± 6.01 *#§ | 4.17 ± 1.30 #§ | 1078.00 ± 132.83 | 1298.00 ± 145.72 * | 3.78 ± 0.73 | 100.67 ± 5.90 |
| recovery | 42.33 ± 4.79 | 7.67 ± 0.33 | 1064.83 ± 51.53 | 1201.50 ± 200.38 * | 3.35 ± 0.63 | 98.00 ± 3.58 |

* $p<0.05$ vs baseline; # $p<0.05$ vs low; § $p<0.05$ vs recovery

## WHAT IS CLAIMED IS:

1. An isolated proteinaceous compound comprising at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals, wherein the compound is represented by the formula:

$$\Delta\text{–}\Theta\text{–}\Omega \qquad \text{(I)}$$

wherein:

$\Delta$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of that portion;

$\Theta$ is the core ring structure of a natriuretic peptide or a variant or derivative of the structure;

$\Omega$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of that portion; and

at least one of $\Delta$, $\Theta$ and $\Omega$ comprises a biologically active portion of TNPc or a variant or derivative thereof.

2. A compound according to claim 1, wherein the biologically active portion comprises a C-terminal portion of TNPc.

3. A compound according to claim 2, wherein the C-terminal portion comprises a sequence selected from any one of SEQ ID NO:1-5, or a variant or derivative thereof.

4. A compound according to claim 1, wherein the biologically active portion comprises the TNPc core ring structure sequence set forth in SEQ ID NO:6, or a variant or derivative thereof.

5. A compound according to claim 1, wherein the biologically active portion comprises a N-terminal portion of TNPc.

6. A compound according to claim 5, wherein the N-terminal portion comprises the sequence set forth in SEQ ID NO:7, or a variant or derivative thereof.

7. A compound according to claim 1, wherein the variant or derivative of the biologically active portion of TNPc comprises a sequence having at least 80, 85, 90, 96, 97, 98 or 99% sequence similarity with, or differs at no more than 1, 2, 3, 4, 5 or 10 amino acid residues from, the corresponding portion represented by any one of SEQ ID NO:1-7, with the proviso that: (a) when the variant or derivative corresponds to a C-terminal portion of TNPc, it comprises a hydrophobic amino acid residue, at position 28 relative to the amino acid sequence of TNPc; and (b) when the variant or derivative corresponds to the core ring structure of TNPc, it comprises a basic amino acid residue, e.g., R or K at position 16 relative to the amino acid sequence of TNPc.

8. A compound according to claim 7, wherein the variant or derivative of the biologically active portion of TNPc shares at least 90% sequence identity with any one of SEQ ID NO:1-7.

9. A compound according to claim 1, wherein when $\Delta$, $\Theta$ or $\Omega$ comprise a biologically active portion of a natriuretic peptide other than TNPc or its variants or derivatives, the natriuretic peptide is selected from ANP, BNP, CNP, DNP, VNP, PNP or an *Oxyuranus* natriuretic peptide other than TNPc, or its variants or derivatives.

10. A compound according to claim 1, having the formula:

$$\Delta_1\text{–}\Theta_1\text{–}\Omega_1 \qquad \textbf{(II)}$$

wherein:

$\Delta_1$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of the portion;

$\Theta_1$ is the core ring structure of a natriuretic peptide or a variant or derivative of the structure; and

$\Omega_1$ is a C-terminal portion comprising an amino acid sequence having the formula:

$$\mathbf{X_1\ X_2\ \Phi_1\ X_3\ X_4\ X_5\ X_6\ \Sigma_1\ X_7\ X_8\ \Lambda} \qquad \textbf{(III)}$$

wherein:

$X_{1\text{-}8}$ are independently selected from any amino acid residue, or modified form thereof;

$\Phi_1$ is selected from any hydrophobic amino acid residue, or modified form thereof; and

$\Sigma_1$ is a small amino acid residue, or modified form thereof; and

$\Lambda$ is absent or is a peptide comprising an amino acid sequence having the formula:

$$\mathbf{X_9\ \Sigma_2\ \Sigma_3\ X_{10}\ X_{11}} \qquad \textbf{(IV)}$$

wherein:

$X_9$ is selected from any amino acid residue, or modified form thereof;

$\Sigma_2$ is absent or is selected from any small amino acid residue, or modified form thereof;

$\Sigma_3$ is absent or is selected from any small amino acid residue, or modified form thereof, when $\Sigma_2$ is present;

$X_{10}$ is selected from any amino acid residue, or modified form thereof, when $\Sigma_3$ is present; and

$X_{11}$ is selected from any amino acid residue, or modified form thereof, when $X_{10}$ is present,

with the proviso that formula III excludes:

SEQ ID NO:8 when $\Delta_1\text{–}\Theta_1$ consists of SEQ ID NO:187;

SEQ ID NO:9 when $\Delta_1$–$\Theta_1$ consists of SEQ ID NO:188;

SEQ ID NO:10 when $\Delta_1$–$\Theta_1$ consists of SEQ ID NO:189;

SEQ ID NO:11 when $\Delta_1$–$\Theta_1$ consists of SEQ ID NO:190;

SEQ ID NO:12 when $\Delta_1$–$\Theta_1$ consists of SEQ ID NO:191; and

SEQ ID NO:13 when $\Delta_1$–$\Theta_1$ consists of SEQ ID NO:192.

11. A compound according to claim 10, wherein $X_1$ is selected from a basic, neutral/polar or a small amino acid residue, or modified form thereof.

12. A compound according to claim 10, wherein $X_2$ is selected from a hydrophobic, basic or a small amino acid residue, or modified form thereof.

13. A compound according to claim 10, wherein $\Phi_1$ is an amino acid residue that comprises an aliphatic or aromatic side chain, or modified form thereof.

14. A compound according to claim 10, wherein $X_3$ is selected from a basic or hydrophobic amino acid residue, or modified form thereof.

15. A compound according to claim 10, wherein $X_4$ is selected from a neutral/polar, basic amino acid residue, hydrophobic or acidic amino acid residue, or modified form thereof.

16. A compound according to claim 10, wherein $X_5$ is selected from a neutral/polar, basic or small amino acid residue, or modified form thereof.

17. A compound according to claim 10, wherein $X_6$ is selected from a basic or small amino acid residue, or modified form thereof.

18. A compound according to claim 10, wherein $\Sigma_1$ is P, or modified form thereof.

19. A compound according to claim 10, wherein $X_7$ is selected from a basic or a neutral/polar amino acid residue, or modified form thereof.

20. A compound according to claim 10, wherein $X_8$ is selected from a basic or small amino acid residue, or modified form thereof.

21. A compound according to claim 10, wherein $X_9$ is selected from a hydrophobic or small amino acid residue, or modified form thereof.

22. A compound according to claim 10, wherein $\Sigma_2$ is S, or modified form thereof.

23. A compound according to claim 10, wherein $\Sigma_3$ is selected from G or T, or modified form thereof.

24. A compound according to claim 10, wherein $X_{10}$ is selected from an acidic or small amino acid residue, or modified form thereof.

25. A compound according to claim 10, wherein $X_{11}$ is A, or modified form thereof.

26. A compound according to claim 10, wherein formula (III) is represented by:

$$\Pi_1\ \Psi_1\ \Phi_2\ \Phi_3\ \Pi_2\ \Pi_3\ \mathbf{X_{12}}\ \Sigma_4\ \Psi_2\ \Psi_3\ \Gamma \qquad \text{(V)}$$

wherein:

$\Pi_1$, $\Pi_2$ and $\Pi_3$ are independently selected from any neutral/polar amino acid residue, or modified form thereof;

$\Psi_1$ $\Psi_2$ and $\Psi_3$ are independently selected from any basic amino acid residue, or modified form thereof;

$\Phi_2$ and $\Phi_3$ are independently selected from any hydrophobic amino acid residue, or modified form thereof;

$X_{12}$ is selected from any amino acid residue, or modified form thereof;

$\Sigma_4$ is a small amino acid residue, or modified form thereof; and

$\Gamma$ is absent or is a peptide of 1-10 amino acid residues.

27. A compound according to claim 26, wherein $\Pi_1$ is a polar/large amino acid residue, or modified form thereof.

28. A compound according to claim 26, wherein $\Psi_1$ is R, or modified form thereof.

29. A compound according to claim 26, wherein $\Phi_2$ is an amino acid residue that comprises an aliphatic side chain, or an aromatic side chain, or modified form thereof.

30. A compound according to claim 26, wherein $\Phi_3$ is selected from M or V, or modified form thereof.

31. A compound according to claim 26, wherein $\Pi_2$ is a polar/large amino acid residue, or modified form thereof.

32. A compound according to claim 26, wherein $\Pi_3$ is a polar/large amino acid residue, or modified form thereof.

33. A compound according to claim 26, wherein $X_{12}$ is a small or basic amino acid residue, or modified form thereof.

34. A compound according to claim 26, wherein $\Sigma_4$ is P, or modified form thereof.

35. A compound according to claim 26, wherein $\Psi_2$ is K, or modified form thereof.

36. A compound according to claim 26, wherein $\Psi_3$ is K, or modified form thereof.

37. A compound according to claim 26, wherein $\Pi_1$ is N, or modified form thereof, $\Psi_1$ is R, or modified form thereof, $\Phi_2$ is I, L or F, or modified form thereof, $\Phi_3$ is M or V, or modified form thereof, $\Pi_2$ is Q, or modified form thereof, $\Pi_3$ is N, or modified form thereof, $X_{12}$ is selected from R or P, or modified form thereof, $\Sigma_4$ is P, or modified form thereof, and each of $\Psi_2$ and $\Psi_3$ are K or modified form thereof.

38. A compound according to claim 26, wherein $\Gamma$ is present and is represented by the formula:

$$\Phi_4 \Sigma_5 \Sigma_6 \Xi \qquad \text{(VI)}$$

wherein:

$\Phi_4$ is a hydrophobic amino acid residue;

$\Sigma_5$ is small amino acid residue, or modified form thereof;

$\Sigma_6$ is small amino acid residue, or modified form thereof; and

$\Xi$ is an acidic amino acid residue, or modified form thereof.

39. A compound according to claim 38, wherein formula (VI) is represented by the sequence set forth in SEQ ID NO:14, or a variant or derivative thereof.

40. A compound according to claim 10, wherein the C-terminal portion represented by $\Omega_1$ is selected from any one of: SEQ ID NO:15-48.

41. A compound according to claim 10, wherein $\Delta_1$ and $\Theta_1$ are independently selected from N-terminal portions and core structures, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP, VNP, PNP or an *Oxyuranus* natriuretic peptide such as a natriuretic peptide from *Oxyuranus microlepidotus*, or their variants or derivatives.

42. A compound according to claim 10, wherein $\Delta_1$ is present and is represented by the formula:

$$\mathbf{O\ \Sigma_1\ \vartheta\ X_1\ X_2\ X_3\ X_4\ X_5\ \Sigma_2} \qquad \textbf{(VII)}$$

wherein:

O is absent or is a peptide of 1-10 amino acid residues;

$\Sigma_{1-2}$ are independently selected from any small amino acid residue, or modified form thereof;

$\vartheta$ is selected from any charged amino acid residue, or modified form thereof; and

$X_{1-5}$ are independently selected from any amino acid residue, or modified form thereof.

43. A compound according to claim 42, wherein O is selected from a small amino acid residue, or modified form thereof, or a protecting moiety.

44. A compound according to claim 42, wherein $\Sigma_1$ is selected from P or S, or modified form thereof.

45. A compound according to claim 42, wherein $\vartheta$ is selected from an acidic or basic amino acid residue, or modified form thereof.

46. A compound according to claim 42, wherein $X_1$ is selected from a hydrophobic, small or acidic amino acid residue, or modified form thereof.

47. A compound according to claim 42, wherein $X_2$ is selected from a basic or hydrophobic amino acid residue, or modified form thereof.

48. A compound according to claim 42, wherein $X_3$ is selected from a hydrophobic, neutral/polar or basic amino acid residue, or modified form thereof.

49. A compound according to claim 42, wherein $X_4$ is selected from a small, basic or hydrophobic amino acid residue, or modified form thereof.

50. A compound according to claim 42, wherein $X_5$ is selected from a neutral/polar, small, basic or acidic amino acid residue, or modified form thereof.

51. A compound according to claim 42, wherein $\Sigma_2$ is selected from P, S or G, or modified form thereof.

52. A compound according to claim 42, wherein $\Sigma_1$ is S, or modified form thereof, 9 is D, or modified form thereof, $X_1$ is selected a small amino acid residue, or modified form thereof, $X_2$ is K, or modified form thereof, $X_3$ is I, or modified form thereof, $X_4$ is G, or modified form thereof, $X_5$ is selected from D or N, or modified form thereof, and $\Sigma_2$ is G, or modified form thereof.

53. A compound according to claim 42, wherein $\Delta_1$ is represented by a sequence selected from any one of SEQ ID NO:49-67.

54. A compound according to claim 10, wherein $\Theta_1$ is represented by the formula:

$$\mathbf{CFGX_1X_2\Phi_1DRIX_3X_4X_5SX_6LGC} \qquad \text{(VIII)}$$

wherein:

$X_{1-6}$ are each independently selected from any amino acid residue, or modified form thereof;

$\Phi_1$ is selected from a hydrophobic amino acid residue, or modified form thereof; and

the two C residues are connected by a bridging bond.

55. A compound according to claim 54, wherein $X_1$ is selected from a basic, hydrophobic, neutral/polar or a small amino acid residue, or modified form thereof.

56. A compound according to claim 54, wherein $X_2$ is selected from a small or basic amino acid residue, or modified form thereof.

57. A compound according to claim 54, wherein $\Phi_1$ is selected from M, L or I, or modified form thereof.

58. A compound according to claim 54, wherein $X_3$ is selected from a small, neutral/polar or basic amino acid residue, or modified form thereof.

59. A compound according to claim 54, wherein $X_4$ is selected from a small or basic amino acid residue, or modified form thereof.

60. A compound according to claim 54, wherein $X_5$ is selected from a small, basic, neutral/polar, or hydrophobic amino acid residue, or modified form thereof.

61. A compound according to claim 54, wherein $X_6$ is selected from a small, basic or neutral/polar amino acid residue, or modified form thereof.

62. A compound according to claim 54, wherein $\Theta_1$ is represented by a sequence selected from any one of SEQ ID NO:68-81.

63. A compound according to claim 1, which is represented by the the formula:

$$\Delta_2\text{–}\Theta_2\text{–}\Omega_2 \qquad \text{(IX)}$$

wherein:

$\Delta_2$ is absent or is a N-terminal portion of a natriuretic peptide or a variant or derivative of that portion;

$\Omega_2$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of that portion; and

$\Theta_2$ comprises an amino acid sequence having the formula:

$$\mathbf{CFG\Phi_1PLDRIX_1SVSGLGC} \qquad \text{(X)}$$

wherein:

$\Phi_1$ is selected from any hydrophobic amino acid residue, or modified form thereof; and

$X_1$ is selected from any small or a basic amino acid residue, or modified form thereof.

64. A compound according to claim 63, wherein $\Phi_1$ is selected from an amino acid residue having an aromatic side chain, or one having an aliphatic side chain, or modified form thereof.

65. A compound according to claim 63, wherein $X_1$ is selected from G, S, R or K, or modified form thereof.

66. A compound according to claim 63, wherein $\Delta_2$ and $\Omega_2$ are independently selected from N- and C-terminal portions, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP, VNP, PNP or an *Oxyuranus* natriuretic peptide, or their variants or derivatives.

67. A compound according to claim 1, which is represented by the formula:

$$\Delta_3\text{–}\Theta_3\text{–}\Omega_3 \qquad \text{(XI)}$$

wherein:

$\Theta_3$ is a core ring structure of a natriuretic peptide or a variant or derivative of the structure;

$\Omega_3$ is a C-terminal portion of a natriuretic peptide or a variant or derivative of the portion; and

$\Delta_3$ comprises an amino acid sequence having the formula:

$$\mathbf{SD\Sigma_1KGX_1G} \qquad \text{(XII)}$$

wherein:

$\Sigma_1$ is selected from any small amino acid residue, or modified form thereof; and

$X_1$ is selected from any amino acid residue, or modified form thereof.

68. A compound according to claim 67, wherein $\Sigma_1$ is S or P, or modified form thereof.

69. A compound according to claim 67, wherein $X_1$ is an acidic or neutral polar amino acid residue, or modified form thereof.

70. A compound according to claim 67, wherein $\Theta_3$ and $\Omega_3$ are independently selected from core structures and C-terminal portions, respectively, of natriuretic peptides selected from ANP, BNP, CNP, DNP or an *Oxyuranus* natriuretic peptide, or their variants or derivatives.

71. An isolated proteinaceous compound having at least one activity selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, in vertebrate animals including mammals, wherein the compound has the formula:

$$Z_1SD\Sigma_1KIGX_1GCFG\Phi_1PLDB_1IX_2SVSGLGCNRX_3\Phi_2QNX_4PKKZ_2 \quad \text{(XIII)}$$

wherein:

$Z_1$ is absent or is a peptide from about 1 to about 50 amino acids or is a protecting moiety;

$\Sigma_1$ is a small amino acid residue, or modified form thereof;

$X_1$ is an acidic or neutral/polar amino acid residue, or modified form thereof;

$\Phi_1$ is a hydrophobic amino acid residue, or modified form thereof;

$B_1$ is a basic amino acid residue, or modified form thereof;

$X_2$ is a basic or small amino acid residue, or modified form thereof;

$X_3$ is a small or hydrophobic amino acid residue, or modified form thereof;

$\Phi_2$ is a hydrophobic amino acid residue, or modified form thereof;

$X_4$ is a basic or small amino acid residue, or modified form thereof; and

$Z_2$ is absent or is a peptide of from about 1 to about 10 amino acids,

the two C residues are connected by a bridging bond,

or a pharmaceutically acceptable salt thereof.

72. A compound according to claim 71, wherein $\Sigma_1$ is selected from P or S, or modified form thereof.

73. A compound according to claim 71, wherein $X_1$ is selected from Q, K, A, S, D or N, or modified form thereof.

74. A compound according to claim 71, wherein $\Phi_1$ is selected from L or F, or modified form thereof.

75. A compound according to claim 71, wherein $B_1$ is selected from H, K or R, or modified form thereof.

76. A compound according to claim 71, wherein $X_2$ is selected from G, S, R or K, or modified form thereof.

77. A compound according to claim 71, wherein $X_3$ is selected from P or I, or modified form thereof.

78. A compound according to claim 71, wherein $\Phi_2$ is selected from V, Nle or M, or modified form thereof.

79. A compound according to claim 71, wherein $X_4$ is selected from R or P, or modified form thereof.

80. A compound according to claim 71, wherein the sequence Asn-Pro is replaced by a sequence selected from Ala-Pro, Gln-Pro, Ala-Hyp or Gln-Hyp.

81. A compound according to claim 71, wherein the sequence Asn-Gly is replaced by a sequence selected from Gln-Gly or Ala-Gly.

82. A compound according to claim 71, wherein M is replaced by Nor or V.

83. A compound according to claim 71, wherein one or both C residues are replaced by selenocysteine

84. A compound according to claim 71, wherein the N-terminus is blocked by pGlu, Pro, Hyp or any N-acetylated residue.

85. A compound according to claim 71, wherein the C-terminus is blocked by amidation or cyclisation.

86. A compound according to claim 71, wherein when the peptide $Z_2$ is present, it comprises the sequence set forth in SEQ ID NO:14, or a variant or derivative thereof.

87. A compound according to claim 71, wherein the bridging bond that connects the two C residues is selected from the group consisting of a disulphide bond, a diselenide or mixed sulphide-selenide bond, a methylene bond, a sulphide/methylene bond, an amide bond and an ester bond.

88. A compound according to claim 71, wherein the bridging bond is a disulphide bond.

89. A compound according to claim 71, wherein the compounds comprise any one of the sequences set forth in SEQ ID NO: 90, 91, 94-114, 117, 118, 120, 123, 124, 126-130, 132-134, 150, 154, 160, 166-169, 173, 176 and 178-192.

90. An isolated polynucleotide that comprises a nucleotide sequence a compound according to claim 1 or claim 71.

91. A vector that comprises a polynucleotide according to claim 90, which is optionally operably connected to a regulatory sequence.

92. A host cell containing a vector according to claim 91.

93. A process for producing a compound according to claim 1 or claim 71, comprising culturing a host cell containing a vector according to claim 91 under conditions sufficient to produce the compound and isolating the compound.

94. An antigen-binding molecule that is immuno-interactive with a compound according to claim 1 or claim 71.

95. A pharmaceutical composition comprising a compound according to claim 1 or claim 71, and a pharmaceutically acceptable carrier, excipient and/or diluent.

96. A method for inducing, stimulating or otherwise promoting in a subject at least one activity in a subject selected from vasodilatory, natriuretic, diuretic, renin-suppressing, bactericidal, weight-reducing or bone growth plate size-increasing activities, comprising administering to the subject an effective amount of a compound according to claim 1 or claim 71 or a composition according to claim 95.

97. A method for treating or preventing at least one of condition selected from heart failure, nephrotic syndrome, cirrhosis of the liver, hypertension, kidney failure, bacterial infections, weight loss, asthma, inflammatory-related diseases; erectile dysfunction; hypercholesterolemia; skeletal dysplasias and as a protectant for toxicity of anti-cancer drugs, in a subject, comprising administering to the subject an effective amount of a according to claim 1 or claim 71 or a composition according to claim 95.

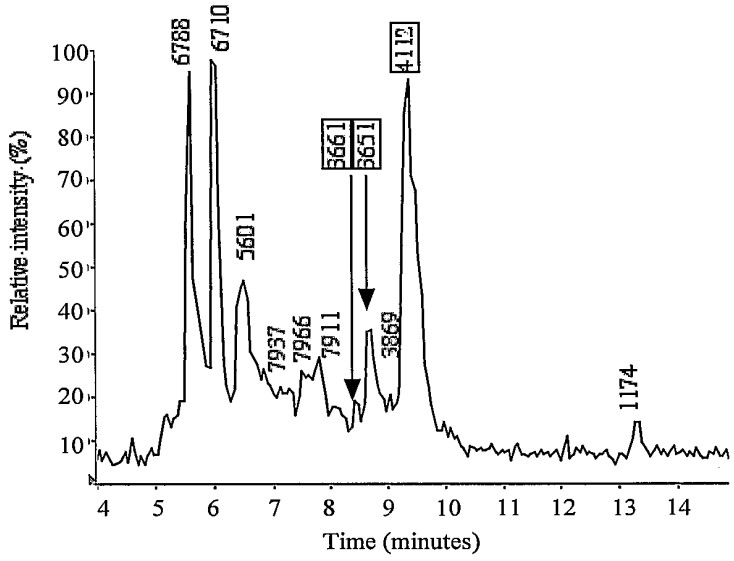

6788
6710
4112
8661
8651
5601
7937
7966
7911
3869
1174
Relative intensity (%)
100
90
80
70
60
50
40
30
20
10
4
5
6
7
8
9
10
11
12
13
14
Time (minutes)

FIGURE 1

| Ring position | 12345678901234567 | |
|---|---|---|
| ANP mouse | SLRRSSCFGGRIDRIGAQSGLGCNSFRYRR | SEQ ID NO: 108 |
| ANP rat | SLRRSSCFGGRIDRIGAQSGLGCNSFRYRR | SEQ ID NO: 109 |
| ANP human | SLRRSSCFGGRMDRIGAQSGLGCNSFRY | SEQ ID NO: 82 |
| BNP human | SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH | SEQ ID NO: 63 |
| BNP rat | IQERLRNSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF | SEQ ID NO: 110 |
| DNP | EVKYDPCFGHKIDRINHVSNLGCPSLRDPRPNAPSTSA | SEQ ID NO: 65 |
| TNPa | SDSKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK | SEQ ID NO: 68 |
| TNPb | SDPKIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK | SEQ ID NO: 69 |
| TNPc | SDSKIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE | SEQ ID NO: 70 |
| VNP | AKTAKSFNSCFGTRMDRIGSWSGLGCNSLKNGTKKKIFGN | SEQ ID NO: 193 |
| PNP | GENEPPKKKAPDGCFGHKIDRIGSHSGLGCNKFKPGH | SEQ ID NO: 87 |
| Lebetin 2 | GDNKPPKKGPPNGCFGHKIDRIGSHSGLGCNKVDDNKG | SEQ ID NO: 86 |

FIGURE 2

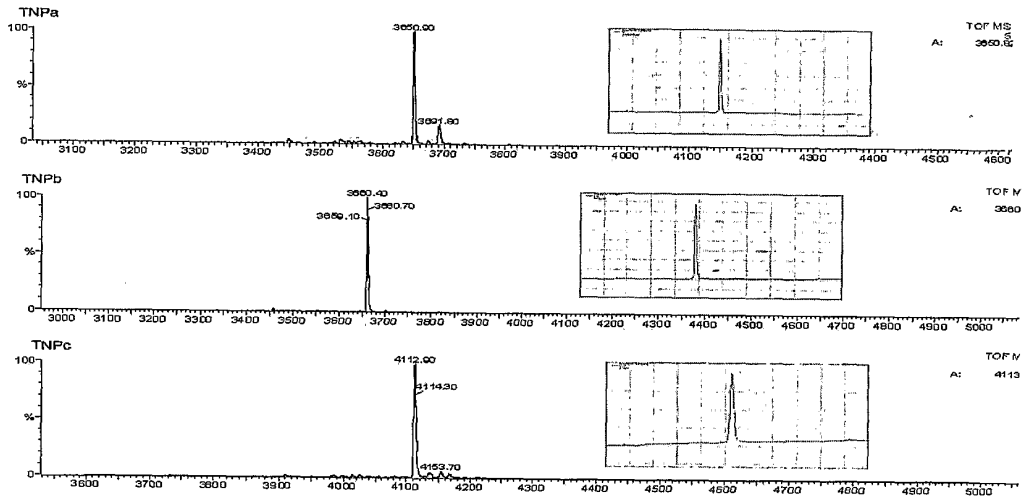



FIGURE 3

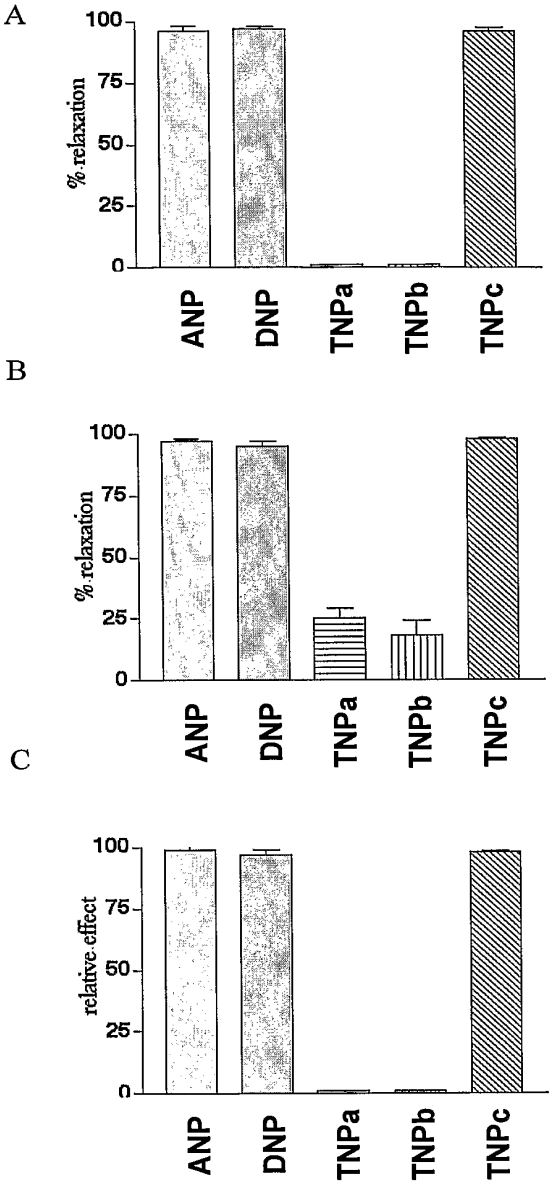

A
100
75
50
25
0
% relaxation
ANP
DNP
TNPa
TNPb
TNPc
B
100
75
50
25
0
% relaxation
ANP
DNP
TNPa
TNPb
TNPc
C
100
75
50
25
0
relative effect
ANP
DNP
TNPa
TNPb
TNPc

FIGURE 4

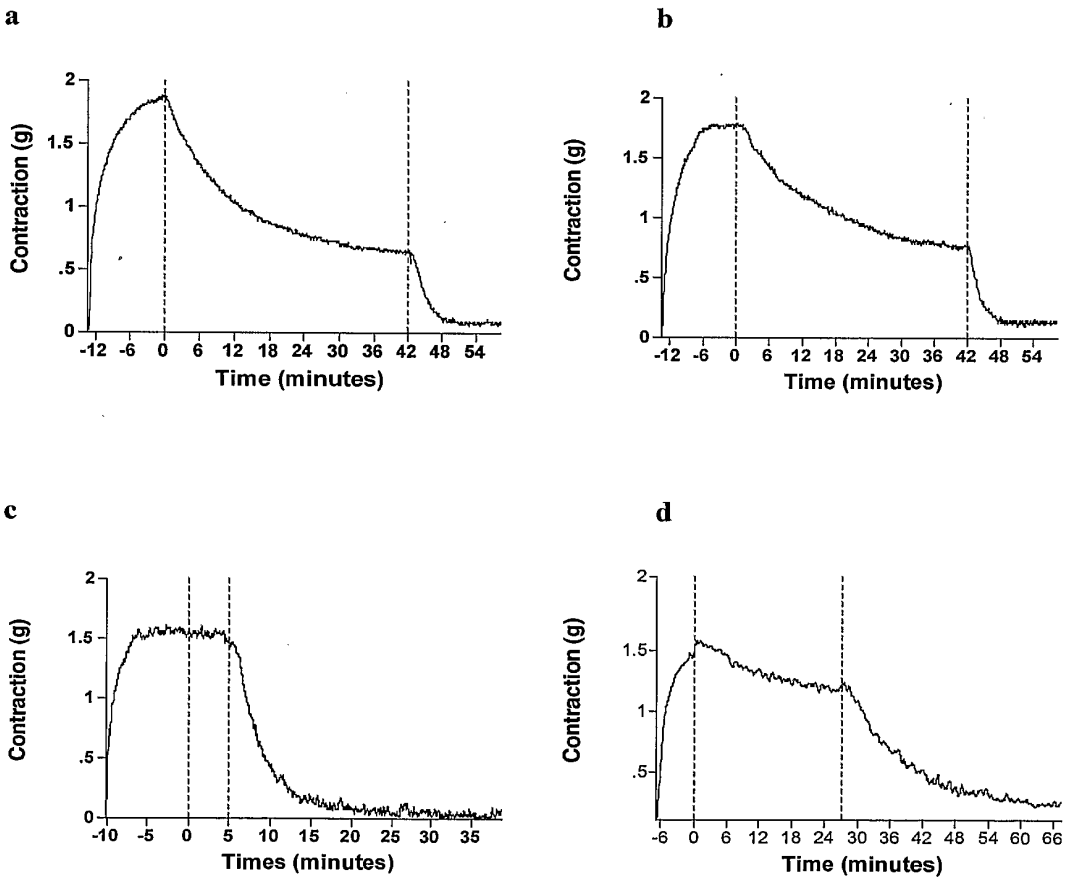
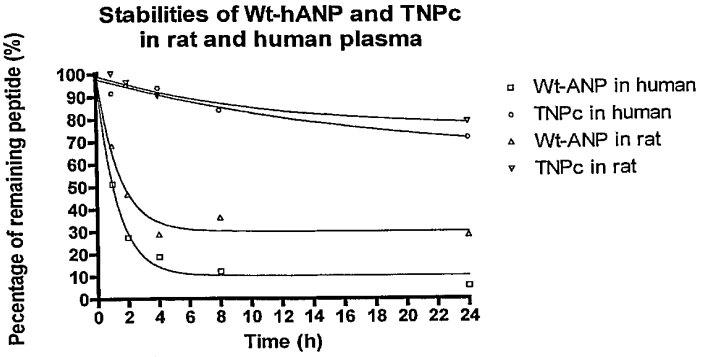
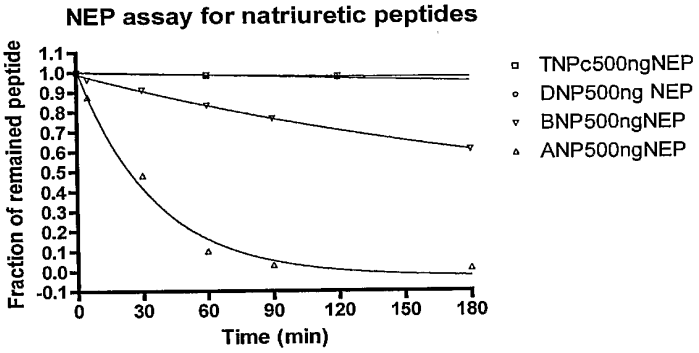

a
Contraction (g)
2
1.5
1
.5
0
-12 -6 0 6 12 18 24 30 36 42 48 54
Time (minutes)
b
Contraction (g)
2
1.5
1
.5
0
-12 -6 0 6 12 18 24 30 36 42 48 54
Time (minutes)
c
Contraction (g)
2
1.5
1
.5
0
-10 -5 0 5 10 15 20 25 30 35
Times (minutes)
d
Contraction (g)
2
1.5
1
.5
-6 0 6 12 18 24 30 36 42 48 54 60 66
Time (minutes)

FIGURE 5

6a




6b




6c

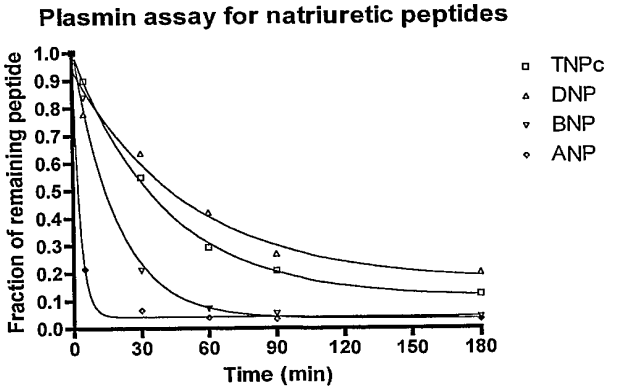



FIGURE 6

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(71) Applicant *(for all designated States except US)*: **MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH** [US/US]; 200 First Street S.W., Rochester, Minnesota 55905 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BURNETT, John, C. Jr.** [US/US]; 1088 Fox Hill Place S.W., Rochester, Minnesota 55902 (US). **LEE, Candace, Y. W.** [CA/US]; 101 Civic Center Drive N.E., Apt. 103, Rochester, Minnesota 55906 (US).

(74) Agents: **KAYTOR, Elizabeth N.** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

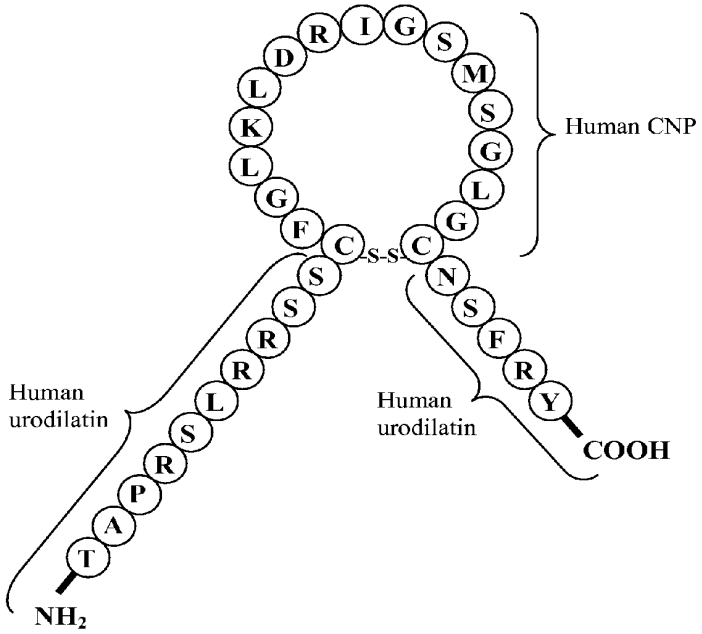
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

*[Continued on next page]*

(54) Title: NATRIURETIC POLYPEPTIDES

(57) Abstract: This document provides natriuretic polypeptides. For example, this document provides polypeptides having a natriuretic activity. In some cases, a polypeptide provided herein can have natriuretic activities, while lacking the ability to lower blood pressure. This document also provides methods and materials for inducing natriuretic activities within a mammal.

Figure 1

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European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

**Published:**

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

Attorney Docket No.: 07039-804WO1

# NATRIURETIC POLYPEPTIDES

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application Serial No. 60/951,117, filed on July 20, 2007.

## STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant HL036634 awarded by the National Institutes of Heart, Lung, and Blood Institute. The government has certain rights in the invention.

## BACKGROUND

*1. Technical Field*

This document relates to natriuretic polypeptides. For example, this document provides methods and materials related to natriuretic polypeptides and the use of natriuretic polypeptides to treat cardiovascular and renal conditions.

*2. Background Information*

Natriuretic polypeptides are polypeptides that can cause natriuresis (increased sodium excretion in the urine). Such polypeptides can be produced by brain, heart, kidney, and/or vasculature tissue.

## SUMMARY

This document relates to natriuretic polypeptides. For example, this document provides methods and materials related to natriuretic polypeptides and the use of natriuretic polypeptides to treat cardiovascular conditions, renal conditions, or both cardiovascular conditions and renal conditions. In some cases, a polypeptide provided herein can have diuretic activity, natriuretic activity, the ability to activate cGMP, the ability to increase glomerular filtration rate, the ability to reduce renin production, the ability to reduce angiotensin production, the ability to reduce aldosterone production, the ability to reduce abnormally elevated cardiac filling pressures, the ability to optimize

renal blood flow, or a combination thereof. In some cases, a polypeptide provided herein can increase endogenous ANP, BNP, and CNP levels. In some cases, a polypeptide provided herein can lack the ability to lower blood pressure and can lack the ability to cause systemic hypotension. In some cases, a polypeptide provided herein can be an agonist for natriuretic peptide receptor-A, natriuretic peptide receptor-B, or both natriuretic peptide receptor-A and natriuretic peptide receptor-B.

In general, one aspect of this document features a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide.

In another aspect, this document features an isolated nucleic acid encoding a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or

substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide.

In another aspect, this document features a vector comprising a nucleic acid encoding a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative

amino acid substitutions. The polypeptide can be a substantially pure polypeptide.

In another aspect, this document features a host cell comprising a nucleic acid encoding a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide. The host cell can be a eukaryotic host cell.

In another aspect, this document features a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension.

The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide.

In another aspect, this document features a method for increasing natriuretic activity within a mammal without lowering blood pressure. The method comprises administering, to the mammal, a polypeptide less than 44 amino acid residues in length, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide.

In another aspect, this document features a method for treating a mammal having a cardiovascular condition or renal condition. The method comprises administering, to the mammal, a polypeptide less than 44 amino acid residues in length under conditions wherein the severity of a manifestation of the cardiovascular condition or renal condition is reduced, wherein the polypeptide comprises, in an order from amino terminus to carboxy terminus: (a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions, (b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and (c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions. The polypeptide can comprise natriuretic activity. The polypeptide can lack the ability to induce systemic hypotension. The polypeptide can comprise the sequence set forth in SEQ ID NO:1. The polypeptide can comprise the sequence set forth in SEQ ID NO:2. The polypeptide can comprise the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3. The polypeptide can comprise the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions. The polypeptide can comprise the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions. The polypeptide can be a substantially pure polypeptide. Administration of the polypeptide to the mammal can be such that it does not lower the blood pressure of the mammal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

## DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of a CU-NP polypeptide that is 32 amino acid residues in length (SEQ ID NO:4). The first ten amino acid residues of SEQ ID NO:4 correspond to amino acid residues 1 to 10 of human urodilatin and are designated as SEQ ID NO:1. Amino acid residues 11 to 27 of SEQ ID NO:4 correspond to amino acid residues 6 to 22 of human mature CNP and are designated as SEQ ID NO:2. Amino acid residues 28 to 32 of SEQ ID NO:4 correspond to amino acid residues 28 to 32 of human urodilatin and are designated as SEQ ID NO:3.

Figure 2 is a graph plotting Renal Perfusion Pressure (as estimated by RPP = MAP – RAP) of normal anesthetized dogs treated with CU-NP or URO as indicated (mean ± SEM; * = $P<0.05$ vs. baseline; ‡ = $P<0.05$ between groups).

Figure 3 is a graph plotting the cGMP response to equimolar concentrations of CU-NP, CNP, and URO in isolated canine glomeruli (n = 3-7 for blanks, i.e., control; n = 5-8 for glomeruli, † = $P<0.0001$ vs. blank; * = $P<0.01$ vs. blank).

Figure 4 is a graph plotting the cGMP response to equimolar concentrations of CU-NP in the presence or absence of an NPR-A antagonist (1 μM of A71915), an NPR-B antagonist (1 μM of P19), or both antagonists (A71915 followed by P19, final concentration 1 μM for both) as assessed in isolated canine glomeruli (n = 2-4 for blanks; n = 3-6 for glomeruli; * = $P<0.05$ vs. blank; † = $P<0.01$ vs. blank; ‡ = $P<0.0001$ vs. blank).

Figure 5 is a graph plotting the cGMP response to CNP or CU-NP in the absence or presence of an NPR-B Antibody (1:100) as assessed in human aortic endothelial cells.

## DETAILED DESCRIPTION

This document relates to natriuretic polypeptides. For example, this document provides methods and materials related to natriuretic polypeptides and the use of

natriuretic polypeptides to treat cardiovascular conditions (e.g., acute decompensated heart failure, acute coronary syndromes, and ventricular remodeling post-myocardial infarction) and renal conditions (e.g., perioperative renal dysfunction, renal dysfunction secondary to heart failure, and diabetic nephropathy).

In some cases, a polypeptide provided herein can have diuretic activity, natriuretic activity, the ability to activate cGMP, the ability to increase glomerular filtration rate, the ability to reduce renin production, the ability to reduce angiotensin production, the ability to reduce aldosterone production, the ability to reduce abnormally elevated cardiac filling pressures, the ability to optimize renal blood flow, or a combination thereof. In some cases, a polypeptide provided herein can increase endogenous ANP, BNP, and CNP levels. In some cases, a polypeptide provided herein can lack the ability to lower blood pressure and cause systemic hypotension. In some cases, a polypeptide provided herein can be an agonist for natriuretic peptide receptor-A, natriuretic peptide receptor-B, or both natriuretic peptide receptor-A and natriuretic peptide receptor-B.

A polypeptide provided herein can have any sequence and can have any length. For example, a polypeptide provided herein can include the sequence set forth in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3. In some cases, a polypeptide provided herein can contain an amino acid sequence that aligns to (a) the sequence set forth in SEQ ID NO:1 with three or less (e.g., two or less, one, or zero) amino acid additions, deletions, substitutions, or combinations thereof, (b) the sequence set forth in SEQ ID NO:2 with five or less (e.g., four or less, three or less, two or less, one, or zero) amino acid additions, deletions, substitutions, or combinations thereof and (c) the sequence set forth in SEQ ID NO:3 with three or less (e.g., two or less, one, or zero) amino acid additions, deletions, substitutions, or combinations thereof. For example, a polypeptide provided herein can contain the sequence set forth in SEQ ID NO:1 with the exception that the first threonine residue or the last serine residue of SEQ ID NO:1 is deleted or replaced with a different amino acid residue.

Amino acid substitutions can be conservative amino acid substitutions. Conservative amino acid substitutions can be, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids;

serine/glycine/alanine/threonine as hydrophilic amino acids. Conservative amino acid substitutions also include groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. After making an amino acid substitution, the activities of the polypeptide containing the amino acid substitution can be assessed using the assays described herein.

In some cases, a polypeptide provided herein can contain (a) a first amino acid sequence that either is set forth in SEQ ID NO:1 or aligns to the sequence set forth in SEQ ID NO:1 with three or less (e.g., two or less, one, or zero) amino acid deletions, substitutions, or combinations thereof, (b) a second amino acid sequence that either is set forth in SEQ ID NO:2 or aligns to the sequence set forth in SEQ ID NO:2 with five or less (e.g., four or less, three or less, two or less, one, or zero) amino acid additions, substitutions, or combinations thereof, and (a) a third amino acid sequence that either is set forth in SEQ ID NO:3 or aligns to the sequence set forth in SEQ ID NO:3 with three or less (e.g., two or less, one, or zero) amino acid deletions, substitutions, or combinations thereof. For example, a polypeptide provided herein can comprise or consist of the sequence set forth in SEQ ID NO:4.

A polypeptide provided herein can have any length. For example, a polypeptide provided herein can be between 25 and 45 (e.g., between 26 and 44, between 27 and 43, between 28 and 42, between 29 and 41, between 30 and 40, between 31 and 39, or between 30 and 35) amino acid residues in length. It will be appreciated that a polypeptide with a length of 25 or 45 amino acid residues is a polypeptide with a length between 25 and 45 amino acid residues.

In some cases, a polypeptide provided herein can be a substantially pure polypeptide. As used herein, the term "substantially pure" with reference to a polypeptide means that the polypeptide is substantially free of other polypeptides, lipids, carbohydrates, and nucleic acid with which it is naturally associated. Thus, a

substantially pure polypeptide is any polypeptide that is removed from its natural environment and is at least 60 percent pure or is any chemically synthesized polypeptide. A substantially pure polypeptide can be at least about 60, 65, 70, 75, 80, 85, 90, 95, or 99 percent pure. Typically, a substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel.

A polypeptide provide herein can be obtained by expression of a recombinant nucleic acid encoding the polypeptide or by chemical synthesis (e.g., using solid phase polypeptide synthesis methods or an peptide synthesizer such as an ABI 431A Peptide Synthesizer; Applied Biosystems; Foster City, CA). For example, standard recombinant technology using expression vectors encoding a polypeptide provide herein can be used. The resulting polypeptides then can be purified using, for example, affinity chromatographic techniques and HPLC. The extent of purification can be measured by any appropriate method, including but not limited to: column chromatography, polyacrylamide gel electrophoresis, or high-performance liquid chromatography. A polypeptide provide herein can be designed or engineered to contain a tag sequence that allows the polypeptide to be purified (e.g., captured onto an affinity matrix). For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ tag (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino termini. Other fusions that can be used include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase.

A polypeptide provided herein can be produced to contain three regions, a first region that includes an N-terminus (e.g., an N-terminus sequence from a human urodilatin polypeptide), a second region that includes a ring structure of a mature natriuretic polypeptide such as a human CNP polypeptide, and third region that includes a C-terminus (e.g., a C-terminus sequence from a human urodilatin polypeptide).

A polypeptide provided herein can be used to treat cardiovascular diseases, congestive heart failure, myocardial infarction, coronary artery diseases, renal diseases, hepatic diseases, cancer, metabolic diseases, or combinations thereof. For example, a CU-NP polypeptide having the amino acid sequence set forth in SEQ ID NO:4 can be

administered to a human having coronary artery disease under conditions wherein the severity of the human's coronary artery disease symptoms is reduced.

A polypeptide provided herein can be formulated as a pharmaceutical composition by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compositions can be administered to a subject in need thereof in an amount effective to treat, for example, heart, liver, kidney, or other sodium retaining conditions. Pharmaceutical compositions can be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions in aqueous physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal drops, or aerosols. Compositions for other routes of administration can be prepared as desired using appropriate methods.

Formulations for parenteral administration can include as common excipients, sterile water, saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and combinations thereof. In some cases, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, polyoxethylene-polyoxypropylene copolymers, or combinations thereof can be used as excipients for controlling the release of the polypeptide *in vivo*. Other suitable parenteral delivery systems that can be used include, without limitation, ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, liposomes, and combinations thereof. Formulations for inhalation administration can include excipients such as lactose. Inhalation formulations can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate, deoxycholate, or combinations thereof, or they can be oily solutions for administration in the form of nasal drops. If desired, a composition containing a polypeptide provided herein can be formulated as gel to be applied intranasally. Formulations for parenteral administration can include glycocholate for buccal administration.

For oral administration, tablets or capsules can be prepared using appropriate methods with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone, or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate);

lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulfate). Tablets can be coated using appropriate methods. Preparations for oral administration can be formulated to give controlled release of the polypeptide.

Nasal preparations can be presented in a liquid form or as a dry product. Nebulised aqueous suspensions or solutions can include carriers or excipients to adjust pH and/or tonicity.

*Nucleic Acids Encoding Polypeptides*

This document also provides isolated nucleic acids that encode one or more of the polypeptides provided herein. The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid (e.g., a nucleic acid encoding a polypeptide comprising or consisting of the amino

acid sequence set forth in SEQ ID NO:4) can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

As used herein, the term "nucleic acid" refers to both RNA and DNA, including mRNA, cDNA, genomic DNA, synthetic (e.g., chemically synthesized) DNA, and nucleic acid analogs. The nucleic acid can be double-stranded or single-stranded, and where single-stranded, can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear. Nucleic acid analogs can be modified at the base moiety, sugar moiety, or phosphate backbone to improve, for example, stability, hybridization, or solubility of a nucleic acid. Modifications at the base moiety include deoxyuridine for deoxythymidine, and 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. Modifications of the sugar moiety can include modification of the 2' hydroxyl of the ribose sugar to form 2'-O-methyl or 2'-O-allyl sugars. The deoxyribose phosphate backbone can be modified to produce morpholino nucleic acids, in which each base moiety is linked to a six-membered, morpholino ring, or peptide nucleic acids, in which the deoxyphosphate backbone is replaced by a pseudopeptide backbone and the four bases are retained. See, for example, Summerton and Weller *Antisense Nucleic Acid Drug Dev.*, 7:187-195 (1997); and Hyrup *et al.* *Bioorgan. Med. Chem.*, 4:5-23 (1996). In addition, the deoxyphosphate backbone can be replaced with, for example, a phosphorothioate or phosphorodithioate backbone, a phosphoroamidite, or an alkyl phosphotriester backbone.

A nucleic acid provided herein can comprise or consist of a sequence that encodes the amino acid sequence set forth in SEQ ID NO:4. For example, such a nucleic acid can contain the human nucleic acid sequence for CNP and urodilatin engineered to encode the amino acid sequence set forth in SEQ ID NO:4.

Typically, an isolated nucleic acid provided herein is at least 10 nucleotides in length (e.g., 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 200, 300, 350, 400, or more nucleotides in length). Nucleic acid molecules that are less than full-length can be useful, for example, as primers or probes for diagnostic purposes. Isolated nucleic acid molecules can be produced by standard techniques, including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, polymerase chain reaction (PCR) techniques can be used. PCR refers to a procedure or technique in which target nucleic acids are enzymatically amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers typically are 15 to 50 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. For example, a primer can be 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, or 45 nucleotides in length. A primer can be purified from a restriction digest by conventional methods, or can be chemically synthesized. Primers typically are single-stranded for maximum efficiency in amplification, but a primer can be double-stranded. Double-stranded primers are first denatured (e.g., treated with heat) to separate the strands before use in amplification. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, ed. by Dieffenbach and Dveksler, Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize a complementary DNA (cDNA) strand. Ligase chain reaction, strand displacement amplification, self-sustained sequence replication or nucleic acid sequence-based amplification also can be used to obtain isolated nucleic acids as described elsewhere (Lewis, *Genetic Engineering News*, 12(9):1 (1992); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874-1878 (1990); and Weiss, *Science*, 254:1292 (1991)).

Isolated nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule (e.g., using automated DNA synthesis in the 3' to 5' direction using phosphoramidite technology) or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that

contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Isolated nucleic acids also can be obtained by mutagenesis. For example, a nucleic acid sequence encoding a polypeptide having the sequence set forth in SEQ ID NO:1, 2, 3, or 4 can be mutated using standard techniques such as, for example, oligonucleotide-directed mutagenesis and/or site-directed mutagenesis through PCR. See, Short Protocols in Molecular Biology, Chapter 8, Green Publishing Associates and John Wiley & Sons, Edited by Ausubel *et al.*, 1992. Such mutations include additions, deletions, substitutions, and combinations thereof.

*Vectors and Host Cells*

This document also provides vectors containing a nucleic acid provided herein. As used herein, a "vector" is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment can be inserted so as to bring about the replication of the inserted segment. A vector can be an expression vector. An "expression vector" is a vector that includes one or more expression control sequences, and an "expression control sequence" is a DNA sequence that controls and regulates the transcription and/or translation of another DNA sequence.

In an expression vector provided herein, the nucleic acid can be operably linked to one or more expression control sequences. As used herein, "operably linked" means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest. Examples of expression control sequences include promoters, enhancers, and transcription terminating regions. A promoter is an expression control sequence composed of a region of a DNA molecule, typically within 100 nucleotides upstream of the point at which transcription starts (generally near the initiation site for RNA polymerase II). To bring a coding sequence under the control of a promoter, it can be necessary to position the translation initiation site of the translational reading frame of the polypeptide between one and about fifty

nucleotides downstream of the promoter. Enhancers provide expression specificity in terms of time, location, and level. Unlike promoters, enhancers can function when located at various distances from the transcription site. An enhancer also can be located downstream from the transcription initiation site. A coding sequence is "operably linked" and "under the control" of expression control sequences in a cell when RNA polymerase is able to transcribe the coding sequence into mRNA, which then can be translated into the polypeptide encoded by the coding sequence.

Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, tobacco mosaic virus, herpes viruses, cytomegalovirus, retroviruses, poxviruses, adenoviruses, and adeno-associated viruses. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, WI), Clontech Laboratories (Mountain View, CA), Stratagene (La Jolla, CA), and Invitrogen/Life Technologies (Carlsbad, CA).

An expression vector can include a tag sequence designed to facilitate subsequent manipulation of the expressed nucleic acid sequence (e.g., purification or localization). Tag sequences, such as green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or Flag™ tag (Kodak, New Haven, CT) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino terminus.

This document also provides host cells containing a nucleic acid molecule and/or nucleic acid vector provided herein. The term "host cell" refers to prokaryotic cells and eukaryotic cells into which a nucleic acid molecule or vector can be introduced. Any method can be used to introduce nucleic acid into a cell. For example, calcium phosphate precipitation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer can be used introduce nucleic acid into cells. In addition, naked DNA can be delivered directly to cells *in vivo* as described elsewhere (U.S. Patent Nos. 5,580,859 and 5,589,466).

*Detecting Polypeptides*

This document provides methods and materials for detecting a polypeptide provided herein. Such methods and materials can be used to monitor polypeptide levels within a mammal receiving the polypeptide as a therapeutic. A polypeptide provided herein (e.g., a CU-NP polypeptide having the amino acid sequence set forth in SEQ ID NO:4) can be detected, for example, immunologically using one or more antibodies. As used herein, the term "antibody" includes intact molecules as well as fragments thereof that are capable of binding to an epitopic determinant of a polypeptide provided herein. The term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains, and typically have specific three-dimensional structural characteristics, as well as specific charge characteristics. Epitopes generally have at least five contiguous amino acids (a continuous epitope), or alternatively can be a set of noncontiguous amino acids that define a particular structure (e.g., a conformational epitope). The term "antibody" includes polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain Fv antibody fragments, Fab fragments, and $F(ab)_2$ fragments. Polyclonal antibodies are heterogenous populations of antibody molecules that are contained in the sera of the immunized animals. Monoclonal antibodies are homogeneous populations of antibodies to a particular epitope of an antigen.

Antibody fragments that have specific binding affinity for a polypeptide provided herein (e.g., a CU-NP polypeptide having the amino acid sequence set forth in SEQ ID NO:4) can be generated by known techniques. For example, F(ab')2 fragments can be produced by pepsin digestion of the antibody molecule; Fab fragments can be generated by reducing the disulfide bridges of F(ab')2 fragments. In some cases, Fab expression libraries can be constructed. See, for example, Huse *et al.*, *Science*, 246:1275 (1989). Once produced, antibodies or fragments thereof can be tested for recognition of a polypeptide provided herein by standard immunoassay methods including ELISA techniques, radioimmunoassays, and Western blotting. See, Short Protocols in Molecular Biology, Chapter 11, Green Publishing Associates and John Wiley & Sons, Edited by Ausubel, F.M et al., 1992.

In immunological assays, an antibody having specific binding affinity for a polypeptide provided herein or a secondary antibody that binds to such an antibody can be labeled, either directly or indirectly. Suitable labels include, without limitation, radionuclides (e.g., $^{125}I$, $^{131}I$, $^{35}S$, $^{3}H$, $^{32}P$, $^{33}P$, or $^{14}C$), fluorescent moieties (e.g., fluorescein, FITC, PerCP, rhodamine, or PE), luminescent moieties (e.g., Qdot™ nanoparticles supplied by Invitrogen (Carlsbad, CA)), compounds that absorb light of a defined wavelength, or enzymes (e.g., alkaline phosphatase or horseradish peroxidase). Antibodies can be indirectly labeled by conjugation with biotin then detected with avidin or streptavidin labeled with a molecule described above. Methods of detecting or quantifying a label depend on the nature of the label and are known in the art. Examples of detectors include, without limitation, x-ray film, radioactivity counters, scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers. Combinations of these approaches (including "multi-layer" assays) familiar to those in the art can be used to enhance the sensitivity of assays.

Immunological assays for detecting a polypeptide provided herein can be performed in a variety of known formats, including sandwich assays, competition assays (competitive RIA), or bridge immunoassays. See, for example, U.S. Patent Nos. 5,296,347; 4,233,402; 4,098,876; and 4,034,074. Methods of detecting a polypeptide provided herein generally include contacting a biological sample with an antibody that binds to a polypeptide provided herein and detecting binding of the polypeptide to the antibody. For example, an antibody having specific binding affinity for a polypeptide provided herein can be immobilized on a solid substrate by any of a variety of methods known in the art and then exposed to the biological sample. Binding of the polypeptide to the antibody on the solid substrate can be detected by exploiting the phenomenon of surface plasmon resonance, which results in a change in the intensity of surface plasmon resonance upon binding that can be detected qualitatively or quantitatively by an appropriate instrument, e.g., a Biacore apparatus (Biacore International AB, Rapsgatan, Sweden). In some cases, the antibody can be labeled and detected as described above. A standard curve using known quantities of a polypeptide provided herein can be generated to aid in the quantitation of the levels of the polypeptide.

In some embodiments, a "sandwich" assay in which a capture antibody is immobilized on a solid substrate can be used to detect the presence, absence, or level of a polypeptide provided herein. The solid substrate can be contacted with the biological sample such that any polypeptide of interest in the sample can bind to the immobilized antibody. The presence, absence, or level of the polypeptide bound to the antibody can be determined using a "detection" antibody having specific binding affinity for the polypeptide. In some embodiments, a capture antibody can be used that has binding affinity for CNP or urodilatin as well as a polypeptide provided herein. In this embodiment, a detection antibody can be used that has specific binding affinity for a particular polypeptide provided herein (e.g., a CU-NP polypeptide having the amino acid sequence set forth in SEQ ID NO:4). It is understood that in sandwich assays, the capture antibody should not bind to the same epitope (or range of epitopes in the case of a polyclonal antibody) as the detection antibody. Thus, if a monoclonal antibody is used as a capture antibody, the detection antibody can be another monoclonal antibody that binds to an epitope that is either physically separated from or only partially overlaps with the epitope to which the capture monoclonal antibody binds, or a polyclonal antibody that binds to epitopes other than or in addition to that to which the capture monoclonal antibody binds. If a polyclonal antibody is used as a capture antibody, the detection antibody can be either a monoclonal antibody that binds to an epitope that is either physically separated from or partially overlaps with any of the epitopes to which the capture polyclonal antibody binds, or a polyclonal antibody that binds to epitopes other than or in addition to that to which the capture polyclonal antibody binds. Sandwich assays can be performed as sandwich ELISA assays, sandwich Western blotting assays, or sandwich immunomagnetic detection assays.

Suitable solid substrates to which an antibody (e.g., a capture antibody) can be bound include, without limitation, microtiter plates, tubes, membranes such as nylon or nitrocellulose membranes, and beads or particles (e.g., agarose, cellulose, glass, polystyrene, polyacrylamide, magnetic, or magnetizable beads or particles). Magnetic or magnetizable particles can be particularly useful when an automated immunoassay system is used.

Antibodies having specific binding affinity for a polypeptide provided herein can be produced through standard methods. For example, a polypeptide can be recombinantly produced as described above, can be purified from a biological sample (e.g., a heterologous expression system), or can be chemically synthesized, and used to immunize host animals, including rabbits, chickens, mice, guinea pigs, or rats. For example, a polypeptide having the amino acid sequence set forth in SEQ ID NO:4, or fragments thereof that are at least six amino acids in length, can be used to immunize an animal. Various adjuvants that can be used to increase the immunological response depend on the host species and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin and dinitrophenol. Monoclonal antibodies can be prepared using a polypeptide provided herein and standard hybridoma technology. In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described by Kohler *et al.*, *Nature*, 256:495 (1975), the human B-cell hybridoma technique (Kosbor *et al.*, *Immunology Today*, 4:72 (1983); Cole *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:2026 (1983)), and the EBV-hybridoma technique (Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96 (1983)). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the monoclonal antibodies can be cultivated *in vitro* and *in vivo*.

Other techniques for detecting a polypeptide provided herein include mass-spectrophotometric techniques such as electrospray ionization (ESI), and matrix-assisted laser desorption-ionization (MALDI). See, for example, Gevaert *et al.*, *Electrophoresis*, 22(9):1645-51 (2001); Chaurand *et al.*, *J. Am. Soc. Mass Spectrom.*, 10(2):91-103 (1999). Mass spectrometers useful for such applications are available from Applied Biosystems (Foster City, CA); Bruker Daltronics (Billerica, MA); and Amersham Pharmacia (Sunnyvale, CA).

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

## EXAMPLES

### Example 1 – Synthesis of CU- NP

A polypeptide with the sequence set forth in Figure 1 was designed and synthesized using an ABI 431A Peptide Synthesizer. This polypeptide is referred to as a CU-NP polypeptide (Figure 1). The synthesized CU-NP polypeptide was confirmed by high-performance liquid chromatography/mass spectrometry. Its molecular weight is 3535.09, and its amino acid sequence is Thr-Ala-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (SEQ ID NO:4) with a disulfide bridge joining the Cys residues (Figure 1).

### Example 2 – *In vivo* effects of CU-NP

Cardiorenal function was assessed in three normal anesthetized dogs. Clearances were obtained at pre-infusion, during intravenous infusion of 10, 50, and 100 ng CU-NP/kg/minute for 45 minutes at each dosage level (i.e., each dog received consecutive 45-minute infusions of 10, 50, and 100 ng/kg/minute), and post-infusion. Tubular $Na^+$ reabsorption and GFR were assessed by clearance of $Li^+$ and inulin, respectively. Neurohormones were quantified by radioimmunoassays. Data were analyzed by repeated measures ANOVA followed by Dunnett's test. The results (mean ± SEM) are presented in Table 1.

In addition, plasma renin activity decreased from 9 ± 2 to 5±1* to 3 ± 1† to 3 ± 1† ng/mL/hour, and angiotensin II levels decreased from 18 ± 1 to 10 ± 0.4† to 5 ± 0.4† to 7 ± 1† pg/mL. No significant change in QTc interval (ms) was observed at the end of infusion at 100 ng/kg/minute (329 ± 15) versus pre-infusion (323 ± 6).

These results demonstrate that a polypeptide containing CNP and urodilatin amino acid sequences can, in a dose dependent manner, (1) increase natriuresis, diuresis, and GFR, (2) decrease cardiac filling pressures, and (3) inhibit renin and angiotensin, without inducing significant hypotension.

**Table 1**

*Renal and cardiovascular data for CU-NP*

| | Pre-Infusion | 10 ng/kg/minute | 50 ng/kg/minute | 100 ng/kg/minute | Post-Infusion |
|---|---|---|---|---|---|
| GFR (mL/min) | 31 ± 5 | 39 ± 1 | 43 ± 4* | 53 ± 3† | 36 ± 2 |
| Urine flow (mL/min) | 0.09 ± 0.01 | 0.3 ± 0.07 | 1.5 ± 0.5† | 1.9 ± 0.3† | 0.3 ± 0.05 |
| $Na^+$ excretion (μEq/ min) | 1.9 ± 0.9 | 33 ± 13 | 235 ± 72† | 342 ± 60† | 66 ± 12 |
| $PFR_{Na+}$ (%) | 92 ± 0.9 | 73 ± 4.5† | 54 ± 1.8† | 55 ± 2.6† | 62 ± 3.5† |
| $DFR_{Na+}$ (%) | 99 ± 0.3 | 98 ± 0.5 | 92 ± 1.6† | 90 ± 0.8† | 97 ± 0.6* |
| Renal cGMP generation (pmol/ min) | 356 ± 36 | 534 ± 94 | 1301 ± 60* | 4608 ± 370† | 1086 ± 27 |
| PCWP (mmHg) | 4.6 ± 0.7 | 3.8 ± 1 | 2.5 ± 1† | 1.7 ± 0.9† | 3.2 ± 1* |
| RAP (mmHg) | 1.5 ± 1 | 0.9 ± 0.9 | 0.6 ± 1* | -0.2 ± 0.8† | 0.1 ± 0.9† |
| MAP (mmHg) | 129 ± 17 | 129 ± 13 | 128 ± 10 | 120 ± 11 | 124 ± 13 |

* = P<0.05; † = P<0.01; GFR = glomerular filtration rate; $PFRNa^+$ = proximal fractional reabsorption of sodium; $DFRNa^+$ = distal fractional reabsorption of sodium; PCWP = pulmonary capillary wedge pressure; RAP = right atrial pressure; MAP = mean arterial pressure.

Example 3 – Further biological effects of CU-NP

CU-NP or CNP polypeptide was intravenously infused into normal anesthetized dogs at 50 ng/kg/minute for 75 minutes. Blood and urine samples were collected pre-infusion (pre-I), at 30 and 60 minutes of infusion, and post-infusion (post-I). Inulin clearance was used for assessment of glomerular filtration rate (GFR). The lithium clearance technique was used for measurement of proximal and distal fractional reabsorption of $Na^+$ ($PFR_{Na}$ and $DFR_{Na}$, respectively). Comparisons at the latter three time points *versus* pre-I were made. Student's t-test was used when comparing results for two time points, whereas repeated measures ANOVA was used to compare four time points. Results (mean ± SEM) are presented in Tables 2A and 2B.

The CU-NP polypeptide significantly increased plasma cGMP and urinary cGMP excretion. CU-NP also increased urine flow and urinary Na+ excretion, and led to enhanced glomerular filtration rate. Pulmonary capillary wedge pressure and right atrial pressure were reduced without reducing systemic hypotension. Pulmonary arterial pressure also was significantly reduced, and cardiac output was preserved. Plasma renin activity, angiotensin II levels, and aldosterone levels were suppressed during CU-NP

infusion, but were followed by an elevation or 'rebound' following cessation of CU-NP infusion. These data suggest that a long-acting form of CU-NP can be used for continued suppression of neurohormones.

CU-NP also significantly increased renal generation of cGMP and urinary $K^+$ excretion. Both $PFR_{Na+}$ and $DFR_{Na+}$ were significantly reduced. These findings were associated with an increase in weight-adjusted renal blood flow and a reduction in renal vascular resistance. An increase in hematocrit also was observed. Plasma ANP, plasma BNP, and plasma CNP were increased, as was urinary excretion of ANP, BNP, and CNP.

These results demonstrate that a CU-NP polypeptide can have cGMP-activating, diuretic, natriuretic, GFR-enhancing, renin-angiotensin-aldosterone system (RAAS)-suppressing, cardiac-unloading, and favorable renal hemodynamic activities while lacking the ability to lower blood pressure and cause systemic hypotension. In addition, these results demonstrate that a CU-NP polypeptide can increase endogenous ANP and BNP levels. The tubular effects of the synthesized CU-NP polypeptide are consistent with actions at the level of proximal tubule and inner medullary collecting duct cells.

**Table 2A**

*Renal and cardiovascular data for CU-NP*

| | Pre-Infusion | 30 minutes | 60 minutes | Post-Infusion |
|---|---|---|---|---|
| GFR (mL/min) | 38.4 ± 3.6 | 50.7 ± 2.6* | 53.8 ± 2.8†‡ | 50.1 ± 3.5* |
| Urine flow (mL/min) | 0.13 ± 0.02 | 1.28 ± 0.25†¶ | 1.34 ± 0.22†¶ | 0.33 ± 0.04 |
| $Na^+$ excretion (μEq/min) | 8.0 ± 3.3 | 216.4 ± 42.3†¶ | 237.7 ± 35.8†¶ | 51.2 ± 9.4 |
| $K^+$ excretion (μeq/min) | 14.9 ± 4.5 | 68.3 ± 12.5† | 74.6 ± 15.3† | 32.9 ± 3.0 |
| $PFR_{Na+}$ (%) | 84.9 ± 2.5 | 62.4 ± 4.0† | 61.3 ± 1.9† | 70.4 ± 2.8† |
| $DFR_{Na+}$ (%) | 99.2 ± 0.2 | 92.4 ± 1.2†¶ | 92.2 ± 1.1†¶ | 97.7 ± 0.4 |
| Renal generation of cGMP (pmol/min) | 469.0 ± 55.4 | 2168 ± 531†§ | 2987 ± 622†¶ | 1394 ± 185 |
| Plasma cGMP (pmol/mL) | 8.2 ± 0.7 | 26.2 ± 1.3†¶ | 29.8 ± 1.5†¶ | 13.0 ± 0.9† |
| Urinary cGMP excretion (pmol/min) | 770 ± 48 | 3508 ± 574†¶ | 4591 ± 664†¶ | 2052 ± 208 |
| PCWP (mmHg) | 5.6 ± 0.9 | 3.9 ± 0.7* | 2.9 ± 0.9† | 4.3 ± 0.8 |
| RAP (mmHg) | 1.1 ± 0.6 | 0.3 ± 0.5 | -0.1 ± 0.5* | 0.7 ± 0.4 |
| Systemic hypotension (mmHg) | 135.9 ± 3.9 | 135.9 ± 2.7 | 133.9 ± 3.6 | 142.3 ± 2.7† |
| PAP (mmHg) | 11.8 ± 0.9 | 10.7 ± 0.8* | 10.5 ± 0.7† | 12.3 ± 0.7 |
| Cardiac output (L/min) | 3.1 ± 0.3 | 3.4 ± 0.5 | 3.0 ±0.5 | 2.8 ± 0.5 |
| Plasma renin activity (ng/mL/hour) | 8.8 ± 2 | 2.5 ± 0.8† | 1.5 ± 0.4† | 14.0 ± 1.3† |
| Angiotensin II (pg/mL) | 13.9 ± 2.0 | 6.9 ± 0.7† | 4.5 ± 0.3† | 22.6 ± 1.6† |
| Aldosterone (ng/dL) | 15.8 ± 2.7 | 14.2 ± 2.2 | 12.1 ± 2.1 | 30.4 ± 3.5† |
| Adjusted RBF (mL/kg/min) | 10.8 ± 0.8 | 11.64 ± 0.5 | 12.21 ± 0.4† | 11.60 ± 0.5 |
| RVR (x $10^{-3}$ mmHg•min•$L^{-1}$) | 0.55 ± 0.05 | 0.50 ± 0.04 | 0.47 ± 0.03† | 0.53 ± 0.04 |
| Hematocrit (%) | 37.8 ± 1.3 | 40.3 ± 1.3† | 41.1 ± 1.4† | 40.3 ± 1.8† |
| Plasma ANP (pg/mL) | 14.1 ± 0.8 | 16.7 ± 0.9* | 16.6 ± 1.1* | 15.2 ± 0.6 |
| Plasma BNP (pg/mL) | 8.2 ± 0.9 | 21.0 ± 1.8† | 17.0 ± 2.2† | 10.1 ± 1.4 |
| Plasma CNP (pg/mL) | 4.0 ± 0.3 | 15.4 ± 0.9† | 15.1 ± 1.4† | 3.2 ± 0.2 |
| ANP excretion (pg/min) | 3.2 ± 1.5 | 21.9 ± 9.5 | 28.3 ± 13.6 | 10.4 ± 2.5 |
| BNP excretion (pg/min) | 13.9 ± 1.5 | 18.2 ± 1.5 | 19.3 ± 1.2 | 28.3 ± 5.4* |
| CNP excretion (pg/min) | 2.0 ± 0.4 | 4.0 ± 1.2 | 9.1 ± 3.9 | 21.5 ±17.4 |

* = P<0.05 vs. pre-I; † = P<0.01 vs. pre-I; ‡ = P<0.05 vs. CNP; § = P<0.01 vs. CNP; ¶ = P<0.001 vs. CNP; GFR = glomerular filtration rate; $PFR_{Na+}$ = proximal fractional reabsorption of sodium; $DFR_{Na+}$ = distal fractional reabsorption of sodium; PCWP = pulmonary capillary wedge pressure; RAP = right atrial pressure; PAP = pulmonary arterial pressure; RBF = renal blood flow; RVR = renal vascular resistance.

**Table 2B**

*Comparative renal and cardiovascular data for CNP*

| | Pre-Infusion | 30 minutes | 60 minutes | Post-Infusion |
|---|---|---|---|---|
| GFR (mL/min) | 38 ± 4 | 42 ± 4 | 39 ± 3 | 44 ± 3 |
| Urine flow (mL/min) | 0.1 ± 0.02 | 0.3 ± 0.06† | 0.4 ± 0.02† | 0.3 ± 0.04 |
| $Na^+$ excretion (μEq/min) | 16 ± 6 | 56 ± 21 | 71 ± 8* | 30 ± 8 |
| $PFR_{Na+}$ (%) | 83 ± 3 | 67 ± 3 | 51 ± 7† | 68 ± 3* |
| $DFR_{Na+}$ (%) | 99 ± 0.5 | 98 ± 0.8 | 98 ± 0.5 | 99 ± 0.2 |
| Renal generation of cGMP (pmol/min) | 491 ± 65 | 452 ± 202 | 603 ± 199 | 582 ± 91 |
| Plasma cGMP (pmol/mL) | 8 ± 1 | 11 ± 1† | 12 ± 1† | 12 ± 1† |
| Urinary cGMP excretion (pmol/min) | 830 ± 74 | 1119 ± 102* | 1338 ± 81† | 1090 ± 76* |

* = P<0.05 vs. pre-I; † = P<0.01 vs. pre-I; GFR = glomerular filtration rate; $PFR_{Na+}$ = proximal fractional reabsorption of sodium; $DFR_{Na+}$ = distal fractional reabsorption of sodium.

Example 3 – Evaluation of CU-NP in human aortic endothelial cells and *in vivo*

CU-NP was tested in human aortic endothelial cells (HAEC), and the time course of cGMP activation *in vivo* was defined. CU-NP was incubated with HAEC (passages 2-5, at 80-90% confluency) at $10^{-10}$, $10^{-8}$ or $10^{-6}$ M for 10 minutes in a $CO_2$ incubator. CU-NP (n = 6) or CNP (n = 3) was intravenously infused into normal anesthetized dogs at 14 pmol/kg/min for 75 minutes. Blood was collected pre-infusion, at 25, 30, 45, 60, 75 minutes during infusion (I) and at 1, 2, 4, 6, 10, 20, 30, 45, 60 minutes post-infusion (post-I). Cyclic GMP was quantified by radioimmunoassay.

As shown in Table 3, CU-NP stimulated cGMP production in HAEC (P<0.01 for $10^{-6}$ M vs. no treatment). Further, CU-NP increased plasma cGMP *in vivo* vs. pre-I (all time points during I and post-I, P<0.01, except at 45 minutes post-I, P<0.05). CU-NP activated cGMP to a greater extent as compared to CNP (P<0.001, 25 minutes I through 30 minutes post-I). Thus, CU-NP significantly activated cGMP in HAEC, and also significantly stimulated cGMP to a greater extent than CNP *in vivo*.

**Table 3**

*CU-NP effects on HAEC*

| CU-NP Concentration | cGMP (mean ± SEM, pmol/mL) |
|---|---|
| $10^{-10}$ M | 0.0007 ± 0.0007 |
| $10^{-8}$ M | 0.03 ± 0.02 |
| $10^{-6}$ M | 0.571 ± 0.05† |
| No treatment | 0.003 ± 0.002 |

† = P<0.01 vs. no treatment.

Example 4 - cGMP stimulating actions of CU-NP in isolated canine glomeruli

Experiments were conducted to determine whether CU-NP directly stimulates cGMP in isolated glomeruli, and to compare the cGMP-activating actions of CU-NP with CNP, URO, and CNP-C, which consisted of the full-length 22-AA of CNP with a duplicate of the N-terminus fused in the C-terminal position. Glomeruli were isolated upon harvest of normal canine kidneys. CU-NP, CNP, URO, and CNP-C ($10^{-5}$ M) were incubated with glomeruli vs. control. Cyclic GMP was measured by RIA, with correction for protein levels. CU-NP and URO elicited greater cGMP responses than respective controls (P<0.01), but without difference between groups. CU-NP and URO stimulated greater cGMP responses vs. CNP and vs. CNP-C (P<0.001). Thus, CU-NP stimulated cGMP in glomeruli to a greater extent than CNP, but to a similar extent as URO. CNP-C did not activate cGMP. These data suggest that enhanced cGMP potentially activated may require N- and/or C- termini of ligands for NP receptor-A.

**Table 4**

*CU-NP effects on cGMP production in isolated glomeruli*

| Treatment | cGMP (mean ± SEM; fmol/μg) |
|---|---|
| CU-NP | 0.73 ± 0.09†‡ |
| CNP | 0.0019 ± 0.0005 |
| URO | 0.69 ± 0.07†‡ |
| CNP-C | 0.00597 ± 0.0053 |

† = P<0.01 vs. corresponding control; ‡ = P<0.001 vs. CNP and CNP-C

Example 5 – Hemoconcentrating effects of CU-NP

Three human NPs (BNP, CNP, URO, and CU-NP were studied to evaluate their effects on vascular permeability as manifested by an increase in hematocrit. Normal

anesthetized dogs received i.v. infusions of BNP (n = 7), CNP (n = 6), URO (n = 5), or CU-NP (n = 6) at 14 pmol/kg/minute. Blood was collected in EDTA tubes on ice and centrifuged. Data are reported for base-line and at 60 minutes of infusion. In addition to increasing hematocrit (Table 5), CU-NP reduced PCW pressure *in vivo* (baseline 6 ± 0.9 mmHg, 60 minutes 3 ± 0.9 mmHg; P<0.05). Thus, hemoconcentrating effects of CU-NP may contribute to its cardiac-unloading actions *in vivo*.

**Table 5**

*CU-NP effects on hematocrit*

| Treatment | Hematocrit (mean ± SEM; %) | |
|---|---|---|
| | **Baseline** | **60 min** |
| BNP | 36 ± 1 | 40 ± 2* |
| CNP | 36 ± 1 | 37 ± 1 |
| URO | 37 ± 1 | 40 ± 1* |
| CU-NP | 38 ± 1 | 41 ± 1† |

(* = P<0.05; † = P<0.01)

Example 6 – Cardiorenal and neurohumoral actions of CU-NP in canine experimental heart failure

CU-NP was evaluated in canine heart failure (HF) to determine whether CU-NP would activate the second messenger cGMP and exert favorable actions without excessive hypotension. Mild HF was induced by pacing (180 bpm for 10 days). CU-NP was intravenously infused into 6 anesthetized dogs at 75 ng/kg/minute for 75 minutes. GFR was measured by inulin clearance. Fractional reabsorption of $Na^+$ (FRNa) was assessed by $Li^+$ clearance. Data were collected at pre-infusion (pre-I), 30 and 60 minutes I, and post-I. Plasma renin activity, angiotensin II , aldosterone, and cGMP also were measured. Results (mean ± SEM) are presented in Table 6.

CU-NP increased plasma cGMP, urinary cGMP excretion, net renal cGMP generation, urine flow, and urinary Na+ excretion. Proximal and distal FRNa were reduced. Renal blood flow and GFR were enhanced, with a mild decrease in MAP. Both CO and SVR were unchanged. PCWP and PAP were reduced. Plasma renin activity, angiotensin II, and aldosterone also were suppressed. Thus, CU-NP activated cGMP in

canine HF and exerted renal-enhancing, cardiac-unloading and Renin-Angiotensin-Aldosterone System-suppressing actions without excessive hypotension.

**Table 6**

*Renal and cardiovascular data for CU-NP in experimental HF*

| | Pre-Infusion | 30 minutes | 60 minutes | Post-Infusion |
|---|---|---|---|---|
| GFR (mL/min) | 40 ± 5 | 51 ± 9 | 51 ± 3 | 53 ± 7* |
| Urine flow (mL/min) | 0.09 ± 0.01 | 0.50 ± 0.15† | 0.56 ± 0.11† | 0.19 ± 0.03 |
| $Na^+$ excretion (μEq/min) | 2.9 ± 0.9 | 80.5 ± 29.4† | 102.0 ± 22.2† | 22.1 ± 7.9 |
| $PFR_{Na+}$ (%) | 89 ± 2 | 68 ± 5† | 67 ± 3† | 79 ± 3* |
| $DFR_{Na+}$ (%) | 99.6 ± 0.1 | 96 ± 1† | 96 ± 1† | 99 ± 0.3 |
| Renal generation of cGMP (pmol/min) | 610 ± 66 | 2977 ± 549† | 3255 ± 662† | 1342 ± 200 |
| Plasma cGMP (pmol/mL) | 17 ± 3 | 41 ± 3† | 43 ± 2† | 21 ± 2 |
| Urinary cGMP excretion (pmol/min) | 1186 ± 152 | 5066 ± 826† | 5476 ± 876† | 2394 ± 197 |
| PCWP (mmHg) | 11 ± 1 | 9 ± 1† | 8 ± 1† | 10 ± 1 |
| PAP (mmHg) | 16 ± 0.8 | 15 ± 0.6* | 14 ± 0.7† | 16 ± 0.7 |
| Plasma renin activity (ng/mL/hour) | 9 ± 2 | 3 ± 1† | 2 ± 1† | 9 ± 2 |
| Angiotensin II (pg/mL) | 32 ± 7 | 9 ± 2† | 9 ± 3† | 19 ± 4 |
| Aldosterone (ng/dL) | 14 ± 5 | 9 ± 2 | 6 ± 2* | 11 ± 3 |
| RBF (mL/min) | 238 ± 39 | 281 ± 42* | 294 ± 42† | 275 ± 42* |
| MAP (mmHg) | 108 ± 9 | 100 ± 8† | 96 ± 8† | 107 ± 8 |
| CO (L/min) | 2.4 ± 0.1 | 2.5 ± 0.1 | 2.4 ± 0.1 | 2.3 ± 0.04 |
| SVR (mmHg•$L^{-1}$•Min) | 42 ± 3 | 39 ± 3 | 39 ± 3 | 45 ± 3 |

* = P<0.05 vs. pre-I; † = P<0.01 vs. pre-I; GFR = glomerular filtration rate; $PFR_{Na+}$ = proximal fractional reabsorption of sodium; $DFR_{Na+}$ = distal fractional reabsorption of sodium; PCWP = pulmonary capillary wedge pressure; PAP = pulmonary arterial pressure; RBF = renal blood flow; MAP = mean arterial pressure; CO = cardiac output; SVR = systemic vascular resistance.

Example 7 – *In vivo* effects of CU-NP on renal perfusion pressure

Studies were conducted to determine the *in vivo* actions of CNP, CU-NP and URO on renal perfusion pressure (RPP, as estimated by MAP-RAP), as RPP is a key determinant of renal function. Given the GFR-enhancing action of CU-NP, *in vitro* studies also were done to test the hypothesis that CU-NP (unlike CNP) also activates NPR-A, a potent renal acting NP receptor.

Equimolar doses of CU-NP, human CNP, or human URO were intravenously infused at 14.14 pmol/kg/minute into 17 normal anesthetized dogs for 75 minutes. RPP was assessed at baseline and at 60 minutes of infusion. Data are expressed as mean ± SEM. Clearances were measured from the 16th to the 45th minute and from the 46th to the 75th minute following initiation of peptide infusion. Within each group, 2-tailed paired t-test was used to compare RPP at 60 minutes of peptide infusion vs. baseline. Comparisons between groups were made by two-way ANOVA followed by Bonferroni post-test.

cGMP responses to the 3 peptides also were assessed in glomeruli isolated from canine kidneys upon harvest, in the presence or absence of an NPR-A antagonist, A719153 (1 μM), or an NPR-B antagonist, P194 (1 μM), or both antagonists added sequentially (A71915 followed by P194, 1 μM final concentration for both). To confirm the involvement of NPR-B in the cGMP response to CU-NP, additional experiments were conducted using human aortic endothelial cells (HAEC). Cyclic GMP response was determined by incubating CU-NP ($10^{-6}$ M) for 10 minutes in the absence or presence of an antibody to the ligand-binding domain of NPR-B. *In vitro* data (mean ± SEM) were analyzed by one-way ANOVA followed by Bonferroni's post-test. Statistical significance was defined as $P<0.05$. GraphPad Prism 4 (GraphPad Software, San Diego, CA) was used for statistical analyses.

In the *in vivo* studies, RPP (mmHg) was preserved with CU-NP as compared to URO. When the two groups were compared, RPP was significantly lower with URO vs. CU-NP at 60 minutes of peptide infusion ($P<0.05$, Figure 2). RPP was unchanged by CNP.

**Table 7**

*CU-NP effects on RPP*

| | **RPP (mean ± SEM; mmHg)** | |
|---|---|---|
| **Treatment** | **Pre-I** | **60 minutes** |
| CNP | 120 ± 4 | 123 ± 8 |
| URO | 127 ± 4 | 119 ± 4* |
| CU-NP | 135 ± 4 | 134 ± 4 |

* = P=0.05 vs. baseline

*In vitro*, $10^{-5}$ M CU-NP increased cGMP 7-fold vs. CNP (0.76 ± 0.05‡ fmol/μg vs. 0.11 ± 0.05 fmol/μg), with a trend to activate cGMP even more than URO (0.54 ± 0.10 fmol/μg; P = 0.086). These results are illustrated in Figure 3. The cGMP-stimulating action of CU-NP was attenuated by blockade of NPR-A with A71915 (0.31 ± 0.05† fmol/μg), blockade of NPR-B with P19 (0.28 ± 0.04† fmol/μg), or blockade of both NPR-A and NPR-B with A71915 and P19 (0.23 ± 0.05† fmol/μg), as shown in Figure 4.

In HAEC, $10^{-6}$ M CU-NP and CNP increased cGMP to 0.30 ± 0.02 pmol/mL and 0.17 ± 0.04 pmol/mL, respectively (P<0.01 for CU-NP vs. CNP; P<0.001 for CU-NP vs. control; P<0.001 for CNP vs. control). In the presence of the NPR-B antibody (1:100), the cGMP responses were attenuated to 0.19 ± 0.02 pmol/mL for CU-NP and 0.08 ± 0.01 pmol/mL for CNP (P<0.01 for CU-NP vs. no antibody and P<0.05 for CNP vs. no antibody). Results are plotted in Figure 5. These data suggest that NPR-B is involved, at least in part, in the cGMP response to CU-NP.

Thus, CU-NP preserves RPP at renal enhancing doses, in contrast to URO, which reduces RPP. Further, in isolated glomeruli the actions of CU-NP involve co-activation of both NPR-A and NPR-B, representing a novel dual NP receptor activator in the kidney. NPR-B is involved in part in the cGMP response to CU-NP in human aortic endothelial cells. Thus, CU-NP represents a novel new peptide technology that is capable of dual NPR-A and NPR-B activation.

## OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A polypeptide less than 44 amino acid residues in length, wherein said polypeptide comprises, in an order from amino terminus to carboxy terminus:

(a) the sequence set forth in SEQ ID NO:1 or the sequence set forth in SEQ ID NO:1 with no more than three additions, subtractions, or substitutions,

(b) the sequence set forth in SEQ ID NO:2 or the sequence set forth in SEQ ID NO:2 with no more than five additions, subtractions, or substitutions, and

(c) the sequence set forth in SEQ ID NO:3 or the sequence set forth in SEQ ID NO:3 with no more than three additions, subtractions, or substitutions.

2. The polypeptide of claim 1, wherein said polypeptide comprises natriuretic activity.

3. The polypeptide of claim 1, wherein said polypeptide lacks the ability to induce systemic hypotension.

4. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:1.

5. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:2.

6. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:3.

7. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:1, the sequence set forth in SEQ ID NO:2, and the sequence set forth in SEQ ID NO:3.

8. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:1 with no more than three conservative amino acid substitutions.

9. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:2 with no more than five conservative amino acid substitutions.

10. The polypeptide of claim 1, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:3 with no more than three conservative amino acid substitutions.

11. The polypeptide of claim 1, wherein said polypeptide is a substantially pure polypeptide.

12. An isolated nucleic acid encoding the polypeptide of claim 1.

13. A vector comprising a nucleic acid encoding the polypeptide of claim 1.

14. A host cell comprising a nucleic acid encoding the polypeptide of claim 1.

15. The host cell of claim 14, wherein said host cell is a eukaryotic host cell.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the polypeptide of claim 1.

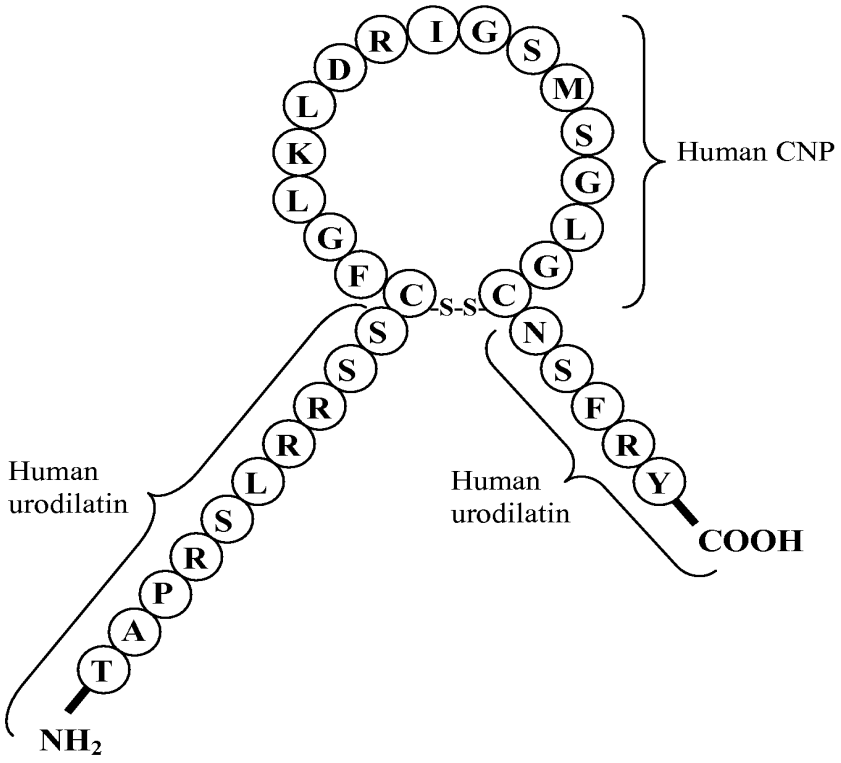
17. A method for increasing natriuretic activity within a mammal without lowering blood pressure, wherein said method comprises administering a polypeptide of claim 1 to said mammal.

18. A method for treating a mammal having a cardiovascular condition or renal condition, wherein said method comprises administering, to said mammal, a polypeptide of claim 1 under conditions wherein the severity of a manifestation of said cardiovascular condition or renal condition is reduced.

19. The method of claim 18, wherein administration of said polypeptide to said mammal does not lower the blood pressure of said mammal.

1/5

**Figure 1**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F
R
R
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Human urodilatin
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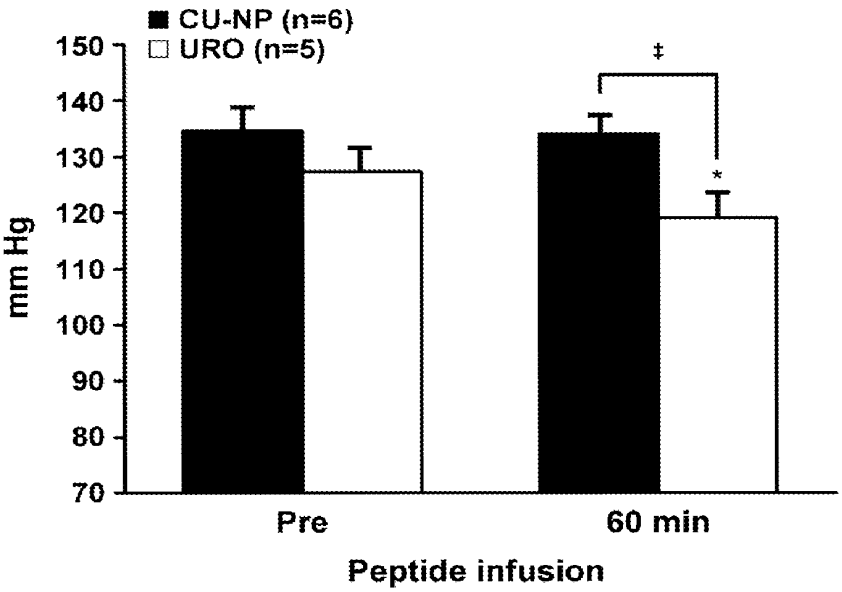
NH2

**Figure 2**


CU-NP (n=6)
URO (n=5)
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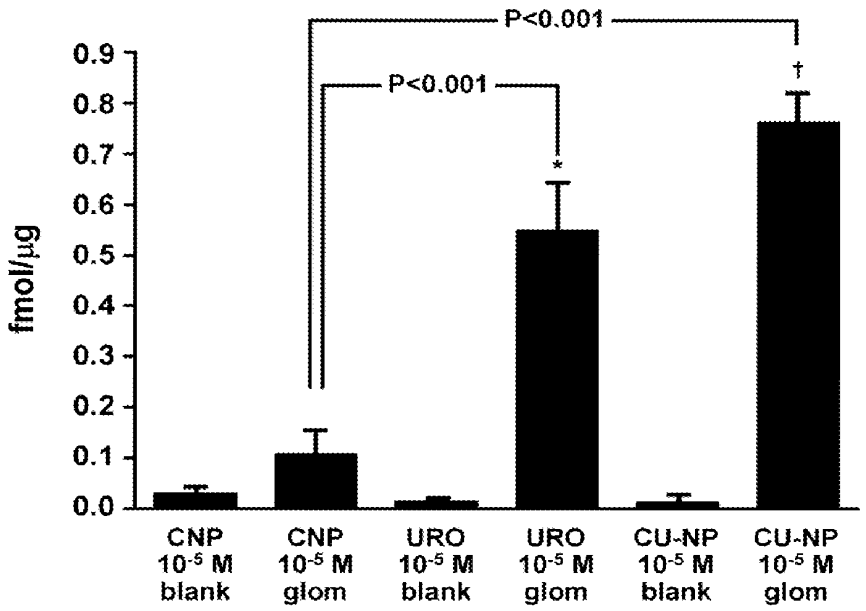
140
130
120
110
100
90
80
70
mm Hg
‡
*
Pre
60 min
Peptide infusion

**Figure 3**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P<0.001
*
†
fmol/µg
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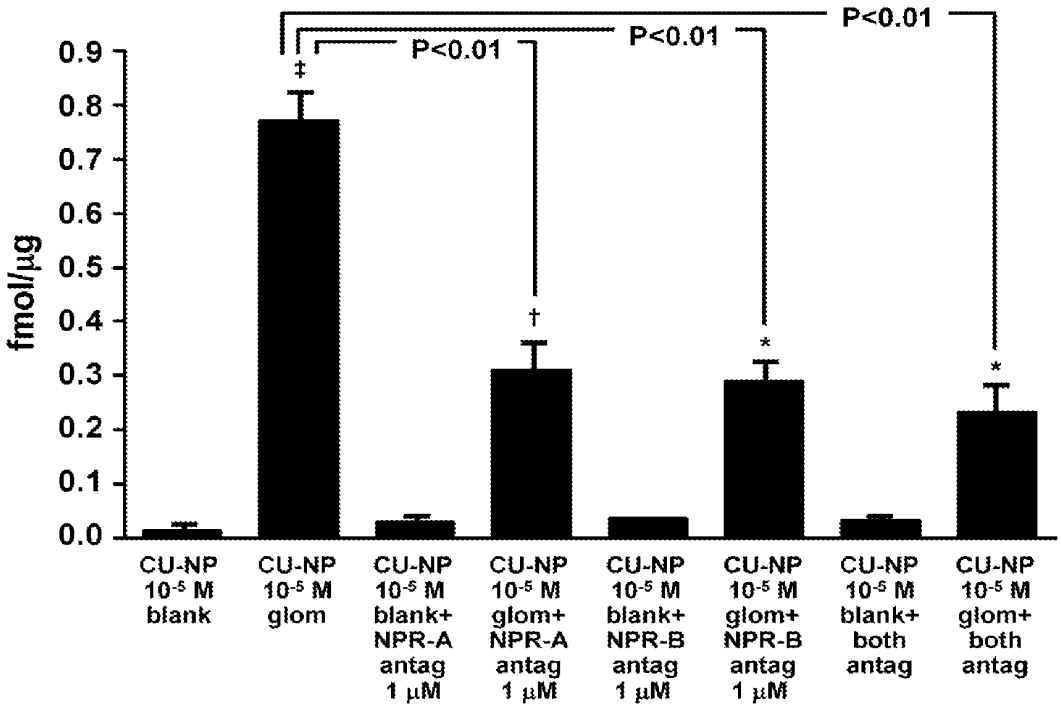
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CNP 10-5 M blank
CNP 10-5 M glom
URO 10-5 M blank
URO 10-5 M glom
CU-NP 10-5 M blank
CU-NP 10-5 M glom

**Figure 4**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P<0.01
P<0.01
fmol/μg
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*
*
CU-NP 10-5 M blank
CU-NP 10-5 M glom
CU-NP 10-5 M blank+ NPR-A antag 1 μM
CU-NP 10-5 M glom+ NPR-A antag 1 μM
CU-NP 10-5 M blank+ NPR-B antag 1 μM
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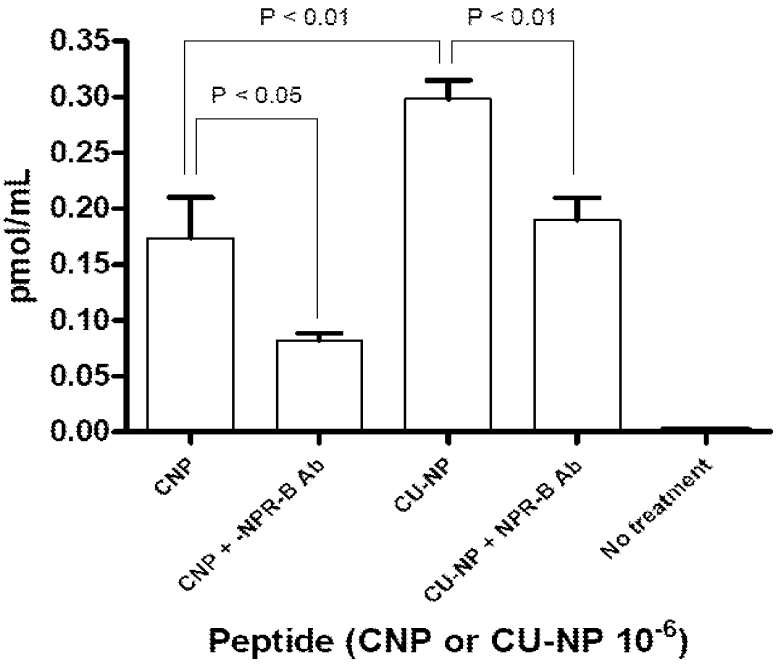
CU-NP 10-5 M blank+ both antag
CU-NP 10-5 M glom+ both antag

**Figure 5**




n = 6 per group

CNP vs no treatment P < 0.001
CU-NP vs no treatment P < 0.001

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2008/070447**

**A. CLASSIFICATION OF SUBJECT MATTER**

***C12N 15/00(2006.01)i, C12P 21/02(2006.01)i, A61K 38/22(2006.01)i***

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC: C12N 15/00, C12P 21/02, A61K 38/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Pubmed, Esp@snet, Delphion

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Lee, C. Y. and Burnett, J. C. Jr. 'Natriuretic peptides and therapeutic applications.' Heart failure Reviews. Vol. 12(2), pp. 131-142 (June 2007) | 1-16 |
| A | WO 01/44284 A2 (Mayo Foundation for Medical Education and Research) (21 June 2001) | 1-16 |
| A | Wei, C. M. et al. 'Vasonatrin peptide: A unique synthetic natriuretic and vasorelaxing peptide.' Journal of Clinical Investigation. Vol. 92(4), pp. 2048-2052 (October 1993) | 1-16 |
| T | Dickey, D. M. et al. 'Novel bifunctional natriuretic peptides as potential therapeutics.' Journal of Biological Chemistry. Vol. 283(50), pp. 35003-35009 (21 October 2008) | 1-16 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 JANUARY 2009 (12.01.2009) | **12 JANUARY 2009 (12.01.2009)** |

| Name and mailing address of the ISA/KR | Authorized officer |
|---|---|
| Korean Intellectual Property Office<br>Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea | KIM, JI YUN |
| Facsimile No. 82-42-472-7140 | Telephone No. 82-42-481-8288 |

Form PCT/ISA/210 (second sheet) (July 2008)

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2008/070447**

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17-19
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 17-19 pertain to methods for treatment of the human or animal body, thus relate to a subject-matter which International Searching Authority is not required to search under PCT Art. 17(2)(a)(i) and Rule 39.1(iv).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2008/070447**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 01/44284 A2 | 21.06.2001 | AT 353916 T | 15.03.2007 |
| | | AU 2001-24339 A1 | 25.06.2001 |
| | | AU 2433901 A | 25.06.2001 |
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| | | DK 1242452 T3 | 11.06.2007 |
| | | EP 1242452 A2 | 25.09.2002 |
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| | | WO 01/44284 A3 | 31.01.2002 |

Docket No.: 135576.010627/REI/CON
(Patent)

**GIN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 | Confirmation No.: 7727

Filed: July 11, 2017 | Art Unit: 3991

For: *VARIANTS OF C-TYPE NATRIURETIC PEPTIDE* | Examiner: Johnny F. Railey

**INFORMATION DISCLOSURE STATEMENT**

**SUBMITTED VIA EFS**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Pursuant to 37 C.F.R. §§1.56, 1.97 and 1.98, Applicants invite the attention of the U.S. Patent and Trademark Office to the references listed on the attached Form PTO/SB/08a. Applicants respectfully request that the information be expressly considered during the prosecution of the above-identified application and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

In accordance with C.F.R. § 1.97(e)(1), the references listed in the attached Form PTO/SB/08a were made of record in the Communication pursuant to Article 94(3) EPC, dated 04 April 2018, as received in European Patent Application No. 16 192 229.9, which corresponds to the subject application.

In accordance with 37 C.F.R. §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 C.F.R. §1.56(a) exists.

In accordance with 37 C.F.R. §1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

Applicants submit that the Information Disclosure Statement is in compliance with 37 C.F.R. §1.98 and respectfully request that the Examiner consider the references listed on the attached Form PTO SB/08.

Applicants believe that no fees are required for consideration and entry of this paper. Nevertheless, Applicants authorize the Director to charge any deficiencies in the fees filed, asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to Deposit Account No. 50-2678, under Order No. 135576.010627/REI/CON.

Respectfully submitted,
Greenberg Traurig LLP

Dated: May 16, 2018

By: /Leslie Serunian/
Leslie Serunian
Registration No.: 35,353
GREENBERG TRAURIG LLP
One International Place
Boston, Massachusetts 02110
Telephone: 617-310-6000
Facsimile: 617-310-6001

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 32639934 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 35893 |
| **Filer:** | Leslie A. Serunian |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 135576.010627/REI/CON |
| **Receipt Date:** | 16-MAY-2018 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 14:20:29 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

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|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Information Disclosure Statement (IDS) Form (SB08) | 135576_010627REICON_SB08.pdf | 1034723<br>c2ef298743d7d125802835688987c416fb24cdf3 | no | 4 |

**Warnings:**

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems. | | | | | |
| 2 | Foreign Reference | EP2277890A1.PDF | 3793162<br>00dec3c5dc0e9635f1d83a8ef9fc771c3dc7a763 | no | 99 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Foreign Reference | WO2006005140A2.PDF | 3007875<br>33c402b2443506f4d092e0c67303e8932b0511e0 | no | 97 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Foreign Reference | WO2009015011A1.PDF | 336606<br>38c5f4cdb8157c397d44a57c0b367348844cce0b | no | 42 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Non Patent Literature | MiltonHariset_et_al_Reference.PDF | 822746<br>093a88989487a1d5c4fc4514b282015e0995c495 | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Non Patent Literature | UniProtKB_citation.PDF | 403808<br>9075bbc22e9b54e7b263563a4d0a960eee3cdb8f | no | 4 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 7 | Transmittal Letter | 135576_010627REICON_IDS.pdf | 109638<br>3392fffb5e8496fcdc89a32c54676f4de1d482f0 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | | **Total Files Size (in bytes):** | 9508558 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 135576.010627/REI/CON | 7727 |

35893 7590 07/10/2018

GREENBERG TRAURIG (BOS)
ONE INTERNATIONAL PLACE, 20th FL
ATTN: PATENT ADMINISTRATOR
BOSTON, MA 02110

| EXAMINER | |
|---|---|
| RAILEY, JOHNNY F | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 07/10/2018 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

BosIpMail@gtlaw.com
baggettj@gtlaw.com
bechetn@gtlaw.com

| Office Action Summary | Application No. 15/646,822 | Applicant(s) WENDT ET AL. | |
|---|---|---|---|
| | Examiner JOHNNY F. RAILEY II | Art Unit 3991 | AIA (First Inventor to File) Status No |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5)☒ Claim(s) *15-30* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *15-30* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *5/16/2018*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

For reissue applications filed before September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the law and rules in effect on September 15, 2012. Where specifically designated, these are "pre-AIA" provisions.

For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.

## DETAILED ACTION

### *3rd Party Protest*

Pursuant to 37 CFR 1.291, a third party protest was filed in this reissue application on 3 March 2018, along with an Information Disclosure Statement.

### *Priority*

This application is a continuation reissue of reissue application 14/604,262, now U.S. Patent RE46,707 E, which is an reissue application for U.S. Patent 8,598,121, filed as application no. 13/466,672 and issued on 3 December 2013, which is a continuation of U.S. Patent Application 12/784,117, now U.S. Patent 8,198,242, issued on 12 June 2012, which claims priority and benefit of U.S. Provisional Application 61/254,563, filed on 23 October 2009, and U.S. Provisional Application 61/180,112, filed on 20 May 2009.

### *Specification*

The amendment to the specification filed on 11 July 2017 is not in compliance with 37 CFR 1.173(d)(1). The amendment contains the phrase "The present application" surrounded by double-brackets. Amendments to the specification are made relative to the patent being reissued and matter to be omitted must be enclosed in brackets. Double brackets are only used when the subject patent already has bracketing. See MPEP 1453, part IV.A. In addition, the amendment should be updated to reflect the fact that reissue application 14/604,262 is now U.S. Patent RE46,707.

### *Claims*

Pending claims are 15-30. Original patent claims 1-14 have been canceled. The independent claims are 15, 18, 21-23, and 27, summarized as follows:

**Claim 15** is drawn to a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 18** is drawn to a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 21** is drawn to a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 22** is drawn to a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 23** is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP is variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 27** is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 145, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

Note: The sequence identifier "SEQ ID NO: 145" as shown in the claims on pages 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 in the amendment as submitted 11 July 2017 is assigned to two different sequences.

***Defective Declaration/Oath***

The reissue oath/declaration filed with this application is defective because the error which is relied upon to support the reissue application is not an error upon which a reissue can be based. See 37 CFR 1.175 and MPEP § 1414. During the prosecution of application 13/466,672, a restriction requirement was held in which claims drawn to the elected species of SEQ ID NO: 185, a C-type natriuretic peptide of 53 amino acids (CNP53) were elected without traverse. The examiner also indicated that the variants of CNP-53 claimed as SEQ ID NOs: 179, 190, and 180 would be rejoined with the elected invention. See the Notice of Allowability mailed on 30 July 2013 in that application. The examiner also rejoined method

claims for the elected composition claims and withdrew from further consideration claims drawn to other non-elected subject matter. The addition of new claims 15-30 is not an error which is relied upon to support the reissue application. See MPEP 1412.01 I:

> Where a restriction (or an election of species) requirement was made in an application and applicant permitted the elected invention to issue as a patent without filing a divisional application on the non-elected invention(s), the non-elected invention(s) cannot be recovered by filing a reissue application. A reissue applicant's failure to timely file a divisional application covering the non-elected invention(s) in response to a restriction (or an election of species) requirement is not considered to be error causing a patent granted on the elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim. Accordingly, this is not correctable by reissue of the original patent under 35 U.S.C. 251. In re Watkinson, 900 F.2d 230, 14 USPQ2d 1407 (Fed. Cir. 1990); In re Orita, 550 F.2d 1277, 1280, 193 USPQ 145, 148 (CCPA 1977); See also In re Mead, 581 F.2d 251, 198 USPQ 412 (CCPA 1978). In this situation, the reissue claims should be rejected under 35 U.S.C. 251 for lack of defect in the original patent and lack of error in obtaining the original patent.

### *Claim Rejections - 35 USC § 251*

Claims 15-30 are rejected as being based upon a defective reissue declaration under 35 U.S.C. 251 as set forth above. See 37 CFR 1.175.

The nature of the defect(s) in the declaration is set forth in the discussion above in this Office action.

### *References*

Tsubery *et al.* "Prolonging the Action of Protein and Peptide Drugs by a Novel Approach of Reversible Polyethylene Glycol Modification," J. Biol. Chem. 279(37):38118-38124 (2004). ["**Tsubery**"]

Komatsu *et al.* "Significance of C-type natriuretic peptide (CNP) in endochondral ossification: analysis of CNP knockout mice," J. Bone Miner. Metab. 20:331-336 (2002). ["**Komatsu**"]*

Krejci *et al.* "Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis," J. Cell Science 118(21):5089-5100 (2005). ["**Krejci**"]

*cited by the examiner

### *Nonstatutory Double Patenting*

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not

identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

**1. Claims 15-20 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 2 of U.S. Patent No. RE46,707 E in view of Tsubery.**

**Claims 15-17** are drawn to a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. The synthetic polymeric group can be a hydrophilic moiety and the hydrophilic moiety can be polyethylene glycol (PEG). **Claim 2** of the '707 patent is drawn to a pharmaceutical composition comprising a CNP peptide variant that is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. Tsubery teaches covalent binding of PEG to a peptide. PEG-conjugated therapeutic proteins exhibit clinical properties superior to those of their corresponding unmodified parent proteins. Covalent binding of PEG to proteins produces steric interference, protecting conjugates from proteolysis and decreasing their rate of clearance from the circulation system via intracellular uptake and kidney filtration. The PEG is released from the conjugate by spontaneous chemical hydrolysis. See page 38118, first and second columns. It would have been obvious to attach PEG to any of SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180 for the purpose of producing a therapeutic with superior clinical properties.

**Claims 18-20** are drawn to a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is

SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. For the reasons given above as taught by Tsubery, it would have been obvious to produce a CNP conjugate comprising a CMP variant that is coupled to PEG through a hydrolysable linkage for the purpose of sustaining the conjugate in the circulatory system by reducing its clearance.

**2. Claim 21 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Komatsu.**

**Claim 21** is drawn to a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP wherein the CNP variant is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. **Claim 5** is described above. Komatsu is a review article that shows that the action of CNP is through binding of the GC-B receptor and activating guanylyl cyclase with the production of cGMP. See Figure 1 on page 332. Note: the specification of the '121 patent teaches at column 2, lines 23-28 that this process was well-known in the art, and that guanylyl cyclase B (GC-B) is also known as the natriuretic peptide receptor B (NPR-B). It would have been obvious and well-known that CNP stimulates cGMP production in a cell expressing the NPR-B (GC-B).

**3. Claim 22 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Krejci.**

**Claim 22** is drawn to a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant wherein the CNP variant is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. **Claim 5** is described above. Krejci teaches that mutations in the FGFR-3 arrests growth of chondrocytes and results in human dwarfisms, including hypochondroplasia. See page 5089, column 1. CNP and FGF oppose each other, so CNP is capable of mediating reversal of FGF signaling. CNP overexpression in cartilage of a mouse model for achondroplasia partially rescues the disease phenotype. See page 5089, column 2. Therefore, it would have been obvious to use a CNP variant as a means of overcoming the arrest of cell growth of chondrocytes thereby treating human dwarfism.

**4. Claims 23-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Tsubery.**

**Claims 23-26** are drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic

polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180.

**Claims 27-30** are drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. **Claim 5** of the '707 patent is drawn to a method of treating a bone-related disorder or skeletal dysplasia, selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia by administering a CNP peptide variant that is SEQ ID NO: 179, SEQ ID NO: 185, SEQ ID NO: 190, or SEQ ID NO: 180. For the reasons given above as taught by Tsubery, it would have been obvious to produce a CNP conjugate comprising a CMP variant that is coupled to PEG through a hydrolysable linkage for the purpose of sustaining the conjugate in the circulatory system by reducing its clearance.

## *Conclusion*

Claims 1-30 are rejected. A shortened statutory period for reply to this office action is set to expire THREE MONTHS from the mailing date of this action.

## ONGOING DUTY TO DISCLOSE

Applicant is reminded of the continuing obligation under 37 CFR 1.178(b), to timely apprise the Office of any prior or concurrent proceeding in which Patent No. RE46,707 E is or was involved. These proceedings would include interferences, reissues, reexaminations, and litigation.

Applicant is further reminded of the continuing obligation under 37 CFR 1.56, to timely apprise the Office of any information which is material to patentability of the claims under consideration in this reissue application.

These obligations rest with each individual associated with the filing and prosecution of this application for reissue. See also MPEP §§ 1404, 1442.01 and 1442.04.

## INTERVIEWS

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice. The fax phone number for the organization where this application or proceeding is assigned is 571-273-9900.

**FUTURE CORRESPONDENCE**

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Johnny F. Railey II** whose telephone number is (571) 272-0985. The examiner can normally be reached on Monday through Thursday from 9:00 a.m. to 6:30 p.m. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Jean Witz**, can be reached on (571) 272-0927.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://www.uspto.gov/patents/process/status/index.jsp . Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

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/Jean C. Witz/
Supervisory Patent Reexamination Specialist
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| Notice of References Cited | Application/Control No. 15/646,822 | Applicant(s)/Patent Under Reexamination WENDT ET AL. | |
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| | Examiner JOHNNY F. RAILEY II | Art Unit 3991 | Page 1 of 1 |

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Doc code: IDS
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**( Not for submission under 37 CFR 1.99)**

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| Application Number | 15646822 |
| Filing Date | 2017-07-11 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 1629 |
| Examiner Name | N/A |
| Attorney Docket Number | 135576-010627/REI/CON |

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The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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| Signature | /Leslie Serunian/ | Date (YYYY-MM-DD) | 2017-10-11 |
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| Name/Print | Leslie Serunian | Registration Number | 35,353 |

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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# Bibliographic Data

Application No: 15/646,822

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /JOHNNY F RAILEY II/ Examiner's Signature | Initials

Title: Variants of C-Type Natriuretic Peptide

| FILING or 371(c) DATE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|
| 07/11/2017 | 514 | 3991 | 135576.010627/REI/CON |
| RULE | | | |

**APPLICANTS**

BioMarin Pharmaceutical Inc., Novato, CA, UNITED STATES

**INVENTORS**

Daniel J. Wendt Novato, CA, UNITED STATES

Shinong Long Novato, CA, UNITED STATES

Sianna Castillo Novato, CA, UNITED STATES

Christopher P. Price Aachen, GERMANY

Mika Aoyagi-Scharber Novato, CA, UNITED STATES

Michel Claude Vellard Novato, CA, UNITED STATES

Augustus O. Okamafe Concord, CA, UNITED STATES

**CONTINUING DATA**

This application is a CON of 14604262 01/23/2015 PAT RE46707

14604262 is a REI of 13466672 05/08/2012 PAT 8598121

13466672 is a CON of 12784117 05/20/2010 PAT 8198242

12784117 has PRO of 61254563 10/23/2009

12784117 has PRO of 61180112 05/20/2009

**FOREIGN APPLICATIONS**

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07/12/2017

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| Index of Claims | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15646822 | WENDT ET AL. |
| | **Examiner** | **Art Unit** |
| | JOHNNY F RAILEY | 3991 |

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| ✓ | Rejected | - | Cancelled | N | Non-Elected | A | Appeal |
| = | Allowed | ÷ | Restricted | I | Interference | O | Objected |

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

| CLAIM | | DATE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Final | Original | 06/15/2018 | | | | | | | | |
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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
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| Application Number | 15646822 |
| Filing Date | 2017-07-11 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | ~~4629~~ 3991 |
| Examiner Name | N/A |
| Attorney Docket Number | 135576-010627/REI/CON |

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| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

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| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
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The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

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| Signature | /Leslie Serunian/ | Date (YYYY-MM-DD) | 2017-10-11 |
|---|---|---|---|
| Name/Print | Leslie Serunian | Registration Number | 35,353 |

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Sheet 1 of 2

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| Application Number | 15/646,822 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 1629 |
| Examiner Name | Not applicable |
| Attorney Docket Number | 065588-495115 |

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| **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** | | | | Application Number | 15/646,822 |
| | | | | Filing Date | July 11, 2017 |
| | | | | First Named Inventor | Daniel J. Wendt |
| | | | | Art Unit | 1629 |
| *(Use as many sheets as necessary)* | | | | Examiner Name | Not applicable |
| Sheet | 2 | of | 2 | Attorney Docket Number | 065588-495115 |

| NON PATENT LITERATURE DOCUMENTS | | | |
|---|---|---|---|
| Examiner Initials * | Cite No.[1] | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T [2] |
| | A | US Application 13/466,672 claims pending as of the mailing of the Restriction Requirement on February 13, 2013. | |
| | B | US Application 13/466,672 Restriction Requirement mailed February 13, 2013. | |
| | C | US Application 13/466,672 Response to Restriction Requirement submitted April 12, 2013. | |
| | D | US Application 13/466,672 Examiner-Initiated Interview Summary mailed July 30, 2013. | |
| | E | US Patent 8,598,121 B2 claims as granted on December 3, 2013. | |
| | F | US Application 15/332,793 claims as amended in a Preliminary Amendment submitted October 24, 2016. | |
| | G1 | *Ex parte Joachim Werres,* Appeal 2006-2966 (BPAI 2007). | ☐ |
| | G2 | *Ex parte Morrison et al.,* Appeal 2012-012266 (PTAB 2013) affirmed (2014-1050 Fed. Cir. 2014). | |
| | G3 | Ex *Parte Solomon*, Appeal 2012-002100 (BPAI 2012), affirmed (2012-1689 Fed. Cir. 2013) | |
| | G4 | *Ex parte Wu et al.*, Appeal 2016-0348 (PTAB 2017). | |
| | H1 | PTO Brief from Federal Circuit appeals in *Ex parte Morrison (2012-1050 Fed. Cir. 2014).* | ☐ |
| | H2 | PTO Brief from Federal Circuit appeals in *Ex parte Solomon (2012-1689 Fed. Cir. 2013).* | |
| TSUBERY | I | TSUBERRY et al., "Prolonging the Action of Protein and Peptide Drugs by a Novel Approach of Reversible Polyethylene Glycol Modification", J. Biol. Chem., Vol. 279, No. 37, pp 38118-3824 (2004). | ☐ |

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

/JOHNNY F RAILEY II/ 06/18/2018

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15646822 | WENDT ET AL. |
| | Examiner | Art Unit |
| | JOHNNY F RAILEY | 3991 |

| CPC- SEARCHED | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| CPC COMBINATION SETS - SEARCHED | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US CLASSIFICATION SEARCHED | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| SEARCH NOTES | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Reviewed prosecution history in parent application 13/466,672 | 6/6/2018 | /J.R./ |
| Reviewed prosecution in RE 46,707 E | 6/6/2018 | /J.R./ |
| Google Search Terms: C-type natriuretic peptide bone review "fibroblast growth factor receptor" | 6/12/2018 | /J.R./ |
| Inventor Search | 6/18/2018 | /J.R./ |

| INTERFERENCE SEARCH | | | |
|---|---|---|---|
| US Class/ CPC Symbol | US Subclass / CPC Group | Date | Examiner |
| | | | |

| | |
|---|---|
| | |

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

**( Not for submission under 37 CFR 1.99)**

| | |
|---|---|
| Application Number | 15646822 |
| Filing Date | 2017-07-11 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | Johnny F. Railey |
| Attorney Docket Number | 135576.010627/REI/CON |

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| Examiner Initial* | Cite No | Foreign Document Number[3] | Country Code[2] i | Kind Code[4] | Publication Date | Name of Patentee or Applicant of cited Document | Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear | T[5] |
|---|---|---|---|---|---|---|---|---|
| /J.R./ | 1 | 2006005140 | WO | A1 | 2006-01-19 | THE UNIVERSITY OF QUEENSLAND, ET AL. | | |
| /J.R./ | 2 | 2009015011 | WO | A1 | 2009-01-29 | MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH | | |
| /J.R./ | 3 | 2 277 890 | EP | A1 | 2011-01-26 | DAIICHI SANKYO COMPANY LTD. | | |

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 3991 |
| | Examiner Name | Johnny F. Railey |
| | Attorney Docket Number | 135576.010627/REI/CON |

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**NON-PATENT LITERATURE DOCUMENTS** [Remove]

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|---|---|---|---|
| /J.R./ | 1 | Anonymous: "UniProtKB - Q9QZ96 (Q9QZ96_CAVPO)", 1 May 2000 (2000-05-01), pages 1-4, SP055463352, Retrieved from the internet: URL:http/www.uniprot.org/uniparc/UPI00000E7708 (retrieved on 2018-03-28) | |
| /J.R./ | 2 | Milton Harris, J et al., "Effect of Pegylation on Pharmaceuticals," Drug Discovery, Nature Publishing Group, GB, Vol. 2, No. 3, 1 March 2003 (2003-03-01), pages 214-221. | |

If you wish to add additional non-patent literature document citation information please click the Add button [Add]

**EXAMINER SIGNATURE**

| Examiner Signature | /JOHNNY F RAILEY II/ | Date Considered | 06/18/2018 |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 3991 |
| | Examiner Name | Johnny F. Railey |
| | Attorney Docket Number | 135576.010627/REI/CON |

**CERTIFICATION STATEMENT**

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

**OR**

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

**SIGNATURE**

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

| Signature | /Leslie Serunian/ | Date (YYYY-MM-DD) | 2018-05-16 |
|---|---|---|---|
| Name/Print | Leslie Serunian | Registration Number | 35353 |

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

## Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 33953998 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 35893 |
| **Filer:** | Leslie A. Serunian |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 135576.010627/REI/CON |
| **Receipt Date:** | 09-OCT-2018 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 14:16:42 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Amendment/Req. Reconsideration-After Non-Final Reject | 135576_010627REI_Response.pdf | 345486<br>2b84ccd3613fe8f8d7d44e75d476d92893f17985 | no | 37 |

**Warnings:**

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| Total Files Size (in bytes): | 345486 |

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**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
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**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

Docket No.: 135576.010627/REI/CON
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 | Confirmation No.: 7727

Filed: July 11, 2017 | Art Unit: 3991

For: *VARIANTS OF C-TYPE NATRIURETIC PEPTIDES* | Examiner: Johnny F. Railey II

**RESPONSE TO THE JULY 10, 2018 NON-FINAL OFFICE ACTION**

**SUBMITTED VIA EFS-Web**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

## INTRODUCTORY COMMENTS

This Response is submitted in reply to the Non-Final Office Action, dated July 10, 2018, ("Office Action"), as received from the U.S. Patent and Trademark Office in the above-identified patent application. Entry and consideration of the amendments and remarks herein are respectfully requested.

An **Amendment to the Specification** begins on page 2 of this paper.

**Amendments to the Claims** are reflected in the **Listing of the Claims** that begins on page 3 of this paper.

**Remarks** begin on page 28 of this paper.

## IN THE SPECIFICATION

Please amend the first paragraph of the specification under "Cross Reference to Related Applications" as follows:

~~The present~~ This application is a continuation reissue of Reissue Application No. 14/604,262, now U.S. Patent No. RE46,707, issued February 13, 2018, which is a reissue of U.S. Patent Application No. 13/466,672, filed May 8, 2012, now U.S. Patent No. 8,598,121, issued December 3, 2013, which is a continuation of U.S. Patent Application No. 12/784,117, filed May 20, 2010, now U.S. Patent No. 8,198,242, issued June 12, 2012, which claims ~~the~~ priority to and benefit of U.S. Provisional Application No. 61/254,563, filed on Oct. 23, 2009, and U.S. Provisional Application No. 61/180,112, filed on May 20, 2009, the disclosure of each of which is incorporated herein by reference in its entirety.

## LISTING OF THE CLAIMS

This listing of the claims replaces and supersedes all prior versions and listings of the claims in the application.

Please amend the claims as follows:

1.-14. **(Canceled)**

15. **(Currently amended)** A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)

(SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)

(SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)

(SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)

(SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);

KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);
KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. **(Previously presented)** The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. **(Previously presented)** The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. **(Currently amended)** A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)

(SEQ ID NO: 150);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);

KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

19. **(Previously presented)** The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. **(Previously presented)** The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. **(Currently amended)** A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B), comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)

(SEQ ID NO: 151);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);

KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

22. **(Currently amended)** A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);

KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);

KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);

KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

23. **(Currently amended)** A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);

KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);

KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);

KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. **(Previously presented)** The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. **(Previously presented)** The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. **(Previously presented)** The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. **(Currently amended)** A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);

KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);

KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: [[~~145~~]] 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

28. **(Previously presented)** The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. **(Previously presented)** The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. **(Previously presented)** The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

## REMARKS

### In the Specification

The specification of the application is amended as set forth hereinabove to comply with 37 C.F.R. §1.173(d)(1) and to reflect the current status of reissue application Serial No. 14/604,262.

### In the Claims

At the time of the Office Action, claims 15-30 were pending and under consideration. In this paper, claims 15, 18, 21-23 and 27 are currently amended, and claims 16, 17, 19, 20, 24-26 and 28-30 are as previously presented. Claims 1-14 are canceled without prejudice or disclaimer. The amended claims are supported by the application as originally filed. No new matter is added by virtue of the amendments to the claims presented herein. Applicants expressly reserve the right to pursue any canceled, withdrawn, non-elected, or otherwise unclaimed subject matter in one or more subsequently filed continuing applications.

The Office Action indicates that the sequence identifier "SEQ ID NO: 145" as shown in the claims on pages 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 in the amendment submitted on July 11, 2017 is assigned to two different sequences. Accordingly, Applicants have amended the claims to correct this inadvertent error. In the claims as currently amended, the sequence of the CNP variant identified as "(Met-Gly-CNP37)" is now correctly identified as SEQ ID NO: 191, rather than as SEQ ID NO: 145 in amended claims 15, 18, 21-23 and 27. Support for the amendment is found in the originally filed specification, for example, *inter alia*, at page 75, lines 9-10, and in the Sequence Listing submitted with the application.

Accordingly, claims 15-30 are currently pending.

### Defective Declaration/Oath

The Office Action indicates that the reissue declaration/oath filed with the instant continuation reissue application is defective because the error which is relied upon to support the

continuation reissue application is allegedly not an error upon which a reissue can be based, citing 37 C.F.R. §1.175 and MPEP §1414.

In support of the objection, the Office Action notes that "[d]uring the prosecution of application 13/466,672, a restriction requirement was held in which claims drawn to the elected species of SEQ ID NO: 185, a C-type natriuretic peptide of 53 amino acids (CNP53) were elected without traverse," and that, although the variants of CNP-53 claimed as SEQ ID NOs: 179, 190, and 180, as well as the method claims directed to the elected compositions, were rejoined, further consideration of claims directed to other, non-elected subject matter was withdrawn. Citing MPEP §1412.01(I), which states that "[a] reissue applicant's failure to timely file a divisional application covering the non-elected invention(s) in response to a restriction (or an election of species) requirement is not considered to be error causing a patent granted on the elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim," the Office Action concludes that the addition of new claims 15-30, which recite previously non-elected species of CNP variants, is not an error which can be relied upon to support the reissue application. (*See*, pages 4-5 of the Office Action).

Applicants respectfully disagree.

The reissue oath/declaration filed with the instant application properly identifies an error upon which a reissue can be based

In accordance with 35 U.S.C. §251, the error upon which a reissue is based must be one which causes the patent to be "deemed wholly or partly inoperative or invalid, by reason of a defective specification or drawing, or by reason of the patentee claiming more or less than he had a right to claim in the patent." *See also*, MPEP §1402.

Contrary to the assertions in the Office Action, Applicants submit that the reissue oath/declaration filed with the instant continuation reissue application properly identifies an error upon which a reissue can be based, namely, by reason of the patentee claiming less than it had the right to claim in the patent, in that the pending broadened claims are directed to embodiments disclosed but not claimed in the 13/466,672 application, which issued as U.S. Patent No. 8,598,121.

As further provided in MPEP §1412.03 (I), "a broadened reissue claim is a claim which enlarges the scope of the claims of the patent." For example, a claim of a reissue application enlarges the scope of the claims of the patent if it is broader in at least one respect, even though it may be narrower in other respects. *Id.*; *see also*, 37 C.F.R. §1.175(b). A claim in the reissue application is also considered to enlarge the scope of the patent claims if it includes subject matter not covered by the patent claims. *Id*; second paragraph. Thus, a claim would be considered a broadening claim if the patent owner would be able to sue any party for infringement who previously could not have been sued for infringement. *Id.* Furthermore, an attorney's failure to appreciate the full scope of the invention was held to be an error correctable through reissue in the decision of *In re Wilder,* 736 F.2d 1516, 222 USPQ 369 (Fed. Cir. 1984), (*see also*, MPEP §1402 (I)).

MPEP § 1412.03 (IV) states that if intent to broaden is indicated in a parent reissue application within two years from the grant of the original patent, a broadened claim can be presented in a continuing (continuation or divisional) reissue application after the two year period. *See also*, *In re Staats*, 671 F.3d 1350, 101 USPQ2d 1930 (Fed. Cir. 2012). *In re Staats* further held that the broadened claims in the continuation reissue application may be directed to an embodiment "alternative" to, and "unrelated" to, the broadened claims of the first reissue application that were filed within the 2-year limit. *Id.* at 1355, 101 USPQ2d at 1934. Thus, the decision provides patentees additional leeway to seek patent protection for previously unclaimed subject matter after the original patent has issued.

The claims of the 13/466,672 application, which issued as U.S. Patent No. 8,598,121, were drawn to a variant of C-type natriuretic peptide (CNP) (specifically, SEQ ID NOs: 179, 185, 190 and 180); a pharmaceutical composition comprising the specified CNP variants; a method of treating a bone-related disorder or skeletal dysplasia; a method for recombinant production of a CNP variant; and a method of increasing long bone growth. By contrast, the independent claims of the instant continuation reissue application are directed a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 15); a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable

linkage (claim 18); a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B), comprising contacting the cell expressing NPR-B with a CNP variant (claim 21); a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant (claim 22); a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant, comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 23); and a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 27).

Applicants submit that pending claims 15-30 represent previously unclaimed subject matter in the form of invention embodiments substantially different from those claimed in U.S. Patent No. 8,598,121. Since these pending continuation reissue claims include subject matter not covered by the patent claims, they can be said to enlarge the scope of the patent claims. Accordingly, because the scope of claims 15-30 sought in the continuation reissue application extends beyond the scope of the claims issued in U.S. Patent No. 8,598,121, the patentee can be considered to have claimed less than it had the right to claim in the patent, which is an error that can be relied upon to support the reissue application.

The pending reissue claims are not substantially identical to those of nonelected groups identified in restriction requirement and represent overlooked aspects of the invention

MPEP §1412.01(I), as cited in the Office Action and which states that "[w]here a restriction (or an election of species) requirement was made in an application, and applicant permitted the elected invention to issue as a patent without filing a divisional application on the non-elected invention(s), the non-elected invention(s) cannot be recovered by filing a reissue application," and "is not considered to be error causing a patent granted on the elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim," relies on the legal precedent commonly referred to as the Orita doctrine. *In re Orita*, 550 F.2d 1277, 193 USPQ 145 (CCPA 1977).

Applicants maintain that the instant case is clearly distinguishable from the *In re Orita case ("Orita")*. In *Orita*, the applicant agreed to a restriction requirement and canceled non-elected claims. Then, after issuance of the patent on the elected claims, an application for reissue was filed asserting claims "substantially identical to the originally non-elected claims." *Id.* at 1278 (emphasis added). The court also rested its decision on the ground that the patentee knowingly acquiesced in the restriction requirement and that such acquiescence, because it is by definition not inadvertent, cannot be error for purposes of the reissue statute. *Id.* By contrast, as noted above, the pending continuation reissue claims 15-30 cover subject matter that was disclosed but had never been presented in the original application and that is substantially different from the non-elected claims.

The claims of the 13/466,672 application, which were subject to a restriction requirement, were directed to the following embodiments identified by the examiner as separate inventions in the application:

*I. Claims 1-4, 30, 45 and 46, drawn to a variant of C-type natriuretic peptide (CNP).*

*II. Claim 7, drawn to a method of treating a bone-related disorder or skeletal dysplasia.*

*Ill. Claim 9, drawn to a method of treating a vascular smooth muscle disorder.*

*IV. Claims 10, 16, 18, 22-24, 31, 35, drawn to a method for recombinant production of a CNP variant and a host cell used in said method.*

*V. Claims 39 and 41, drawn to a method of treating a CNP-responsive condition or disorder.*

*VI. Claim 44, drawn to a method of increasing long bone growth.*

By contrast, and as noted hereinabove, the independent claims of the instant continuation reissue application are directed a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 15); a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 18); a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B), comprising

contacting the cell expressing NPR-B with a CNP variant (claim 21); a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3), comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant (claim 22); a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 23); and a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage (claim 27).

Once again, Applicants emphasize that pending claims 15-30 represent previously unclaimed subject matter in the form of invention embodiments that are substantially different from those originally claimed in the 13/466,672 application. Since claims 15-30 pending in the subject continuation reissue application represent subject matter not covered by the claims of the 13/466,672 application, they are neither identical nor substantially similar to the non-elected claims. As such, the *Orita* doctrine does not apply.

Rather, the pending claims in the subject continuation reissue application are drawn to overlooked aspects of the invention. According to MPEP §1412.01(II), claims to overlooked aspects are not subject to recapture because the claims are, by definition, unrelated to subject matter that was surrendered during the prosecution of the original application. In the decision *In re Youman*, 679 F.3d 1335, 102 USPQ2d 1862 (Fed. Cir. 2012), the Federal Circuit explained:

> *Whereas the recapture rule applies when surrendered subject matter is being reclaimed, overlooked aspects by definition were never claimed and thus never surrendered. See Mostafazadeh, 643 F.3d at 1360 [98 USPQ2d at 1644]. Rather, as we explained in Mostafazadeh, "overlooked aspects" is a separate inquiry under reissue that is independent of whether or not the recapture rule applies.* 679 F.3d at 1347, 102 USPQ2d at 1870.

The overlooked aspects inquiry is only applicable when an examiner determines that the broadening aspect(s) of that reissue claim relate(s) to subject matter that applicant previously surrendered during the prosecution of the original application (which became the patent to be reissued) in step 2 of the recapture analysis. *See*, MPEP §1412.02, subsection II.B. If the

examiner determines that claims are drawn to overlooked aspects, Applicants respectfully request that it be stated in the record of the subject application which claims are drawn to overlooked aspects. A recapture analysis would not need to be continued for claims that are drawn to overlooked aspects.

The pending reissue claims are broad enough to read on or link the invention elected together with the invention not elected

Applicants submit that the pending continuation reissue claims should be evaluated as overlooked aspects of the invention and more properly compared with the case of *In re Doyle* ("*Doyle*") 293 F.3d 1355, 63 USPQ2d 1161 (Fed. Cir. 2002), which presents numerous counter-arguments to the overbroad application of the *Orita* doctrine and is also recited in MPEP §1412.01(I). With respect to *Doyle*, the MPEP states that "the court permitted the patentee to file a reissue application to present a so-called linking claim, a claim broad enough to read on or link the invention elected (and patented) together with the invention not elected." *Id.* Of note, in the very next paragraph, the MPEP further states that "[c]laims to separate inventions/embodiments/species that were disclosed but never covered by the claims in the original application prosecution are claims to overlooked aspects." *See*, MPEP §1412.01(II).

Doyle's original application describes a genus of chiral transition metal catalysts and methods of using the catalysts to perform enantioselective reactions with prochiral starting materials. During prosecution of the original application, the Examiner issued a nine-way restriction requirement. In response, Doyle elected to prosecute claims of a single group directed to a method of using the genus of catalysts to insert carbenes and canceled claims directed to other non-elected chemical modifications. The original application eventually issued as U.S. Patent No. 5,296,595. After failing to file any divisional applications directed to the cancelled claims during the pendency of the original application, Doyle filed a request for reissue of the '595 patent within the two-year time limit for broadening reissues with claims that encompassed a genus of methods that included the methods of the original claims, as well as those of three groups of claims that were not elected in the original application. While the PTO argued that *In re Orita* prevented a patentee from seeking through reissue claims that it had failed to pursue after a restriction requirement, the Federal Circuit was not convinced that this reasoning should

apply to Doyle's reissue claims; however, noting that the reissue claims were neither identical to nor substantially similar to the nonelected, cancelled claims. Instead, the reissue claims were genus claims that read on both elected and nonelected subject matter and no claims of individually similar scope were presented in the original application. The new claims were thus linking claims -- claims that read on both elected and nonelected claims -- which could have, and indeed should have, been presented in the original application. On this basis, the Federal Circuit concluded that the failure to file the claims in the original application was indeed an error.

As in *Doyle*, the instant continuation reissue claims are neither identical to nor substantially similar to the non-elected claims (see discussion above). Moreover, the new claims are genus claims, whereas the nonelected claims, most notably with respect to the composition claims directed to CNP variants, are species that fall within the new genus claims, directed to, for example, macromolecules capable of releasing a CNP variants, sustained release CNP variant formulations, methods of stimulating cGMP production in a cell, and methods of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3). As such, the continuation reissue claims are substantially broader than the claims of the nonelected groups with species elections. In contrast to *Orita* and similar to *Doyle*, Applicants never asserted the continuation reissue claims or anything similar to them in the original application, and also never agreed to prosecute the reissue claims in a divisional application. Accordingly, the estoppel rationale underlying *In re Orita* cannot support a similar result here, because there is simply no agreement as to the particular claims whereby Applicants may be estopped.

The court in *In re Doyle* also dispelled the notion that acquiescence to a restriction requirement forecloses the applicant's right to assert claims in reissue to any portion of the subject matter of the nonelected groups. In response to the assertion that Dr. Doyle's linking claims read on the subject matter of some of the nonelected groups and that he was allegedly estopped from seeking in reissue to add those claims to the patent claiming the elected group, the court held that "*In re Orita* did not create a broad rule disallowing any reissue claims that read on nonelected subject matter," again pointing out that the rationale underlying the holding in *In re*

*Orita* extends only to claims that are identical to or of substantially similar scope to those of the nonelected group. *Id.* at 1361.

In view of the above, Applicants submit that the pending continuation reissue claims are substantially different from the non-elected inventions, and thus, the reissue oath/declaration filed with the instant application properly relies upon an error which a reissue can be based. Accordingly, Applicants respectfully request that the objection be withdrawn.

## Claim Rejections

### Rejection under 35 U.S.C. §251

Claims 15-30 are rejected as allegedly being based upon a defective reissue declaration under 35 U.S.C. §251 as set forth above. See 37 C.F.R. §1.175.

In view of the remarks and discussion presented hereinabove, Applicants respectfully request that the rejection under 35 U.S.C. §251 be reconsidered and withdrawn.

### Nonstatutory Double Patenting

Claims 15-20 are rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claim 2 of U.S. Patent No. RE46,707 E in view of Tsubery et al. (*J. Biol. Chem.*, 279(37):38118-38124 (2004)).

Claim 21 is rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Komatsu et al. (*J. Bone Miner. Metab.*, 20:331-336 (2002)) for the reasons set forth on page 7 of the Office Action.

Claim 22 is rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Krejci et al. (*J. Cell Science*, 118(21):5089-5100 (2005)) for the reasons set forth on page 6 of the Office Action for the reasons set forth on page 7 of the Office Action.

Claims 23-30 are rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Tsubery for the reasons set forth on pages 7-8 of the Office Action.

Applicants respectfully request that these rejections be held in abeyance until allowable subject matter has been identified in the subject application, at which time Applicants will present further remarks and/or consider filing one or more terminal disclaimers.

## CONCLUSION

Applicants submit that the pending claims are in condition for allowance and respectfully requested expedited notification of the same.

## AUTHORIZATION

The Commissioner is hereby authorized to charge any fees, or additional fees, that may be required for the entry and consideration of this paper to Deposit Account No. 50-2678, Order No. 135576.010627/REI/CON. Should an extension of time be required in addition to that requested in a petition for extension of time submitted herewith, the Commissioner is requested to grant a petition for that extension of time which is required to make this paper and accompanying submissions timely, and is hereby authorized to charge any fee for such an extension of time, or to credit any overpayment, to Deposit Account No. 50-2678, Order No. 135576.010627/REI/CON.

Respectfully submitted,
Greenberg Traurig, LLP

Dated: October 9, 2018

By: /Melissa Hunter-Ensor, Ph.D./
Melissa Hunter-Ensor, Ph.D.
Registration No.: 55,289
Leslie Serunian
Registration No.: 35,353
GREENBERG TRAURIG, LLP
One International Place, Suite 2000
Boston, MA 02110
Telephone: (617) 310-6000
Facsimile: (617) 310-6001
Customer No. 35893

PTO/SB/06 (09-11)
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| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/646,822 | Filing Date<br>07/11/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | 10/09/2018 | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * 16 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 6 | Minus | *** 6 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | Patricia F Lewis |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

Doc Code: PA..
Document Description: Power of Attorney

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# TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

| | |
|---|---|
| Application Number | 15/646,822 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Title | Variants of C-Type Natriuretic Peptide |
| Art Unit | 3991 |
| Examiner Name | Johnny F. Railey |
| Attorney Docket Number | 30610/44556C1-R2 |

**SIGNATURE of Applicant or Patent Practitioner**

| | | | |
|---|---|---|---|
| Signature | /Katherine L. Neville/ | Date (Optional) | March 15, 2019 |
| Name | Katherine L. Neville | Registration Number | 53,379 |
| Title (if Applicant is a juristic entity) | | | |
| Applicant Name (if Applicant is a juristic entity) | | | |

**NOTE:** This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of 1 forms are submitted.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
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# POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

| Application Number | Filing Date |
|---|---|
| | |

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 90849

**OR**

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

**Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:**

[ ] The address associated with the above-mentioned Customer Number

**OR**

[ ] The address associated with Customer Number:

**OR**

| | | | | | |
|---|---|---|---|---|---|
| Firm or Individual Name | | | | | |
| Address | | | | | |
| City | | State | | Zip | |
| Country | | | | | |
| Telephone | | Email | | | |

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

BIOMARIN PHARMACEUTICAL INC.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

**SIGNATURE of Applicant for Patent**

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

| | | | |
|---|---|---|---|
| Signature | Luisa Bigornia | Date (Optional) | |
| Name | Luisa Bigornia | | |
| Title | Vice President, Intellectual Property, BioMarin Pharmaceutical Inc. | | |

**NOTE:** Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[X] Total of 1 forms are submitted.

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 35433946 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 35893 |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Filer Authorized By:** | Katherine Lynn Neville |
| **Attorney Docket Number:** | 135576.010627/REI/CON |
| **Receipt Date:** | 15-MAR-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 11:27:11 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Assignee showing of ownership per 37 CFR 3.73 | Statement_By_Assignee_to_Establish_Ownership_37_CFR_373c.PDF | 25111<br>a1d1571f3b344b34c681380d9056271ef70a3dd3 | no | 2 |
| **Warnings:** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| 2 | Power of Attorney | Transmittal_for_Power_of_Attorney.PDF | 16370<br>84da99d1390c3b8c73955990cabba41cc30e6af2 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Power of Attorney | 44556C1-R2_Executed_General_POA.PDF | 57983<br>f9df49ff3b99475076003bd944a2ae1f32e077f9 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 99464 | | |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

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# STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: BioMarin Pharmaceutical Inc.

Application No./Patent No.: 15/646,822 Filed/Issue Date: July 11, 2017

Titled: Variants of C-Type Natriuretic Peptide

BioMarin Pharmaceutical Inc. , a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. [x] The assignee of the entire right, title, and interest.

2. [ ] An assignee of less than the entire right, title, and interest (check applicable box):

   [ ] The extent (by percentage) of its ownership interest is _____ %). Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   [ ] There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. [ ] The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. [ ] The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. [ ] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____ , Frame _____ , or for which a copy thereof is attached.

B. [x] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Daniel J. Wendt To: BioMarin Pharmaceutical Inc.
   The document was recorded in the United States Patent and Trademark Office at
   Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

2. From: Shinong Long To: BioMarin Pharmaceutical Inc.
   The document was recorded in the United States Patent and Trademark Office at
   Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

PTO/AIA/96 (08-12)
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## STATEMENT UNDER 37 CFR 3.73(c)

3. From: Sianna Castillo To: BioMarin Pharmaceutical Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

4. From: Christopher P. Price To: BioMarin Pharmaceutical Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

5. From: Mika Aoyagi-Scharber To: BioMarin Pharmaceutical Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

6. From: Augustus O. Okhamafe To: BioMarin Pharmaceutical Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 044054 , Frame 0097 , or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

| /Katherine L. Neville/ | March 15, 2019 |
|---|---|
| Signature | Date |
| Katherine L. Neville | 53,379 |
| Printed or Typed Name | Title or Registration Number |

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
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www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 |

**CONFIRMATION NO. 7727**

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

**POA ACCEPTANCE LETTER**

*OC000000106703287*

Date Mailed: 03/20/2019

## NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/15/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

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| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 135576.010627/REI/CON |

**CONFIRMATION NO. 7727**

**POWER OF ATTORNEY NOTICE**

35893
GREENBERG TRAURIG (BOS)
ONE INTERNATIONAL PLACE, 20th FL
ATTN: PATENT ADMINISTRATOR
BOSTON, MA 02110

*OC000000106703277*

Date Mailed: 03/20/2019

## NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/15/2019.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 05/10/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| RAILEY, JOHNNY F | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 05/10/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

| *Office Action Summary* | Application No. 15/646,822 | Applicant(s) Wendt et al. | |
|---|---|---|---|
| | Examiner JOHNNY F RAILEY II | Art Unit 3991 | AIA (FITF) Status No |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 09 October 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 15-30 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

## DETAILED ACTION

### *Notice of Pre-AIA or AIA Status*

The present application is being examined under the pre-AIA first to invent provisions.

For reissue applications filed before September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the law and rules in effect on September 15, 2012. Where specifically designated, these are "pre-AIA" provisions.

For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.

### *Priority*

This application is a continuation reissue of reissue application 14/604,262, now U.S. Patent RE46,707 E, which is an reissue application for U.S. Patent 8,598,121, filed as application no. 13/466,672 and issued on 3 December 2013, which is a continuation of U.S. Patent Application 12/784,117, now U.S. Patent 8,198,242, issued on 12 June 2012, which claims priority and benefit of U.S. Provisional Application 61/254,563, filed on 23 October 2009, and U.S. Provisional Application 61/180,112, filed on 20 May 2009.

### *Status*

The amendments to the specification and claims filed on 9 October 2018 are not in compliance with 37 CFR 1.173. The amendment to the specification as presented at page 2 of the response contains crossed-through words. The claims 15, 18, 21-23, and 27 contain crossed-through and double-bracketed material as follows: [[145]] 191. New claims 15-30 should remain underlined. All amendments in reissue applications are made relative to the patent. See MPEP 1453.

### *Claims*

Pending claims are 15-30. Original patent claims 1-14 have been canceled. The independent claims are 15, 18, 21-23, and 27 are amended and summarized as follows:

**Claim 15** is drawn to a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 18** is drawn to a sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 21** is drawn to a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 22** is drawn to a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 23** is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP is variant selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

**Claim 27** is drawn to a method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:

SEQ ID Nos: 145, 179, 185, 190, 180, 146-160, 60, 161-178, 182, 186, 192, 75, 181, 191, 144, 183, 188, 193, 36, 184, 189, 194;

PEG1K-forms of 36, 184, 189, 194

PEO12-forms of 36, 184, 189, 194; and

PEO24-forms of 36, 184, 189, 194.

## *Defective Declaration/Oath*

The reissue oath/declaration filed with this application is defective because the error which is relied upon to support the reissue application is not an error upon which a reissue can be based. See 37 CFR 1.175 and MPEP § 1414. During the prosecution of application 13/466,672, a restriction requirement was held in which claims drawn to the elected species of SEQ ID NO: 185, a C-type natriuretic peptide of 53 amino acids (CNP53) were elected without traverse. The examiner also indicated that the variants of CNP-53 claimed as SEQ ID NOs: 179, 190, and 180 would be rejoined with the elected invention. See the Notice of Allowability mailed on 30 July 2013 in that application. The examiner also rejoined method claims for the elected composition claims and withdrew from further consideration claims drawn to other non-elected subject matter. The addition of new claims 15-30 is not an error which is relied upon to support the reissue application. See MPEP 1412.01 I:

> Where a restriction (or an election of species) requirement was made in an application and applicant permitted the elected invention to issue as a patent without filing a divisional application on the non-elected invention(s), the non-elected invention(s) cannot be recovered by filing a reissue application. A reissue applicant's failure to timely file a divisional application covering the non-elected invention(s) in response to a restriction (or an election of species) requirement is not considered to be error causing a patent granted on the elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim. Accordingly, this is not correctable by reissue of the original patent under 35 U.S.C. 251. In re Watkinson, 900 F.2d 230, 14 USPQ2d 1407 (Fed. Cir. 1990); In re Orita, 550 F.2d 1277, 1280, 193 USPQ 145, 148 (CCPA 1977); See also In re Mead, 581 F.2d 251, 198 USPQ 412 (CCPA 1978). In this situation, the reissue claims should be rejected under 35 U.S.C. 251 for lack of defect in the original patent and lack of error in obtaining the original patent.

### *Claim Rejections - 35 USC § 251*

Claims 15-30 are rejected as being based upon a defective reissue declaration under 35 U.S.C. 251 as set forth above. See 37 CFR 1.175.

The nature of the defect(s) in the declaration is set forth in the discussion above in this Office action. This rejection is maintained from the previous office action.

### *Applicant's Response*

Beginning at page 29 of the response, applicants argue that the reissue oath/declaration properly identifies an error upon which a reissue can be based, wherein that reason is that patentee is claiming less than it had a right to claim in the patent. The pending claims are broadened and are directed to embodiments disclosed but not claimed in the 13/466,672 application that issued as U.S. Patent No. 8,598,121. Applicants cite MPEP §1412.03 (I) and (IV) to support the broadening aspect of a reissue.

Applicant notes that claims in the 13/466,672 application (which issued as U.S. Patent 8,598,121) were drawn to a variant of C-type natriuretic peptide (CNP) with the specific SEQ ID NOs: 179; 185; 190; and 180. Pharmaceutical compositions and methods of treating a bone-related disorder or skeletal dysplasia were claimed. A method for recombinant production of a CNP variants and a method of increasing long bone growth were also claimed. Compositions and methods were limited to the species having SEQ ID NOs: 179; 185; 190; and 180. Applicants argue that the independent claims of the current continuation reissue application are directed to a macromolecule capable of releasing a CNP variant, in particular comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage. Sustained release CNP variants having the hydrolysable linkage are also claimed, as well as various methods of using these CNP variants to treat cells, conditions or disorders. See pages 30-31 in the response.

It is argued that pending claims 15-30 represent previously unclaimed subject matter in the form of invention embodiments substantially different from those claimed in U.S. Patent No. 8,598,121. Further, applicant states that subject matter included in this continuation reissue includes subject matter not covered by patent claims, therefore the claims at reissue can be said to enlarge the scope of the patent claims. The reissue claims are held to be not substantially identical to those of the nonelected groups identified in the restriction requirement and represent overlooked aspects of the invention. Applicant distinguishes the instant case from the decision *In re Orita*, 550 F.2d 1277, 193 USPQ145 (CCPA 1977) by arguing that the claims at reissue in *Orita* were substantially identical to the originally non-elected claims. Applicant states: "By contrast, as noted above, the pending continuation reissue claims 15-30 cover subject matter that was disclosed but had never been presented in the original application and that is substantially different from the non-elected claims." Applicant argues that the independent claims of the instant continuation reissue comprise CNP variants with a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage. This is previously unclaimed subject

matter that is substantially different from those originally claimed in the '672 application. Citing *In re Youman*, 679 F.3d 1335, 102 USPQ2d 1862 (Fed. Cir. 2012), applicant argues that the recapture rule applies when surrendered subject matter is being reclaimed, overlooked aspects by definition were never claimed and thus never surrendered. See pages 32-33 in the response.

Applicant submits that the current reissue claims should be evaluated in view of the findings of *In re Doyle* 293 F.3d 1355, 63 USPQ2d 1161 (Fed. Cir. 2002). With respect to *Doyle*, the MPEP states that "the court permitted the patentee to file a reissue application to present a so-called linking claim, a claim broad enough to read on or link the invention elected (and patented) together with the invention not elected." Further, MPEP §1412.01(II) states that "[c]laims to separate inventions/embodiments/species that were disclosed but never covered by the claims in the original application prosecution are claims to overlooked aspects." Applicant argues:

> As in *Doyle*, the instant continuation reissue claims are neither identical to nor substantially similar to the non-elected claims, most notably with respect to the composition claims directed to CNP variants, are species that fall within the new genus claims, directed to, for example, macromolecules capable of releasing a CNP variants, sustained release CNP variant formulations, methods of stimulating cGMP production in a cell, and methods of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3). As such, the continuation reissue claims are substantially broader than the claims of the nonelected groups with species elections. In contrast to *Orita*, and similar to *Doyle*, Applicants never asserted the continuation reissue claims or anything similar to them in the original application, and also never agreed to prosecute the reissue claims in a divisional application.

See pages 34-35 in the response. Finally, applicant notes on page 36: "*Orita* extends only to claims that are identical to or of substantially similar scope to those of the non-elected group."

### *Examiner's Comments*

A review of the content of the claims and the restriction requirement as presented in application 13/366,672 is in order:

Claims 1-4, 7, 9, 10, 16, 18, 22-24, 30, 31, 35, 39, 41, and 44-46 were subject to a restriction requirement in the paper mailed on 13 February 2013. The restriction requirement set forth the following groups of inventions as independent and distinct:

> Restriction to one of the following inventions is required under 35 U.S.C. 121:
>
> I. Claims 1-4, 30, 45 and 46, drawn to a variant of C-type natriuretic peptide (CNP), classified in class 530, subclass 300.
>
> II. Claim 7, drawn to a method of treating a bone-related disorder or skeletal dysplasia, classified in class 514, subclass 12.4.
>
> III. Claim 9, drawn to a method of treating a vascular smooth muscle disorder, classified in class 514, subclass 12.4.

IV. Claims 10, 16, 18, 22-24, 31,35, drawn to a method for recombinant production of a CNP variant and a host cell used in said method, classified in class 435, subclass 69.1/325.

V. Claims 39 and 41, drawn to a method of treating a CNP-responsive condition or disorder, classified in class 514, subclass 12.4.

VI. Claim 44, drawn to a method of increasing long bone growth, classified in class 514, subclass 12.4.

The subject matter of claims 1-4, 30, 45, and 46, drawn to a variant of C-type natriuretic peptide (CNP), were regarded and the invention of Group I.

Claim 1 requires that the CNP variant be conjugated to a moiety selected from a hydrophobic acid and an amino acid sequence from 1 to 20 amino acids. The CNP variants are identified specifically by the following SEQ ID NOs: 60, 75, 144, 146-180, 183-185, 188-190, 193 and 194.

Claim 30 requires that the CNP variant is produced from a polynucleotide sequence (encoding the CNP variant polypeptide) linked to a second polynucleotide sequence (encoding a cleavable peptide) whereby a fusion protein is produced. The CNP variants are identified specifically by the following SEQ ID NOs: 34, 36, 37, 39, 41, 60-63, 68, 69, 71, 75-77, 79, 81, 87, 144, 179, 180, 183-185, 188-190, 193, 194, and 146-178.

Claim 46 requires that the CNP variant be conjugated to a moiety selected from a hydrophobic acid and an amino acid sequence from 1 to 20 amino acids. The CNP variants are identified specifically by the following SEQ ID NOs: 145, 181, 182, 186, 191, and 192.

In the restriction requirement on page 4, it was noted:

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

On page 5, applicant was also required to select a patentably distinct species:

This application contains claims directed to the following patentably distinct species: sequences as disclosed in claims 1, 9, 10, 30, 31, 39, and 46. The species are independent or distinct because each sequence is a distinct separate polypeptide. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, no claim is generic.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

The species are independent or distinct because each sequence is a distinct separate polypeptide; a search for the recited sequences would not be coextensive.

In the response to the restriction requirement that was filed on 12 April 2013, applicant elected Group I and the species Pro-CNP53 (SEQ ID NO: 185) for examination. Applicant advised that claim 45 should be in Group VI, and therefore the claims that read upon the elected subject matter are claims 1-4, 30, and 46. "Applicants request rejoinder of the non-elected method claims (Groups II-VI) upon notification of allowance of the elected composition claims." There was no traversal of the restriction and election of species requirements.

At allowance mailed on 30 July 2013 held:

Applicants' election, of Group I, claims 1-4, 30, 45 and 46, drawn to a variant of C-type natriuretic peptide (CNP), in the reply filed on 12 April 2013, is acknowledged. In response to a requirement for species election, Applicants elect the species Pro-CNP53 (SEQ ID NO: 185) for continued examination. Because Applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Additionally, Applicants request rejoinder of the non-elected method claims (Groups ll-VI) upon notification of allowance of the elected composition claims. Said Groups will be rejoined and examined upon the identification of allowable subject matter.

Claims 1-4, 7, 9, 10, 16, 18, 22-24, 30, 35, 39, 41 and 44-46 are pending in the instant application. Claims 7, 9,10,16,18, 22-24, 31,35, 39, 41,45 and 46 have been withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Claims 1-4, 30 and 45 are under consideration to the extent they read on the elected invention, SEQ ID NO: 185.

Based upon this review of the file record in the '672 application, applicant was given an opportunity to traverse the restriction and election of species requirements. This was not done. Applicant was free to file any divisional applications on the non-elected subject matter drawn to independent and distinct inventions. This also was not done. The claims in the current reissue are drawn now to that non-elected subject matter, including all of the species of CNP variants listed by SEQ ID NO as was found in the original claims of the '672 application. The methods of treatment in the current reissue claims also are not substantially different from the methods of the non-elected inventions. The same CNP variants as originally presented in the '672 application are used to the same effects as the current claims: all involve using these CNP variants to treat a subject. There is no structural difference in the variants found in the original claims of the '672 application and the effects of administering these variants to a subject involve the same inherent properties. Note, for example, original claim 10 in the '672 application which broadly requires administering the CNP peptide or variant to a subject to produce a "therapeutic effect" in the subject by increasing the level of at least one bone- or cartilage-associated biomarker. Applicant's

attempts to distinguish the instant CNP variants from those of the '672 application are unpersuasive. See page 30, last paragraph in the response. The presence of a "hydrolysable linkage" is not distinct from the claims of the '672 application. See claims 10, 23, 24, 30, and 46. The CNP variants contain a hydrophobic acid or an amino acid sequence (cleavable peptide) conjugated to the CNP protein. That "cleavable peptide" can be removed by a hydrolysis reaction. Long-chain peptides contain amino acids linked via peptide bonds, a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule. Living organisms have enzymes capable of both forming and breaking peptide bonds. Therefore any claims to a "hydrolysable linkage" does not distinguish the reissue claims from the "cleavable peptides" of the '672 claims.

The reissue claims are substantially identical to those of the nonelected groups identified in the restriction requirement of the '672 application. In this regard, the findings of *Orita* are relevant. *Orita* precludes a reissue applicant from obtaining substantially identical claims to those of nonelected groups identified in a restriction requirement when such claims could not have been prosecuted in the application from which they were restricted. The claims in *Doyle* were different from those of the applicant's in *Orita* because *Doyle*'s new claims were neither identical nor substantially similar to the nonelected claims. The current reissue claims do not embrace any "overlooked aspects of the invention," while they are substantially similar if not identical to the nonelected canceled claims of the '672 application. The reissue claims are drawn to one or more of the non-elected groups and species of the original restriction requirement. The claims are not genus claims reading on elected and non-elected claims, but are claims comprised of a Markush groupings of specific SEQ ID NOs, which were present in the non-elected inventions in the '672 application. The current claims are not broader than the original non-elected claims of the '672 application, but rather represent species of the non-elected inventions. Unlike in *Doyle*, the claims presented in the current reissue application are not linking claims, but fall within the scope of the non-elected claims in the '672 application.

### ***Claim Rejections – Nonstatutory Double Patenting***

The previous office action held the following nonstatutory double patenting rejections:

1. Claims 15-20 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 2 of U.S. Patent No. RE46,707 E in view of Tsubery.

2. Claim 21 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Komatsu.

3. Claim 22 is rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Krejci.

4. Claims 23-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claim 5 of U.S. Patent No. RE46,707 E in view of Tsubery.

***Applicant's Response***

Applicant requests that these rejections be held in abeyance until allowable subject matter has been identified.

***Examiner's Comments***

The nonstatutory double patenting rejections remain of record, as no allowable subject matter has been indicated.

**CONCLUSION**

Claims 15-30 are rejected. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

**ONGOING DUTY TO DISCLOSE**

Applicant is reminded of the continuing obligation under 37 CFR 1.178(b), to timely apprise the Office of any prior or concurrent proceeding in which Patent No. RE46,707 E is or was involved. These proceedings would include interferences, reissues, reexaminations, and litigation.

Applicant is further reminded of the continuing obligation under 37 CFR 1.56, to timely apprise the Office of any information which is material to patentability of the claims under consideration in this reissue application.

These obligations rest with each individual associated with the filing and prosecution of this application for reissue. See also MPEP §§ 1404, 1442.01 and 1442.04.

**INTERVIEWS**

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice. The fax phone number for the organization where this application or proceeding is assigned is 571-273-9900.

**FUTURE CORRESPONDENCE**

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Johnny F. Railey II** whose telephone number is (571)272-0985. The examiner can

normally be reached on Monday through Thursday from 9:00 a.m. to 6:30 p.m. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Jean Witz**, can be reached on (571) 272-0927.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://www.uspto.gov/patents/process/status/index.jsp . Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

All correspondence relating to this proceeding should be directed to:

**By EFS:** Registered users may submit via the electronic filing system EFS-Web at:

https://efs.uspto.gov/efile/myportal/efs-registered

**By mail to:** Mail Stop Reexam/Reissue
Central Reexamination Unit
Commissioner for Patents
United States Patent & Trademark Office
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Alexandria, VA 22313-1450

**By hand:** Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

/Johnny F. Railey II/
Patent Reexamination Specialist
CRU - Art Unit 3991

/Padmashri Ponnaluri/
Patent Reexamination Specialist
CRU - Art Unit 3991

/Jean C. Witz/
Supervisory Patent Reexamination Specialist
CRU - Art Unit 3991

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | JOHNNY F RAILEY II | 3991 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Reviewed prosecution history in parent application 13/466,672 | 6/6/2018 | /J.R./ |
| Reviewed prosecution in RE 46,707 E | 6/6/2018 | /J.R./ |
| Google Search Terms: C-type natriuretic peptide bone review "fibroblast growth factor receptor" | 6/12/2018 | /J.R./ |
| Inventor Search | 6/18/2018 | /J.R./ |
| updated Inventor Search | 04/03/2019 | /J.R,/ |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | | | |

| Index of Claims | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | JOHNNY F RAILEY II | 3991 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ✓ | Rejected | - | Cancelled | N | Non-Elected | A | Appeal |
| = | Allowed | ÷ | Restricted | I | Interference | O | Objected |

**CLAIMS**

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

| CLAIM | | DATE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Final | Original | 06/15/2018 | 04/17/2019 | | | | | | | |
| | 1 | - | - | | | | | | | |
| | 2 | - | - | | | | | | | |
| | 3 | - | - | | | | | | | |
| | 4 | - | - | | | | | | | |
| | 5 | - | - | | | | | | | |
| | 6 | - | - | | | | | | | |
| | 7 | - | - | | | | | | | |
| | 8 | - | - | | | | | | | |
| | 9 | - | - | | | | | | | |
| | 10 | - | - | | | | | | | |
| | 11 | - | - | | | | | | | |
| | 12 | - | - | | | | | | | |
| | 13 | - | - | | | | | | | |
| | 14 | - | - | | | | | | | |
| | 15 | - | - | | | | | | | |
| | 16 | ✓ | ✓ | | | | | | | |
| | 17 | ✓ | ✓ | | | | | | | |
| | 18 | ✓ | ✓ | | | | | | | |
| | 19 | ✓ | ✓ | | | | | | | |
| | 20 | ✓ | ✓ | | | | | | | |
| | 21 | ✓ | ✓ | | | | | | | |
| | 22 | ✓ | ✓ | | | | | | | |
| | 23 | ✓ | ✓ | | | | | | | |
| | 24 | ✓ | ✓ | | | | | | | |
| | 25 | ✓ | ✓ | | | | | | | |
| | 26 | ✓ | ✓ | | | | | | | |
| | 27 | ✓ | ✓ | | | | | | | |
| | 28 | ✓ | ✓ | | | | | | | |
| | 29 | ✓ | ✓ | | | | | | | |
| | 30 | ✓ | ✓ | | | | | | | |

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 07/03/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| RAILEY, JOHNNY F | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 07/03/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

| *Applicant-Initiated Interview Summary* | **Application No.** 15/646,822 | **Applicant(s)** Wendt et al. | |
|---|---|---|---|
| | **Examiner** JOHNNY F RAILEY II | **Art Unit** 3991 | **AIA (FITF) Status** No |

All participants (applicant, applicants representative, PTO personnel):

(1) JOHNNY F. RAILEY II.

(2) JEAN WITZ.

(3) KATE NEVILLE.

(4) AJAY SINGH.

Date of Interview: 25 June 2019.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☑Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 15-30.

Identification of prior art discussed: NONE.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Discussed the rejection of claims 15-30 based upon a defective declaration under 35 USC 251. Applicant noted that claim 15 requires that the CNP variant comprise a synthetic polymeric group coupled to a hydrolysable linkage. This is a feature that was previously not claimed in the original patent and was not subject to restriction by the examiner in application 13/466672. Previous unclaimed methods, i.e. claims 21 and 22, do not require the presence of a synthetic polymeric group coupled to a hydrolysable linkage. These methods were not subject to the restriction requirement. Other method claims, i.e. claims 23 and 27 require the presence of the synthetic polymeric group coupled to a hydrolysable linkage. Applicant's response after final will address these issues and will be considered. The rejection of claims 15-30 based upon a defective declaration should be withdrawn. Whether or not a restriction/election by original presentation of claims 15-30 will be addressed upon the response. The non-statutory double patenting rejections of claims 15-30 remain of record and should be addressed in applicant's response..

**Applicant recordation instructions:** The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

**Examiner recordation instructions**: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

| /JOHNNY F RAILEY II/<br>Patent Reexamination Specialist, Art Unit 3991 | /Jean C. Witz/ Supervisory Patent Reexamination Specialist CRU 3991 |
|---|---|

# Summary of Record of Interview Requirements

**Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record**
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

**Title 37 Code of Federal Regulations (CFR) 1.133 Interviews**
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

---

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

**Examiner to Check for Accuracy**

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

MARSHALL, GERSTEIN & BORUN LLP
ATTORNEYS AT LAW
233 SOUTH WACKER DRIVE
6300 WILLIS TOWER
CHICAGO, ILLINOIS 60606-6357
(312) 474-6300
FAX: (312) 474-0448

6/24/2019

**FACSIMILE TRANSMISSION SHEET**

TO: 1-571-273-0985

FROM: TSEVCIK

RE: USSN 15646822 - Interview Agenda

PAGES (INCLUDING THIS PAGE): 2

**********

Please see the attached agenda for an interview on June 25th at 2pm Eastern with attorney Kate Neville regarding USSN 1564622.

*If you do not receive all pages of this fax in good condition, please contact Tiffany M. Sevcik at (312) 474-6300.*

********

*This transmission contains confidential information intended only for the addressee. If you are not the*
Continuation of partially transmitted fax.
*facsimile in error, please notify us by telephone immediately.*

Agenda for Examiner Interview

U.S. Patent Application No. 15/646,822
Examiner Johnny Railey II, Supervisor Jean Witz
Applicant representative Katherine L. Neville
June 25, 2PM EST

I. Discuss Objection to Oath and Declaration

A. Patent Office position

1. Declaration does not describe sufficient error in claims as prescribed under 37 CFR 1.175

-asserted that restriction requirement in parent application 13/366,672 required election of CNP species, no generic claim

-claims of '672 application, CNP variant could be conjugated to a hydrophobic acid and an amino acid sequence from 1-20 amino acids, reissue claims are substantially identical to those of the non-elected groups identified in the '672 application

B. Points for discussion

1. USPTO position that pending claims are substantially identical to non-elected subject matter in parent

2. USPTO position that pending claims represent species of non-elected inventions in parent claims

3. USPTO position that "hydrolysable linkage" in pending claims does not distinguish the reissue claims from the "cleavable peptide" in '672 claims

4. Difference in subject matter of parent claims and present claims directed to macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage

MARSHALL, GERSTEIN & BORUN LLP
ATTORNEYS AT LAW
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4. Difference in subject matter of parent claims and present claims directed to macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

| | |
|---|---|
| Application No.: 15/646,822 | Confirmation No.: 7727 |
| Filed: July 11, 2017 | Art Unit: 3991 |
| For: Variants of C-Type Natriuretic Peptide | Examiner: J. F. Railey |

**AMENDMENT AFTER FINAL ACTION UNDER 37 C.F.R. § 1.116**

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

**INTRODUCTORY COMMENTS**

This paper is filed in response to the Final Office Action ("the Action") dated May 10, 2019, in the above-identified U.S. patent application.

This paper is timely filed within two-months of receipt of the Final Action. No fees are believed due in connection with this filing. If any fees are deemed necessary, the Commissioner is hereby authorized to charge such fees to our deposit account no. 13-2855.

**Amendments to the Claims: none.** A listing of the claims begins on page **2** of this paper.

**Remarks/Arguments** begin on page **22** of this paper.

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 36542364 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 10-JUL-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 15:21:32 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | | 44556C1-R2_response_to_Final.PDF | 79589<br>435fc7ab89ebfc799674ec98bd5bc4296dfe0650 | yes | 23 |

| | Multipart Description/PDF files in .zip description | | |
|---|---|---|---|
| | **Document Description** | **Start** | **End** |
| | Applicant Arguments/Remarks Made in an Amendment | 22 | 23 |
| | Claims | 2 | 21 |
| | Response After Final Action | 1 | 1 |
| **Warnings:** | | | |
| **Information:** | | | |
| | **Total Files Size (in bytes):** | 79589 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**<u>New Applications Under 35 U.S.C. 111</u>**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**<u>National Stage of an International Application under 35 U.S.C. 371</u>**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**<u>New International Application Filed with the USPTO as a Receiving Office</u>**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

## REMARKS

Claims 15-30 are pending and under examination. In the Action, the Examiner rejected claims 15-30 as assertedly being based on a defective reissue Oath or Declaration. The claims were also rejected on grounds of non-statutory double patenting. Reconsideration is requested in light of the following remarks.

### I. Summary of Interview Pursuant to MPEP §713.04 and 37 CFR §1.133

Applicant would like to thank Examiners Railey and Witz for the courtesy of the telephone discussion on June 25, 2019.

During the interview, the defective Oath and Declaration rejection was discussed. As noted in the Interview Summary of July 3, 2019, the Examiners indicated that the subject matter of claims 15-30 was previously not claimed nor subject to a restriction requirement in a previous application. Applicant thanks the Examiners for the helpful discussion.

### II. The Rejection of the Claims in View of a Defective Oath or Declaration Should Be Withdrawn

In the Action, the Examiner maintained the rejection of claims 15-30 on the basis that the Oath or Declaration filed in the reissue application was defective under 35 USC §251. The Examiner asserted that the declaration does not describe a sufficient error in the patent claims as prescribed under 37 CFR 1.175, and asserted that the restriction requirement in parent application 13/366,672 ("the '672 application") required election of CNP species, having no generic or linking claim, and that the CNP variant could be conjugated to a hydrophobic acid and an amino acid sequence from 1-20 amino acids, which is substantially identical to the reissue claims. Applicant respectfully disagrees.

35 USC §251 states that the error upon which a reissue is based must be one which causes the patent to be "deemed wholly or partly inoperative or invalid, by reason of a defective specification or drawing, or by reason of the patentee claiming more or less than he had a right to claim in the patent." See also, MPEP §1402.

The Oath filed with the present reissue application indicated that the patent was defective by virtue of patentee claiming less than he had a right to claim. Pending claims 15 and 18 are directed to a macromolecule or sustained release composition, respectively, comprising a CNP variant and a synthetic polymeric group coupled to the CNP

variant through a hydrolysable linkage. As noted in the Interview Summary, the feature of a synthetic polymeric group is a feature that was previously not claimed in the original patent and was not subject to restriction by the examiner in the '672 application. Currently pending claims 21 and 22 were not presented in the '672 application (i.e., were previously unclaimed as acknowledged by the Examiner in the Interview Summary) and were not subject to a restriction requirement. Further currently pending method claims 23 and 27 require the presence of the synthetic polymeric group coupled to a hydrolysable linkage, which was also not the subject of a restriction requirement. As such, the claims are directed to subject matter that was not previously claimed in or subject to a restriction requirement in a parent application, and Applicant is entitled to pursue the overlooked aspects of the invention claimed herein. (MPEP §1412.01(II)).

Therefore, the Oath accurately reflects that patentee is claiming less than he had a right to claim, and the rejection of the Oath or Declaration as defective under 35 USC §251 should be withdrawn and the application examined accordingly.

## III. The Rejection of the Claims on the Grounds of Non-statutory Double Patenting

Applicant acknowledges the rejections of the claims on the grounds of non-statutory double patenting and maintains the request that the rejections be held in abeyance until notification of allowable subject matter.

## IV. Conclusion

Applicant submits that the pending application is in condition for allowance and respectfully requests notification of the same.

Dated: July 10, 2019

Respectfully submitted,

By /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

## AMENDMENTS TO THE CLAIMS

1-14 (Cancelled)

15. (Previously presented) A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. (Previously presented) The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. (Previously presented) The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. (Previously presented) A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)

(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC

(CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

19. (Previously presented) The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. (Previously presented) The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. (Previously presented) A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC

[CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-
50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ
ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID
NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]

(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

22. (Previously presented) A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-
50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ
ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

23. (Previously presented) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant

comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. (Previously presented) The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. (Previously presented) The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. (Previously presented) The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. (Previously presented) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

28. (Previously presented) The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. (Previously presented) The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. (Previously presented) The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/646,822 | Filing Date<br>07/11/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | 07/10/2019 | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * 16 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 6 | Minus | *** 6 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /PATRICIA F LEWIS/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 09/17/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| RAILEY, JOHNNY F | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 09/17/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

| ***Advisory Action Before the Filing of an Appeal Brief*** | **Application No.** 15/646,822 | **Applicant(s)** Wendt et al. | |
|---|---|---|---|
| | **Examiner** JEAN C WITZ | **Art Unit** 3991 | **AIA (FITF) Status** No |

***--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --***

THE REPLY FILED 10 July 2019 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☑ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☐ The period for reply expires ____ months from the mailing date of the final rejection.
   b) ☑ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection.The current period for reply expires ____ months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   *Examiner Note*: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANTS FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37CFR 41.37(a).

AMENDMENTS

3. ☐ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: _____ (See 37CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☑ Applicants reply has overcome the following rejection(s): Rejection of the claims under 35 USC 251 as having a defective declaration.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☐ For purposes of appeal, the proposed amendment(s):(a)☐ will not be entered, or (b)☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☑ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☐ Other: _____.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed:_____.
    Claim(s) objected to:_____.
    Claim(s) rejected:_____.
    Claim(s) withdrawn from consideration:_____.

| /JEAN C WITZ/<br>Supervisory Patent Examiner, Art Unit 3991 | |
|---|---|

Continuation of REQUEST FOR RECONSIDERATION/OTHER 12. The request for reconsideration has been considered but does NOT place the application in condition for allowance because: The non-statutory double patenting rejections have not been addressed by persuasive argument, amendment or by the filing of a terminal disclaimer and therefore remain of record.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/646,822 | Filing Date<br>07/11/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| AMENDMENT | 09/26/2019 | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * 16 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 6 | Minus | *** 6 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| AMENDMENT | | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /LINDA HUMES/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

| **Doc Code: DIST.E.FILE**<br>**Document Description: Electronic Terminal Disclaimer - Filed** | PTO/SB/26<br>U.S. Patent and Trademark Office<br>Department of Commerce |
|---|---|

| | |
|---|---|
| Electronic Petition Request | **TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT** |
| Application Number | 15646822 |
| Filing Date | 11-Jul-2017 |
| First Named Inventor | Daniel Wendt |
| Attorney Docket Number | 30610/44556C1-R2 |
| Title of Invention | Variants of C-Type Natriuretic Peptide |

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

| Owner | Percent Interest |
|---|---|
| BIOMARIN PHARMACEUTICAL INC. | 100% |

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

RE46707

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

◉ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

| | |
|---|---|
| ○ | I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application. |

Applicant claims the following fee status:

○ Small Entity

○ Micro Entity

◉ Regular Undiscounted

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

◉ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Registration Number 53379

○ A sole inventor

○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

○ A joint inventor; all of whom are signing this request

| | |
|---|---|
| Signature | /Katherine L. Neville/ |
| Name | Katherine L. Neville |

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| STATUTORY OR TERMINAL DISCLAIMER | 1814 | 1 | 160 | 160 |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **160** |

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 15646822

Filing Date: 11-Jul-2017

Applicant/Patent under Reexamination: Wendt

Electronic Terminal Disclaimer filed on September 26, 2019

☒ APPROVED

**This patent is subject to a terminal disclaimer**

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 37291042 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Filer Authorized By:** | Katherine Lynn Neville |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 26-SEP-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 17:09:39 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $160 |
| RAM confirmation Number | E20199PH09360233 |
| Deposit Account | |
| Authorized User | |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Terminal Disclaimer-Filed (Electronic) | eTerminal-Disclaimer.pdf | 33553<br>f8df89809574d29a00358460d2c342229fcef280 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Fee Worksheet (SB06) | fee-info.pdf | 30571<br>5d07387e260628fdff723a60793997259c934189 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 64124 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 Confirmation No.: 7727

Filed: July 11, 2017 Art Unit: 3991

For: Variants of C-Type Natriuretic Peptide Examiner: Johnny F. Railey

**SUPPLEMENTAL RESPONSE AFTER FINAL ACTION UNDER 37 C.F.R. § 1.116**

MS Re-exam/Re-issue
Central Reexamination Unit
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

**INTRODUCTORY COMMENTS**

This paper is filed in response to an Advisory Action (hereafter "the Action"), mailed September 17, 2019, in the above-mentioned application.

This paper is filed with a Petition for Two-Month Extension of time and the requisite fee. If any additional fees are deemed necessary, the Commissioner is hereby authorized to charge such fees to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

**Amendments to the Claims: None.** A complete listing of the claims begins on page **2** of this paper.

**Remarks** begin on page **22** of this paper.

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| Extension - 2 months with $0 paid | 1252 | 1 | 600 | 600 |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **600** |

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 37291707 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 26-SEP-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 21:42:24 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $600 |
| RAM confirmation Number | E20199PL43113571 |
| Deposit Account | 132855 |
| Authorized User | Katherine Neville |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:<br>37 CFR 1.17 (Patent application and reexamination processing fees) | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Extension of Time | Two_Month_Request_for_Extension_of_Time_Under_37_CFR_1136a.PDF | 22673<br>db70aaea5a65dcccc1939fb89fa87ca695039128 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | | 44556C1-R2_supplemental_response_to_FOA.PDF | 73901<br>d3ce30a3855cdc1dc001c67a17deecc5099bdfb0 | yes | 22 |

| | Multipart Description/PDF files in .zip description | | |
|---|---|---|---|
| | **Document Description** | **Start** | **End** |
| | Applicant Arguments/Remarks Made in an Amendment | 22 | 22 |
| | Claims | 2 | 21 |
| | Supplemental Response or Supplemental Amendment | 1 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Applicant Arguments/Remarks Made in an Amendment | Exhibit_-_eTD_Approval__44556C1-R2.PDF | 72969<br>ac4fe9655ab7d9b84fb551c7757f7d99d12cc2bb | no | 5 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Fee Worksheet (SB06) | fee-info.pdf | 30645<br>6f7d43c9159965aa1e5b8f46568091393120 8c2e | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 200188 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

PTO/AIA/22 (03-13)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

| **PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)** | Docket Number (Optional) 30610/44556C1-R2 |
|---|---|
| Application Number 15/646,822-Conf. #7727 | Filed July 11, 2017 |
| For Variants of C-Type Natriuretic Peptide | |
| Art Unit 3991 | Examiner Johnny F. Railey |

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

| | Fee | Small Entity Fee | Micro Entity Fee | |
|---|---|---|---|---|
| ☐ One month (37 CFR 1.17(a)(1)) | $200 | $100 | $50 | $ |
| ☒ Two months (37 CFR 1.17(a)(2)) | $600 | $300 | $150 | $ 600.00 |
| ☐ Three months (37 CFR 1.17(a)(3)) | $1,400 | $700 | $350 | $ |
| ☐ Four months (37 CFR 1.17(a)(4)) | $2,200 | $1,100 | $550 | $ |
| ☐ Five months (37 CFR 1.17(a)(5)) | $3,000 | $1,500 | $750 | $ |

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 13-2855 .

☒ Payment made via EFS-Web.

**WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 53,379 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Katherine L. Neville/ — Signature
September 26, 2019 — Date

Katherine L. Neville — Typed or printed name
(312) 474-6300 — Telephone Number

**NOTE:** This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

## REMARKS

Claims 15-30 are pending and under examination. In the Advisory Action, the Examiner maintained the rejection of claims 15-30 on grounds of non-statutory double patenting. Reconsideration is requested in light of the filing of the following remarks.

### I. The Rejection of the Claims on the Grounds of Non-statutory Double Patenting

The Examiner had previously issued a rejection of the pending claims in view of claims 2 and 5 of U.S. Patent No. RE46,707, with respect to recitation of SEQ ID NOs: 179, 185, 190 or 180. In order to expedite prosecution, Applicants have submitted a terminal disclaimer with respect to the '707 patent. A copy of the eTerminal Disclaimer and the approval form is included herewith.

As such, Applicants respectfully request that the rejection be withdrawn.

### II. Conclusion

Applicants submit that the pending application is in condition for allowance and respectfully request notification of the same.

Dated: September 26, 2019

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

## AMENDMENTS TO THE CLAIMS

1-14 (Cancelled)

15. (Previously presented) A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. (Previously presented) The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. (Previously presented) The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. (Previously presented) A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)

(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC

(CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

19. (Previously presented) The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. (Previously presented) The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. (Previously presented) A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC

[CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-
50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ
ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID
NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]

(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

22. (Previously presented) A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-
50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ
ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

23. (Previously presented) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant

comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC
(CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO:
182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID
NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ
ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO:
144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. (Previously presented) The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. (Previously presented) The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. (Previously presented) The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. (Previously presented) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);

PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

28. (Previously presented) The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. (Previously presented) The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. (Previously presented) The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

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| Electronic Petition Request | **TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT** |
| Application Number | 15646822 |
| Filing Date | 11-Jul-2017 |
| First Named Inventor | Daniel Wendt |
| Attorney Docket Number | 30610/44556C1-R2 |
| Title of Invention | Variants of C-Type Natriuretic Peptide |

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

| Owner | Percent Interest |
|---|---|
| BIOMARIN PHARMACEUTICAL INC. | 100% |

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

RE46707

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

◉ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

| | |
|---|---|
| ○ | I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application. |

Applicant claims the following fee status:

○ Small Entity

○ Micro Entity

◉ Regular Undiscounted

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

◉ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Registration Number 53379

○ A sole inventor

○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

○ A joint inventor; all of whom are signing this request

| | |
|---|---|
| Signature | /Katherine L. Neville/ |
| Name | Katherine L. Neville |

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 15646822

Filing Date: 11-Jul-2017

Applicant/Patent under Reexamination: Wendt

Electronic Terminal Disclaimer filed on September 26, 2019

☒ APPROVED

**This patent is subject to a terminal disclaimer**

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 37291042 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Filer Authorized By:** | Katherine Lynn Neville |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 26-SEP-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 17:09:39 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $160 |
| RAM confirmation Number | E20199PH09360233 |
| Deposit Account | |
| Authorized User | |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Terminal Disclaimer-Filed (Electronic) | eTerminal-Disclaimer.pdf | 33553<br>f8df89809574d29a00358460d2c342229fce f280 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Fee Worksheet (SB06) | fee-info.pdf | 30571<br>5d07387e260628fdff723a60793997259c93 4189 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 64124 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

| **PATENT APPLICATION FEE DETERMINATION RECORD**<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/646,822 | Filing Date<br>07/11/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| AMENDMENT | 10/10/2019 | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * 16 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 6 | Minus | *** 6 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| AMENDMENT | | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /PATRICIA F LEWIS/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

PTO/SB/30 (11-17)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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# Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

| | |
|---|---|
| Application Number | 15/646,822-Conf. #7727 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

**This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.**
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
   - a. [ ] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
     - ii. [ ] Other _____
   - b. [x] Enclosed
     - i. [x] Amendment/Reply
     - ii. [ ] Affidavit(s)/ Declaration(s)
     - iii. [x] Information Disclosure Statement (IDS)
     - iv. [ ] Other _____

2. Miscellaneous
   - a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. [ ] Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   - a. [ ] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 13-2855 .
     - i. [x] RCE fee required under 37 CFR 1.17(e)
     - ii. [ ] Extension of time fee (37 CFR 1.136 and 1.17)
     - iii. [ ] Other _____
   - b. [ ] Check in the amount of $ _____ enclosed
   - c. [x] Payment by credit card ~~(Form PTO-2038 enclosed)~~
   - d. [x] Payment by EFS-Web

**WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**

***SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED***

| | | | |
|---|---|---|---|
| Signature | /Katherine L. Neville/ | Date | October 10, 2019 |
| Name (Print/Type) | Katherine L. Neville | Registration No. | 53,379 |

***CERTIFICATE OF MAILING OR TRANSMISSION***

I hereby certify that this correspondence is being EFS-Web transmitted to the United States Patent and Trademark Office (USPTO), deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the USPTO on the date shown below.

| | | | |
|---|---|---|---|
| Signature | | | |
| Name (Print/Type) | | Date | |

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 Confirmation No.: 7727

Filed: July 11, 2017 Art Unit: 3991

For: Variants of C-Type Natriuretic Peptide Examiner: Bruce Campell

**SECOND SUPPLEMENTAL RESPONSE AFTER FINAL ACTION**

MS Re-exam/Re-issue
Central Reexamination Unit
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

**INTRODUCTORY COMMENTS**

This paper is filed as a supplemental response in the above-mentioned application.

This paper is filed with a Request for Continued Examination and an IDS and the requisite fee. A petition for Two-Month Extension of time has been filed previously. If any additional fees are deemed necessary, the Commissioner is hereby authorized to charge such fees to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

**Amendments to the Claims: None.** A complete listing of the claims begins on page **2** of this paper.

**Remarks** begin on page **22** of this paper.

## AMENDMENTS TO THE CLAIMS

Cancel claim 1.

Cancel claim 2.

Cancel claim 3.

Cancel claim 4.

Cancel claim 5.

Cancel claim 6.

Cancel claim 7.

Cancel claim 8.

Cancel claim 9.

Cancel claim 10.

Cancel claim 11.

Cancel claim 12.

Cancel claim 13.

Cancel claim 14.

15. (New) A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. (New) The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. (New) The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. (New) A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC
[CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-
50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ
ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]

(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

19. (New) The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. (New) The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. (New) A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

22. (New) A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

23. (New) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48)
(SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47)
(SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC

(CNP-46)
(SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-45) (SEQ ID NO: 153);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ
ID NO: 154);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
155);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:
156);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC
(CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. (New) The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. (New) The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. (New) The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. (New) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190); DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180); LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146); RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147); VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176); KGCFGLKLDRIGSMSGLGC (CNP- 19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);
MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)]
(SEQ ID NO: 189);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)]
(SEQ ID NO: 184);
PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)]
(SEQ ID NO: 194);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);
PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);
PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and
PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

28. (New) The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. (New) The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. (New) The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

## REMARKS

Claims 15-30 are pending and under examination. It was noted by the Examiner that the previous response presented the claims in incorrect reissue format.

### I. Status of the Claims

No claims are amended herein. This paper is submitted to bring the format of the pending claims into compliance with the rules for reissue applications.

Claims 15-30 were newly added with the filing of the application and are properly designated by underlining of the new claim.

During prosecution, in a response filed October 9, 2018, claims 15, 18, 21-23 and 27 were amended to correct the sequence identifier for (Met-Gly-CNP37), which was listed as SEQ ID NO: 145, but is properly listed herein as SEQ ID NO: 191.

Nothing is this paper constitutes new matter.

### II. Conclusion

Applicants submit that the pending application is in condition for allowance and respectfully request notification of the same.

Dated: October 10, 2019

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

| | |
|---|---|
| Application No.: 15/646,822 | Confirmation No.: 7727 |
| Filed: July 11, 2017 | Art Unit: 3991 |
| For: Variants of C-Type Natriuretic Peptide | Examiner: B. Campell |

## INFORMATION DISCLOSURE STATEMENT (IDS)

MS RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 C.F.R. §§ 1.56, 1.97, and 1.98, the attention of the United States Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of the above-identified application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement, pursuant to 37 C.F.R. § 1.114(c), accompanies the Request for Continued Examination (37 C.F.R. § 1.114) submitted herewith.

In accordance with 37 C.F.R. § 1.98(a)(2)(ii), copies of U.S. patents and U.S. patent application publications are not submitted. Submitted herewith are copies of foreign patents and non-patent literature in accordance with 37 C.F.R. § 1.98(a)(2).

In accordance with 37 C.F.R. § 1.97(g), the filing of this Information Disclosure Statement shall not be construed as a representation that a search has been made. In accordance with 37 C.F.R. § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in this

Information Disclosure Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

It is submitted that the Information Disclosure Statement is in compliance with 37 C.F.R. § 1.98, and the Examiner is respectfully requested to consider the listed references.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2 from which the undersigned is authorized to draw.

Dated: October 10, 2019

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

PTO/SB/08a (07-09)
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Sheet 1 of 2

| ***Complete if Known*** | |
|---|---|
| Application Number | 15/646,822-Conf. #7727 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

## U. S. PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Document Number<br>Number-Kind Code[2] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear |
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## FOREIGN PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Foreign Patent Document<br>Country Code[3]-Number[4]-Kind Code[5] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
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| | | WO-2005/116655-A2 | 12-08-2005 | Nobex Corp | | |
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| Examiner Signature | | Date Considered | |
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| Sheet | 2 | of | 2 |
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| *Complete if Known* | |
|---|---|
| **Application Number** | 15/646,822-Conf. #7727 |
| **Filing Date** | July 11, 2017 |
| **First Named Inventor** | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

**NON PATENT LITERATURE DOCUMENTS**

| Examiner Initials* | Cite No.[1] | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T[2] |
|---|---|---|---|
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| Examiner Signature | | Date Considered | |
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[1] Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached.

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(71) Applicant *(for all designated States except US)*: **NEKTAR THERAPEUTICS** [US/US]; 201 Industrial Road, San Carlos, California 94070 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **BOSSARD, Mary J.** [US/US]; 120 Kelvingrove Drive, Madison, Alabama 35758 (US).

(74) Agents: **DESAI, Naishadh** et al.; Nektar Therapeutics, 201 Industrial Road, San Carlos, California 94070 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published**:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/033217 A1

(54) Title: POLYMER CONJUGATES OF CD-NP PEPTIDES

(57) Abstract: The invention provides CD-NP peptides that are chemically modified by covalent attachment of a water soluble oligomer. A conjugate of the invention, when administered by any of a number of administration routes, exhibits characteristics that are different from the characteristics of the peptide not attached to the water soluble oligomer.

# POLYMER CONJUGATES OF CD-NP PEPTIDES

## CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application Serial No. 61/192,533, filed 19 September 2008, the disclosure of which is incorporated herein by reference in its entirety.

## FIELD OF THE INVENTION

[0002] Among other things, the present invention relates to conjugates comprising a CD-NP peptide moiety covalently attached to one or more water-soluble polymers.

## BACKGROUND OF THE INVENTION

[0003] Brain-type natriuretic peptide (BNP, also called nesiritide) is one of a family of peptides that are involved in cardiovascular, renal, and endocrine homeostasis. It was discovered in 1988, almost a decade after the discovery of atrial natriuretic peptide (ANP). Although it was first isolated from porcine brain, it is known for its activity at receptors in vascular smooth muscle and endothelial cells. BNP is an endogenous peptide produced by the heart. It is first produced as prepro-BNP and is subsequently shortened twice to the active form, a 32-amino acid peptide with one disulfide bond.

[0004] BNP binds to the natriuretic peptide receptor A (NPR-A), a membrane bound protein on the cell surface. The binding event triggers the synthesis of cGMP in the cytosol by guanylate cyclase. It is through this secondary messenger that BNP accomplishes the cardio-vascular, renal, and endocrine effects with which it is associated. Regulation of BNP is accomplished by several different means. BNP molecules that bind to NPR-A and stimulate cGMP production are removed from circulation, but there are other means by which BNP is eliminated without invoking a response. The most common means of removal is through binding to the clearance receptor, natriuretic peptide receptor C (NPR-C). Upon binding to NPR-C, the peptide is taken into the cell and cleaved enzymatically. The next major means of clearance is degradation by neutral endopeptidase (NEP), which is a membrane-bound enzyme on the cell surface. Finally, BNP is removed to a small extent by renal filtration.

[0005] Under normal conditions, BNP is produced in low amounts in the atria and ventricles. However, when the ventricles are stretched during cardiac decompensation, the amount of BNP that is produced increases greatly. Although the atria are still involved, the ventricles become the main site of production. The heart produces BNP in response to a

stretching of the ventricles that occurs during decompensation at the outset of congestive heart failure (CHF). The effects of BNP include natriuresis, diuresis, vasodilation, and a lowering of diastolic blood pressure. These effects are brought about through the actions of a secondary messenger, cyclic guanosine monophosphate (cGMP). Production of cGMP is triggered when BNP interacts with the natriuretic peptide receptor A (NPR-A) which is a membrane-bound receptor located on the surface of endothelial cells in blood vessels, kidneys, and lungs. Plasma concentration of BNP incrementally increases with increased severity of CHF. Despite this increase, the beneficial effects of BNP are blunted in severe CHF, raising the possibility of a relative deficiency state in overt CHF. Alternatively, as the assays currently employed to measure plasma concentration of BNP do not specifically differentiate between pre-pro BNP and the mature form, this pro-hormone may not be adequately processed to its mature form in overt CHF. Therefore, either the amount of BNP that the heart can produce is overcome or prepro-BNP is not adequately converted into its active form, thus reducing its beneficial actions. Because of its early production at the onset of heart disease, BNP has become important as a diagnostic marker to detect patients who are at high risk of developing CHF.

**[0006]** BNP functions to relieve cardiac decompensation in several ways. BNP leads to the excretion of sodium and an increase in urine output, which lessen congestion. It also functions as a vasodilator, the effects of which are enhanced by several other actions. Most notable of these functions are the roles BNP plays in the interference of the renin-angiotensin-aldosterone system (RAAS). It leads to inhibition of renin, which is a key enzyme in the generation of the vasoconstrictive peptide angiotensin. It inhibits the overgrowth of epithelial cells lining vascular tissue, which left unchecked, can greatly reduce blood flow. A final way that BNP functions to relieve cardiac decompensation is its lusitropic effects. It improves myocardial relaxation of the ventricles, resulting in lower diastolic blood pressure.

**[0007]** In 2001, human BNP was approved by the FDA under the trade name NATRECOR (nesiritide), for the treatment of acute congestive heart failure. NATRECOR was the first drug approved for the treatment of CHF in over twelve years. It is administered by intravenous continuous infusion over a period of 48 hours. As the drug is expensive and requires hospitalization, NATRECOR is only used for the most acute cases.

**[0008]** A novel chimeric version of natriuretic peptide has been created by combining portions of C-type natriuretic peptide (CNP) with Dendroaspis natriuretic peptide. CD-NP consists of the 22-amino acid human C-type natriuretic peptide and the 15-amino acid C-terminus of Dendroaspis

NP. CNP is less hypotensive than the B-type natriuretic peptide but affects venodilation. Dendroaspis is a potent natriuretic and diuretic peptide that is hypotensive but functions via a separate guanylyl cyclase receptor compared to CNP. Another chimeric peptide, CU-NP, which is a combination of C-type natriuretic peptide and urodilantin, has also been identified through in vivo studies to have renal benefits without inducing significant hypotension and is in pre-clinical development.

**[0009]** Normally, peptides suffer from a short in vivo half life, sometimes mere minutes, making them generally impractical, in their native form, for administration. Thus there exists a need in the art for modified CD-NP peptides having an enhanced half-life and/or reduced clearance as well as additional advantages as compared to the CD-NP peptides in their unmodified form.

## SUMMARY OF THE INVENTION

**[0010]** Accordingly, the present invention provides conjugates comprising a CD-NP or CU-NP peptide moiety covalently attached to one or more water-soluble polymers. The water-soluble polymer may be stably bound to the CD-NP or CU-NP peptide moiety, or it may be releasably attached to the CD-NP or CU-NP peptide moiety.

**[0011]** The invention further provides methods of synthesizing such CD-NP or CU-NP peptide polymer conjugates and compositions comprising such conjugates. The invention further provides methods of treating, preventing, or ameliorating a disease, disorder or condition in a mammal comprising administering a therapeutically effective amount of a CD-NP or CU-NP peptide polymer conjugate of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. BNP2.1. PEGylation rate of BNP-32 with mPEG2-40kDa Butyr-ALD.

Fig. BNP2.2. Typical purification profile for the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32.

Fig BNP2.3. HPLC analysis of the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32.

Fig. BNP2.4. MALDI-TOF analysis of the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32.

Fig. BNP2.5. SDS-PAGE analysis of BNP-32 and purified [mono]-[mPEG2-Butyr-ALD-40K]-[BNP-32] conjugate. .

Fig. BNP4.1. Typical cation-exchange purification profile of [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32].

Fig. BNP4.2. SDS-PAGE (4-12% Bis-Tris-Nu-PAGE, Invitrogen) analysis of BNP-32 and the purified [mono]-[mPEG2-Butyr-ALD-40K]-[BNP-32] conjugate..

Fig BNP4.3. RP-HPLC analysis of the purified [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32] conjugate..

Fig. BNP4.4. MALDI-TOF analysis of the purified [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32] conjugate.

Fig. BNP5.1. Typical first cation-exchange purification profile for [mono]-[mPEG-SBC-30K]-[BNP-32].

Fig. BNP5.2. SDS-PAGE analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate.

Fig BNP5.3. RP-HPLC analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate.

Fig. BNP5.4. MALDI-TOF analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate.

Fig. BNP6.1. Typical first cation-exchange purification profile of [mPEG2-C2-fmoc-NHS-40K].

Fig. BNP6.2. SDS-PAGE analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate.

Fig BNP6.3. RP-HPLC analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate..

Fig. BNP6.4. MALDI-TOF analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate.

Figure BNP7.1 shows the mean plasma concentration-time profiles of for C2-FMOC-PEG2-40K-BNP, its corresponding metabolite and released BNP.

Figure BNP7.2 shows the non-released PEG-BNP levels after the administration of the two non-releasable PEG constructs (ButyrALD-40K-BNP, ButyrALD-10K-BNP).

## DETAILED DESCRIPTION

**[0012]** As used in this specification and the intended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymer" includes a single polymer as well as two or more of the same or different polymers, reference to "an optional excipient" or to "a pharmaceutically acceptable excipient" refers to a single optional excipient as well as two or more of the same or different optional excipients, and the like.

[0013] In describing and claiming one or more embodiments of the present invention, the following terminology will be used in accordance with the definitions described below.

[0014] As used herein, the terms "CD-NP peptide" and "CD-NP peptides" mean one or more peptides having demonstrated or potential use in treating, preventing, or ameliorating one or more diseases, disorders, or conditions in a subject in need thereof, as well as related peptides. These terms may be used to refer to CD-NP peptides prior to conjugation to a water-soluble polymer as well as following the conjugation. CD-NP peptides include, but are not limited to, those disclosed herein, including in Table 1. CD-NP peptides include peptides found to have use in treating, preventing, or ameliorating one or more diseases, disorders, or conditions after the time of filing of this application. Related peptides include fragments of CD-NP peptides, CD-NP peptide variants, and CD-NP peptide derivatives that retain some or all of the CD-NP activities of the CD-NP peptide. As will be known to one of skill in the art, as a general principle, modifications may be made to peptides that do not alter, or only partially abrogate, the properties and activities of those peptides. In some instances, modifications may be made that result in an increase in CD-NP activities. Thus, in the spirit of the invention, the terms "CD-NP peptide" and "CD-NP peptides" are meant to encompass modifications to the CD-NP peptides defined and/or disclosed herein that do not alter, only partially abrogate, or increase the CD-NP activities of the parent peptide. The embodiments herein refer to CD-NP. However, it is envisaged that other netriuretic peptides, including, but not limited to, CU-NP, may be used in these embodiments.

Table 1

| Name | Sequence | SEQ ID NO: |
|---|---|---|
| BNP | SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH | 1 |
| CD-NP | GLSKGCFGLKLDRIGSMSGLGCPSLRDPRPNAPS TSA | 2 |
| CU-NP | Thr-Ala-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr with a disulfide bridge. | 3 |

[0015] The term "CD-NP activity" as used herein refers to a demonstrated or potential biological activity whose effect is consistent with a desirable CD-NP outcome in humans, or to desired effects in non-human mammals or in other species or organisms. A given CD-NP peptide may have one or more CD-NP activities, however the term "CD-NP activities" as

used herein may refer to a single CD-NP activity or multiple CD-NP activities. "CD-NP activity" includes the ability to induce a response *in vitro*, and may be measured *in vivo* or *in vitro*. For example, a desirable effect may be assayed in cell culture, or by clinical evaluation, $EC_{50}$ assays, $IC_{50}$ assays, or dose response curves. *In vitro* or cell culture assays, for example, are commonly available and known to one of skill in the art for many CD-NP peptides as defined and/or disclosed herein. CD-NP activity includes treatment, which may be prophylactic or ameliorative, or prevention of a disease, disorder, or condition. Treatment of a disease, disorder or condition can include improvement of a disease, disorder or condition by any amount, including elimination of a disease, disorder or condition.

**[0016]** Natriuretic peptide conjugates of the invention may induce the cardiovascular, renal, and/or endocrine effects that are associated with the native peptide. Cell based assays may be used to show which conjugates are proficient agonists of the human natriuretic peptide receptor A, leading to the suitable production of cGMP. Biochemical assays may be used to show which conjugates offer the suitable protection against proteolytic enzymes. In vivo experiments may be used to show which conjugates afford a desirable bioavailability. Conjugates can be tested in established dog models. Conjugates may be subjected to detailed pharmacokinetic, pharmacodynamic, and toxicity studies in rats and dogs. Conjugates according to embodiments of the present invention will be useful for the treatment of early-stage, chronic, and acute congestive heart failure.

**[0017]** Conjugates of the invention can be tested for agonist activity at the human natriuretic peptide receptor A (NPR-A) in vitro. The vasorelaxant, natriuretic, and diuretic properties of BNP are ascribed to a secondary messenger, cyclic GMP (cGMP). The production of cGMP is accomplished by guanylate cyclase, an enzyme that is activated when BNP binds to NPR-A. cGMP production can be measured in cultures of human aortic endothelial cells that endogenously express NPR-A. Thus, the relative activity of the CD-NP conjugates can be determined by the level of cGMP production in these cells.

**[0018]** The conjugates can be tested for oral bioavailability. Oral bioavailability of the conjugates can be tested in rats, for example. The conjugates can be administered to the gastrointestinal tract by oral gavage and the presence of conjugates in the bloodstream can be assayed using available radioimmunoassay procedures.

**[0019]** As used herein, the terms "peptide," "polypeptide," and "protein," refer to polymers comprised of amino acid monomers linked by amide bonds. Peptides may include the standard 20 α-amino acids that are used in protein synthesis by cells (*i.e.* natural amino acids), as well as non-natural amino acids (non-natural amino acids nay be found in nature,

but not used in protein synthesis by cells, *e.g.*, ornithine, citrulline, and sarcosine, or may be chemically synthesized), amino acid analogs, and peptidomimetics. Spatola, (1983) in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, Weinstein, ed., Marcel Dekker, New York, p. 267. The amino acids may be D- or L-optical isomers. Peptides may be formed by a condensation or coupling reaction between the α-carbon carboxyl group of one amino acid and the amino group of another amino acid. The terminal amino acid at one end of the chain (amino terminal) therefore has a free amino group, while the terminal amino acid at the other end of the chain (carboxy terminal) has a free carboxyl group. Alternatively, the peptides may be non-linear, branched peptides or cyclic peptides. Moreover, the peptides may optionally be modified or protected with a variety of functional groups or protecting groups, including on the amino and/or carboxy terminus.

**[0020]** Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

**[0021]** The terms "CD-NP peptide fragment" or "fragments of CD-NP peptides" refer to a polypeptide that comprises a truncation at the amino-terminus and/or a truncation at the carboxyl-terminus of a CD-NP peptide as defined herein. The terms "CD-NP peptide fragment" or "fragments of CD-NP peptides" also encompasses amino-terminal and/or carboxyl-terminal truncations of CD-NP peptide variants and CD-NP peptide derivatives. CD-NP peptide fragments may be produced by synthetic techniques known in the art or may arise from *in vivo* protease activity on longer peptide sequences. It will be understood that CD-NP peptide fragments retain some or all of the CD-NP activities of the CD-NP peptides.

**[0022]** As used herein, the terms "CD-NP peptide variants" or "variants of CD-NP peptides" refer to CD-NP peptides having one or more amino acid substitutions, including conservative substitutions and non-conservative substitutions, amino acid deletions (either internal deletions and/or *C*- and/or *N*- terminal truncations), amino acid additions (either internal additions and/or *C*- and/or *N*- terminal additions, *e.g.*, fusion peptides), or any combination thereof. Variants may be naturally occurring (*e.g.* homologs or orthologs), or non-natural in origin. The term "CD-NP peptide variants" may also be used to refer to CD-NP peptides incorporating one or more non-natural amino acids, amino acid analogs, and

peptidomimetics. It will be understood that, in accordance with the invention, CD-NP peptide fragments retain some or all of the CD-NP activities of the CD-NP peptides.

**[0023]** The terms "CD-NP peptide derivatives" or "derivatives of CD-NP peptides" as used herein refer to CD-NP peptides, CD-NP peptide fragments, and CD-NP peptide variants that have been chemically altered other than through covalent attachment of a water-soluble polymer. It will be understood that, in accordance with the invention, CD-NP peptide derivatives retain some or all of the CD-NP activities of the CD-NP peptides.

**[0024]** As used herein, the terms "amino terminus protecting group" or "N-terminal protecting group," "carboxy terminus protecting group" or "C-terminal protecting group;" or "side chain protecting group" refer to any chemical moiety capable of addition to and optionally removal from a functional group on a peptide (*e.g.*, the *N*-terminus, the *C*-terminus, or a functional group associated with the side chain of an amino acid located within the peptide) to allow for chemical manipulation of the peptide.

**[0025]** "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are interchangeable and encompass any nonpeptidic water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure "-$(OCH_2CH_2)_n$-" where (n) is 2 to 4000. As used herein, PEG also includes "-$CH_2CH_2$-$O(CH_2CH_2O)_n$-$CH_2CH_2$-" and "-$(OCH_2CH_2)_nO$-," depending upon whether or not the terminal oxygens have been displaced. Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups and so forth. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of -$OCH_2CH_2$- repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below.

**[0026]** The terms "end-capped" and "terminally capped" are interchangeably used herein to refer to a terminal or endpoint of a polymer having an end-capping moiety. Typically, although not necessarily, the end-capping moiety comprises a hydroxy or $C_{1-20}$ alkoxy group, more preferably a $C_{1-10}$ alkoxy group, and still more preferably a $C_{1-5}$ alkoxy group. Thus, examples of end-capping moieties include alkoxy (*e.g.*, methoxy, ethoxy and benzyloxy), as well as aryl, heteroaryl, cyclo, heterocyclo, and the like. It must be remembered that the end-capping moiety may include one or more atoms of the terminal monomer in the polymer [*e.g.*, the end-capping moiety "methoxy" in $CH_3O(CH_2CH_2O)_n$- and

$CH_3(OCH_2CH_2)_n$-]. In addition, saturated, unsaturated, substituted and unsubstituted forms of each of the foregoing are envisioned. Moreover, the end-capping group can also be a silane. The end-capping group can also advantageously comprise a detectable label. When the polymer has an end-capping group comprising a detectable label, the amount or location of the polymer and/or the moiety (*e.g.*, active agent) to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemiluminescers, moieties used in enzyme labeling, colorimetric (*e.g.*, dyes), metal ions, radioactive moieties, gold particles, quantum dots, and the like. Suitable detectors include photometers, films, spectrometers, and the like. The end-capping group can also advantageously comprise a phospholipid. When the polymer has an end-capping group comprising a phospholipid, unique properties are imparted to the polymer and the resulting conjugate. Exemplary phospholipids include, without limitation, those selected from the class of phospholipids called phosphatidylcholines. Specific phospholipids include, without limitation, those selected from the group consisting of dilauroylphosphatidylcholine, dioleylphosphatidylcholine, dipalmitoylphosphatidylcholine, disteroylphosphatidylcholine, behenoylphosphatidylcholine, arachidoylphosphatidylcholine, and lecithin.

**[0027]** The term "targeting moiety" is used herein to refer to a molecular structure that helps the conjugates of the invention to localize to a targeting area, e.g., help enter a cell, or bind a receptor. Preferably, the targeting moiety comprises of vitamin, antibody, antigen, receptor, DNA, RNA, sialyl Lewis X antigen, hyaluronic acid, sugars, cell specific lectins, steroid or steroid derivative, RGD peptide, ligand for a cell surface receptor, serum component, or combinatorial molecule directed against various intra- or extracellular receptors. The targeting moiety may also comprise a lipid or a phospholipid. Exemplary phospholipids include, without limitation, phosphatidylcholines, phospatidylserine, phospatidylinositol, phospatidylglycerol, and phospatidylethanolamine. These lipids may be in the form of micelles or liposomes and the like. The targeting moiety may further comprise a detectable label or alternately a detectable label may serve as a targeting moiety. When the conjugate has a targeting group comprising a detectable label, the amount and/or distribution/location of the polymer and/or the moiety (e.g., active agent) to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemiluminescers, moieties used in enzyme labeling, colorimetric (e.g., dyes), metal ions, radioactive moieties, gold particles, quantum dots, and the like.

**[0028]** "Non-naturally occurring" with respect to a polymer as described herein, means a polymer that in its entirety is not found in nature. A non-naturally occurring polymer of the invention may, however, contain one or more monomers or segments of monomers that are naturally occurring, so long as the overall polymer structure is not found in nature.

**[0029]** The term "water soluble" as in a "water-soluble polymer" is any polymer that is soluble in water at room temperature. Typically, a water-soluble polymer will transmit at least about 75%, more preferably at least about 95%, of light transmitted by the same solution after filtering. On a weight basis, a water-soluble polymer will preferably be at least about 35% (by weight) soluble in water, more preferably at least about 50% (by weight) soluble in water, still more preferably about 70% (by weight) soluble in water, and still more preferably about 85% (by weight) soluble in water. It is most preferred, however, that the water-soluble polymer is about 95% (by weight) soluble in water or completely soluble in water.

**[0030]** "Hydrophilic," *e.g*, in reference to a "hydrophilic polymer," refers to a polymer that is characterized by its solubility in and compatability with water. In non-cross linked form, a hydrophilic polymer is able to dissolve in, or be dispersed in water. Typically, a hydrophilic polymer possesses a polymer backbone composed of carbon and hydrogen, and generally possesses a high percentage of oxygen in either the main polymer backbone or in pendent groups substituted along the polymer backbone, thereby leading to its "water-loving" nature. The water-soluble polymers of the present invention are typically hydrophilic, *e.g.,* non-naturally occurring hydrophilic.

**[0031]** Molecular weight in the context of a water-soluble polymer, such as PEG, can be expressed as either a number average molecular weight or a weight average molecular weight. Unless otherwise indicated, all references to molecular weight herein refer to the weight average molecular weight. Both molecular weight determinations, number average and weight average, can be measured using gel permeation chromatography or other liquid chromatography techniques. Other methods for measuring molecular weight values can also be used, such as the use of end-group analysis or the measurement of colligative properties (*e.g.*, freezing-point depression, boiling-point elevation, and osmotic pressure) to determine number average molecular weight, or the use of light scattering techniques, ultracentrifugation or viscometry to determine weight average molecular weight. The polymers of the invention are typically polydisperse (i.e., number average molecular weight and weight average molecular weight of the polymers are not equal), possessing low

polydispersity values of preferably less than about 1.2, more preferably less than about 1.15, still more preferably less than about 1.10, yet still more preferably less than about 1.05, and most preferably less than about 1.03.

**[0032]** The term "active" or "activated" when used in conjunction with a particular functional group refers to a reactive functional group that reacts readily with an electrophile or a nucleophile on another molecule. This is in contrast to those groups that require strong catalysts or highly impractical reaction conditions in order to react (i.e., a "non-reactive" or "inert" group).

**[0033]** As used herein, the term "functional group" or any synonym thereof is meant to encompass protected forms thereof as well as unprotected forms.

**[0034]** The terms "spacer moiety," "linkage" and "linker" are used herein to refer to an atom or a collection of atoms optionally used to link interconnecting moieties such as a terminus of a polymer segment and a CD-NP peptide or an electrophile or nucleophile of a CD-NP peptide. The spacer moiety may be hydrolytically stable or may include a physiologically hydrolyzable or enzymatically degradable linkage. Unless the context clearly dictates otherwise, a spacer moiety optionally exists between any two elements of a compound (e.g., the provided conjugates comprising a residue of a CD-NP peptide and a water-soluble polymer that can be attached directly or indirectly through a spacer moiety).

**[0035]** A "monomer" or "mono-conjugate," in reference to a polymer conjugate of a CD-NP peptide, refers to a CD-NP peptide having only one water-soluble polymer molecule covalently attached thereto, whereas a CD-NP peptide "dimer" or "di-conjugate" is a polymer conjugate of a CD-NP peptide having two water-soluble polymer molecules covalently attached thereto, and so forth.

**[0036]** "Alkyl" refers to a hydrocarbon, typically ranging from about 1 to 15 atoms in length. Such hydrocarbons are preferably but not necessarily saturated and may be branched or straight chain, although typically straight chain is preferred. Exemplary alkyl groups include methyl, ethyl, propyl, butyl, pentyl, 2-methylbutyl, 2-ethylpropyl, 3-methylpentyl, and the like. As used herein, "alkyl" includes cycloalkyl as well as cycloalkylene-containing alkyl.

**[0037]** "Lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms, and may be straight chain or branched, as exemplified by methyl, ethyl, *n*-butyl, *i*-butyl, and *t*-butyl.

**[0038]** "Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon chain, including bridged, fused, or spiro cyclic compounds, preferably made up of 3 to about 12 carbon atoms, more preferably 3 to about 8 carbon atoms. "Cycloalkylene" refers to a cycloalkyl group that is inserted into an alkyl chain by bonding of the chain at any two carbons in the cyclic ring system.

**[0039]** "Alkoxy" refers to an -O-R group, wherein R is alkyl or substituted alkyl, preferably $C_{1-6}$ alkyl (*e.g.*, methoxy, ethoxy, propyloxy, and so forth).

**[0040]** The term "substituted" as in, for example, "substituted alkyl," refers to a moiety (*e.g.*, an alkyl group) substituted with one or more noninterfering substituents, such as, but not limited to: alkyl; $C_{3-8}$ cycloalkyl, *e.g.*, cyclopropyl, cyclobutyl, and the like; halo, *e.g.*, fluoro, chloro, bromo, and iodo; cyano; alkoxy, lower phenyl; substituted phenyl; and the like. "Substituted aryl" is aryl having one or more noninterfering groups as a substituent. For substitutions on a phenyl ring, the substituents may be in any orientation (*i.e.*, ortho, meta, or para).

**[0041]** "Noninterfering substituents" are those groups that, when present in a molecule, are typically nonreactive with other functional groups contained within the molecule.

**[0042]** "Aryl" means one or more aromatic rings, each of 5 or 6 core carbon atoms. Aryl includes multiple aryl rings that may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings. As used herein, "aryl" includes heteroaryl.

**[0043]** "Heteroaryl" is an aryl group containing from one to four heteroatoms, preferably sulfur, oxygen, or nitrogen, or a combination thereof. Heteroaryl rings may also be fused with one or more cyclic hydrocarbon, heterocyclic, aryl, or heteroaryl rings.

**[0044]** "Heterocycle" or "heterocyclic" means one or more rings of 5-12 atoms, preferably 5-7 atoms, with or without unsaturation or aromatic character and having at least one ring atom that is not a carbon. Preferred heteroatoms include sulfur, oxygen, and nitrogen.

**[0045]** "Substituted heteroaryl" is heteroaryl having one or more noninterfering groups as substituents.

**[0046]** "Substituted heterocycle" is a heterocycle having one or more side chains formed from noninterfering substituents.

**[0047]** An "organic radical" as used herein shall include alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, and substituted aryl.

**[0048]** "Electrophile" and "electrophilic group" refer to an ion or atom or collection of atoms, that may be ionic, having an electrophilic center, *i.e.*, a center that is electron seeking, capable of reacting with a nucleophile.

**[0049]** "Nucleophile" and "nucleophilic group" refers to an ion or atom or collection of atoms that may be ionic having a nucleophilic center, *i.e.*, a center that is seeking an electrophilic center or with an electrophile.

**[0050]** A "physiologically cleavable" or "hydrolyzable" or "degradable" bond is a bond that reacts with water (*i.e.*, is hydrolyzed) under physiological conditions. The tendency of a bond to hydrolyze in water will depend not only on the general type of linkage connecting two central atoms but also on the substituents attached to these central atoms. Appropriate hydrolytically unstable or weak linkages include but are not limited to carboxylate ester, phosphate ester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides.

**[0051]** "Releasably attached," *e.g.*, in reference to a CD-NP peptide releasably attached to a water-soluble polymer, refers to a CD-NP peptide that is covalently attached via a linker that includes a degradable linkage as disclosed herein, wherein upon degradation (*e.g.*, hydrolysis), the CD-NP peptide is released. The CD-NP peptide thus released will typically correspond to the unmodified parent or native CD-NP peptide, or may be slightly altered, *e.g.*, possessing a short organic tag. Preferably, the unmodified parent CD-NP peptide is released.

**[0052]** An "enzymatically degradable linkage" means a linkage that is subject to degradation by one or more enzymes.

**[0053]** A "hydrolytically stable" linkage or bond refers to a chemical bond, typically a covalent bond, that is substantially stable in water, that is to say, does not undergo hydrolysis under physiological conditions to any appreciable extent over an extended period of time. Examples of hydrolytically stable linkages include, but are not limited to, the following: carbon-carbon bonds (*e.g.*, in aliphatic chains), ethers, amides, urethanes, and the like. Generally, a hydrolytically stable linkage is one that exhibits a rate of hydrolysis of less than about 1-2% per day under physiological conditions. Hydrolysis rates of representative chemical bonds can be found in most standard chemistry textbooks. It must be pointed out that some linkages can be hydrolytically stable or hydrolyzable, depending upon (for example) adjacent and neighboring atoms and ambient conditions. One of ordinary skill in

the art can determine whether a given linkage or bond is hydrolytically stable or hydrolyzable in a given context by, for example, placing a linkage-containing molecule of interest under conditions of interest and testing for evidence of hydrolysis (*e.g.*, the presence and amount of two molecules resulting from the cleavage of a single molecule). Other approaches known to those of ordinary skill in the art for determining whether a given linkage or bond is hydrolytically stable or hydrolyzable can also be used.

**[0054]** The terms "pharmaceutically acceptable excipient" and "pharmaceutically acceptable carrier" refer to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient.

**[0055]** "Pharmacologically effective amount," "physiologically effective amount," and "therapeutically effective amount" are used interchangeably herein to mean the amount of a polymer-(CD-NP peptide) conjugate that is needed to provide a desired level of the conjugate (or corresponding unconjugated CD-NP peptide) in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, *e.g.*, the particular CD-NP peptide, the components and physical characteristics of the CD-NP composition, intended patient population, individual patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein.

**[0056]** "Multi-functional" means a polymer having three or more functional groups contained therein, where the functional groups may be the same or different. Multi-functional polymeric reagents of the invention will typically contain from about 3-100 functional groups, or from 3-50 functional groups, or from 3-25 functional groups, or from 3-15 functional groups, or from 3 to 10 functional groups, or will contain 3, 4, 5, 6, 7, 8, 9 or 10 functional groups within the polymer backbone. A "difunctional" polymer means a polymer having two functional groups contained therein, either the same (*i.e.*, homodifunctional) or different (*i.e.*, heterodifunctional).

**[0057]** The terms "subject," "individual," or "patient" are used interchangeably herein and refer to a vertebrate, preferably a mammal. Mammals include, but are not limited to, murines, rodents, simians, humans, farm animals, sport animals, and pets.

**[0058]** "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

**[0059]** "Substantially" (unless specifically defined for a particular context elsewhere or the context clearly dictates otherwise) means nearly totally or completely, for instance,

satisfying one or more of the following: greater than 50%, 51% or greater, 75% or greater, 80% or greater, 90% or greater, and 95% or greater of the condition.

**[0060]** Unless the context clearly dictates otherwise, when the term "about" precedes a numerical value, the numerical value is understood to mean the stated numerical value and also ± 10% of the stated numerical value.

**[0061]** Turning now to one or more aspects of the invention, conjugates are provided, the conjugates comprising a CD-NP peptide covalently attached (either directly or through a spacer moiety or linker) to a water-soluble polymer. The conjugates generally have the following formula:

$$\text{CD-NP} —[— \text{X} — \text{POLY} ]_k$$

wherein CD-NP is a CD-NP peptide as defined herein, X is a covalent bond or is a spacer moiety or linker, POLY is a water soluble polymer, and k in an integer ranging from 1-10, preferably 1-5, and more preferably 1-3.

**CD-NP Peptides**

**[0062]** As previously stated, the conjugates of the invention comprise a CD-NP peptide as disclosed and/or defined herein. CD-NP peptides include those currently known to have demonstrated or potential use in treating, preventing, or ameliorating one or more diseases, disorders, or conditions in a subject in need thereof as well as those discovered after the filing of this application. CD-NP peptides also include related peptides.

**[0063]** The CD-NP peptides of the invention may comprise any of the 20 natural amino acids, and/or non-natural amino acids, amino acid analogs, and peptidomimetics, in any combination. The peptides may be composed of D- amino acids or L-amino acids, or a combination of both in any proportion. In addition to natural amino acids, the CD-NP peptides may contain, or may be modified to include, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, or more non-natural amino acids. Exemplary non-natural amino acids and amino acid analogs that can be use with the invention include, but are not limited to, 2-aminobutyric acid, 2-aminoisobutyric acid, 3-(1-naphthyl)alanine, 3-(2-naphthyl)alanine, 3-methylhistidine, 3-pyridylalanine, 4-chlorophenylalanine, 4-fluorophenylalanine, 4-hydroxyproline, 5-hydroxylysine, alloisoleucine, citrulline, dehydroalanine, homoarginine, homocysteine, homoserine, hydroxyproline, N-acetylserine, N-formylmethionine, N-methylglycine, N-methylisoleucine, norleucine, N-α-methylarginine, O-phosphoserine, ornithine,

phenylglycine, pipecolinic acid, piperazic acid, pyroglutamine, sarcosine, valanine, β-alanine, and β-cyclohexylalanine.

**[0064]** The CD-NP peptides may be, or may be modified to be, linear, branched, or cyclic, with our without branching.

**[0065]** Additionally, the CD-NP peptides may optionally be modified or protected with a variety of functional groups or protecting groups, including amino terminus protecting groups and/or carboxy terminus protecting groups. Protecting groups, and the manner in which they are introduced and removed are described, for example, in "Protective Groups in Organic Chemistry," Plenum Press, London, N.Y. 1973; and. Greene *et al.*, "PROTECTIVE GROUPS IN ORGANIC SYNTHESIS" 3rd Edition, John Wiley and Sons, Inc., New York, 1999. Numerous protecting groups are known in the art. An illustrative, non-limiting list of protecting groups includes methyl, formyl, ethyl, acetyl, t-butyl, anisyl, benzyl, trifluoroacetyl, N-hydroxysuccinimide, t-butoxycarbonyl, benzoyl, 4-methylbenzyl, thioanizyl, thiocresyl, benzyloxymethyl, 4-nitrophenyl, benzyloxycarbonyl, 2-nitrobenzoyl, 2-nitrophenylsulphenyl, 4-toluenesulphonyl, pentafluorophenyl, diphenylmethyl, 2-chlorobenzyloxycarbonyl, 2,4,5-trichlorophenyl, 2-bromobenzyloxycarbonyl, 9-fluorenylmethyloxycarbonyl, triphenylmethyl, and 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl. For discussions of various different types of amino- and carboxy-protecting groups, see, for example, U.S. Pat. No. 5,221,736 (issued Jun. 22, 1993); U.S. Pat. No. 5,256,549 (issued Oct. 26, 1993); U.S. Pat. No. 5,049,656 (issued Sep. 17, 1991); and U.S. Pat. No. 5,521,184 (issued May 28, 1996).

**[0066]** The CD-NP peptides contain, or may be modified to contain, functional groups to which a water-soluble polymer may be attached, either directly or through a spacer moiety or linker. Functional groups include, but are not limited to, the *N*-terminus of the CD-NP peptide, the *C*-terminus of the CD-NP peptide, and any functional groups on the side chain of an amino acid, *e.g.* lysine, cysteine, histidine, aspartic acid, glutamic acid, tyrosine, arginine, serine, methionine, and threonine, present in the CD-NP peptide.

**[0067]** The CD-NP peptides can be prepared by any means known in the art, including non-recombinant and recombinant methods, or they may, in some instances, be commercially available. Chemical or non-recombinant methods include, but are not limited to, solid phase peptide synthesis (SPPS), solution phase peptide synthesis, native chemical ligation, intein-mediated protein ligation, and chemical ligation, or a combination thereof. In a preferred embodiment, the CD-NP peptides are synthesized using standard SPPS, either manually or by using commercially available automated SPPS synthesizers.

**[0068]** SPPS has been known in the art since the early 1960's (Merrifield, R. B., *J. Am. Chem. Soc.*, 85:2149-2154 (1963)), and is widely employed. (*See also*, Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Heidelberg (1984)). There are several known variations on the general approach. (*See*, for example, "Peptide Synthesis, Structures, and Applications" © 1995 by Academic Press, Chapter 3 and White (2003) *Fmoc Solid Phase Peptide Synthesis, A practical Approach*, Oxford University Press, Oxford). Very briefly, in solid phase peptide synthesis, the desired C-terminal amino acid residue is coupled to a solid support. The subsequent amino acid to be added to the peptide chain is protected on its amino terminus with Boc, Fmoc, or other suitable protecting group, and its carboxy terminus is activated with a standard coupling reagent. The free amino terminus of the support-bound amino acid is allowed to react with the carboxy-terminus of the subsequent amino acid, coupling the two amino acids. The amino terminus of the growing peptide chain is deprotected, and the process is repeated until the desired polypeptide is completed. Side chain protecting groups may be utilized as needed.

**[0069]** Alternatively, the CD-NP peptides may be prepared recombinantly. Exemplary recombinant methods used to prepare CD-NP peptides include the following, among others, as will be apparent to one skilled in the art. Typically, a CD-NP peptide as defined and/or described herein is prepared by constructing the nucleic acid encoding the desired peptide or fragment, cloning the nucleic acid into an expression vector, transforming a host cell (*e.g.*, plant, bacteria such as *Escherichia coli*, yeast such as *Saccharomyces cerevisiae*, or mammalian cell such as Chinese hamster ovary cell or baby hamster kidney cell), and expressing the nucleic acid to produce the desired peptide or fragment. The expression can occur via exogenous expression or via endogenous expression (when the host cell naturally contains the desired genetic coding). Methods for producing and expressing recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are known to those of ordinary skill in the art. See, for example, U.S. Patent No. 4,868,122, and Sambrook et al., Molecular Cloning--A Laboratory Manual (Third Edition), Cold Spring Harbor Laboratory Press (2001).

**[0070]** To facilitate identification and purification of the recombinant peptide, nucleic acid sequences that encode an epitope tag or other affinity binding sequence can be inserted or added in-frame with the coding sequence, thereby producing a fusion peptide comprised of the desired CD-NP peptide and a peptide suited for binding. Fusion peptides can be identified and purified by first running a mixture containing the fusion peptide through an

affinity column bearing binding moieties (*e.g.*, antibodies) directed against the epitope tag or other binding sequence in the fusion peptide, thereby binding the fusion peptide within the column. Thereafter, the fusion peptide can be recovered by washing the column with the appropriate solution (*e.g.*, acid) to release the bound fusion peptide. Optionally, the tag may subsequently be removed by techniques known in the art. The recombinant peptide can also be identified and purified by lysing the host cells, separating the peptide, *e.g.*, by size exclusion chromatography, and collecting the peptide. These and other methods for identifying and purifying recombinant peptides are known to those of ordinary skill in the art.

**Related Peptides**

**[0071]** It will be appreciated and understood by one of skill in the art that certain modifications can be made to the CD-NP peptides defined and/or disclosed herein that do not alter, or only partially abrogate, the properties and activities of these CD-NP peptides. In some instances, modifications may be made that result in an increase in CD-NP activities. Additionally, modifications may be made that increase certain biological and chemical properties of the CD-NP peptides in a beneficial way, *e.g.* increased *in vivo* half life, increased stability, decreased susceptibility to proteolytic cleavage, etc. Thus, in the spirit and scope of the invention, the term "CD-NP peptide" is used herein in a manner to include not only the CD-NP peptides defined and/or disclosed herein, but also related peptides, *i.e.* peptides that contain one or more modifications relative to the CD-NP peptides defined and/or disclosed herein, wherein the modification(s) do not alter, only partially abrogate, or increase the CD-NP activities as compared to the parent peptide.

**[0072]** Related peptides include, but are not limited to, fragments of CD-NP peptides, CD-NP peptide variants, and CD-NP peptide derivatives. Related peptides also include any and all combinations of these modifications. In a non-limiting example, a related peptide may be a fragment of a CD-NP peptide as disclosed herein having one or more amino acid substitutions. Thus it will be understood that any reference to a particular type of related peptide is not limited to a CD-NP peptide having only that particular modification, but rather encompasses a CD-NP peptide having that particular modification and optionally any other modification.

**[0073]** Related peptides may be prepared by action on a parent peptide or a parent protein (*e.g.* proteolytic digestion to generate fragments) or through *de novo* preparation (*e.g.* solid phase synthesis of a peptide having a conservative amino acid substitution relative to

the parent peptide). Related peptides may arise by natural processes (*e.g.* processing and other post-translational modifications) or may be made by chemical modification techniques. Such modifications are well-known to those of skill in the art.

**[0074]** A related peptide may have a single alteration or multiple alterations relative to the parent peptide. Where multiple alterations are present, the alterations may be of the same type or a given related peptide may contain different types of modifications. Furthermore, modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the *N*- or *C*- termini.

**[0075]** As previously noted, related peptides include fragments of the CD-NP peptides defined and/or disclosed herein, wherein the fragment retains some of or all of at least one CD-NP activity of the parent peptide. The fragment may also exhibit an increase in at least one CD-NP activity of the parent peptide. In certain embodiments of the invention, CD-NP peptides include related peptides having at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 contiguous amino acid residues, or more than 125 contiguous amino acid residues, of any of the CD-NP peptides disclosed, herein, including in Table 1. In other embodiments of the invention, CD-NP peptides include related peptides having 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acid residues deleted from the *N*-terminus and/or having 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acid residues deleted from the *C*-terminus of any of the CD-NP peptides disclosed herein, including in Table 1.

**[0076]** Related peptides also include variants of the CD-NP peptides defined and/or disclosed herein, wherein the variant retains some of or all of at least one CD-NP activity of the parent peptide. The variant may also exhibit an increase in at least one CD-NP activity of the parent peptide. In certain embodiments of the invention, CD-NP peptides include variants having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 conservative and/or non-conservative amino acid substitutions relative to the CD-NP peptides disclosed herein, including in Table 1. Desired amino acid substitutions, whether conservative or non-conservative, can be determined by those skilled in the art.

**[0077]** In certain embodiments of the invention, CD-NP peptides include variants having conservative amino substitutions; these substitutions will produce a CD-NP peptide having functional and chemical characteristics similar to those of the parent peptide. In other embodiments, CD-NP peptides include variants having non-conservative amino substitutions; these substitutions will produce a CD-NP peptide having functional and chemical characteristics that may differ substantially from those of the parent peptide. In certain

embodiments of the invention, CD-NP peptide variants have both conservative and non-conservative amino acid substitutions. In other embodiments, each amino acid residue may be substituted with alanine.

**[0078]** Natural amino acids may be divided into classes based on common side chain properties: nonpolar (Gly, Ala, Val, Leu, Ile, Met); polar neutral (Cys, Ser, Thr, Pro, Asn, Gln); acidic (Asp, Glu); basic (His, Lys, Arg); and aromatic (Trp, Tyr, Phe). By way of example, non-conservative amino acid substitutions may involve the substitution of an amino acid of one class for that of another, and may be introduced in regions of the peptide not critical for CD-NP activity.

**[0079]** Preferably, amino acid substitutions are conservative. Conservative amino acid substitutions may involve the substitution of an amino acid of one class for that of the same class. Conservative amino acid substitutions may also encompass non-natural amino acid residues, including peptidomimetics and other atypical forms of amino acid moieties, and may be incorporated through chemical peptide synthesis,

**[0080]** Amino acid substitutions may be made with consideration to the hydropathic index of amino acids. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte *et al.*, 1982, *J. Mol. Biol.* 157:105-31). Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. The hydropathic indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

**[0081]** It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

**[0082]** It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its biological properties. According to U.S. Patent No. 4,554,101, incorporated herein by reference, the following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine

(+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

**[0083]** In certain embodiments of the invention, CD-NP peptides include variants having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acid deletions relative to the CD-NP peptides disclosed herein, including in Table 1. The deleted amino acid(s) may be at the *N*- or *C*- terminus of the peptide, at both termini, at an internal location or locations within the peptide, or both internally and at one or both termini. Where the variant has more than one amino acid deletion, the deletions may be of contiguous amino acids or of amino acids at different locations within the primary amino acid sequence of the parent peptide.

**[0084]** In other embodiments of the invention, CD-NP peptides include variants having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acid additions relative to the CD-NP peptides disclosed herein, including in Table 1. The added amino acid(s) may be at the *N*- or *C*- terminus of the peptide, at both termini, at an internal location or locations within the peptide, or both internally and at one or both termini. Where the variant has more than one amino acid addition, the amino acids may be added contiguously, or the amino acids may be added at different locations within the primary amino acid sequence of the parent peptide.

**[0085]** Addition variants also include fusion peptides. Fusions can be made either at the *N*-terminus or at the *C*-terminus of the CD-NP peptides disclosed herein, including in Table 1. In certain embodiments, the fusion peptides have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acid additions relative to the CD-NP peptides disclosed herein, including in Table 1. Fusions may be attached directly to the CD-NP peptide with no connector molecule or may be through a connector molecule. As used in this context, a connector molecule may be an atom or a collection of atoms optionally used to link a CD-NP peptide to another peptide. Alternatively, the connector may be an amino acid sequence designed for cleavage by a protease to allow for the separation of the fused peptides.

**[0086]** The CD-NP peptides of the invention may be fused to peptides designed to improve certain qualities of the CD-NP peptide, such as CD-NP activity, circulation time, or reduced aggregation. CD-NP peptides may be fused to an immunologically active domain,

*e.g.* an antibody epitope, to facilitate purification of the peptide, or to increase the *in vivo* half life of the peptide. Additionally, CD-NP peptides may be fused to known functional domains, cellular localization sequences, or peptide permeant motifs known to improve membrane transfer properties.

**[0087]** In certain embodiments of the invention, CD-NP peptides also include variants incorporating one or more non-natural amino acids, amino acid analogs, and peptidomimetics. Thus the present invention encompasses compounds structurally similar to the CD-NP peptides defined and/or disclosed herein, which are formulated to mimic the key portions of the CD-NP peptides of the present invention. Such compounds may be used in the same manner as the CD-NP peptides of the invention. Certain mimetics that mimic elements of protein secondary and tertiary structure have been previously described. Johnson *et al.*, Biotechnology and Pharmacy, Pezzuto et al. (Eds.), Chapman and Hall, NY, 1993. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions. A peptide mimetic is thus designed to permit molecular interactions similar to the parent peptide. Mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains. Methods for generating specific structures have been disclosed in the art. For example, U.S. Patent Nos. 5,446,128, 5,710,245, 5,840,833, 5,859,184, 5,440,013; 5,618,914, 5,670,155, 5,475,085, 5,929,237, 5,672,681 and 5,674,976, the contents of which are hereby incorporated by reference, all disclose peptidomimetics structures that may have improved properties over the parent peptide, for example they may be conformationally restricted, be more thermally stable, exhibit increased resistance to degredation, etc.

**[0088]** In another embodiment, related peptides comprise or consist of a peptide sequence that is at least 70% identical to any of the CD-NP peptides disclosed herein, including in Table 1. In additional embodiments, related peptides are at least 75% identical, at least 80% identical, at least 85% identical, 90% identical, at least 91% identical, at least 92% identical, 93% identical, at least 94% identical, at least 95% identical, 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical to any of the CD-NP peptides disclosed herein, including in Table 1.

**[0089]** Sequence identity (also known as % homology) of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to those described in *Computational Molecular Biology* (A.M. Lesk, ed., Oxford University Press 1988); *Biocomputing: Informatics and Genome Projects* (D.W. Smith, ed., Academic Press

1993); *Computer Analysis of Sequence Data* (Part 1, A.M. Griffin and H.G. Griffin, eds., Humana Press 1994); G. von Heinle, *Sequence Analysis in Molecular Biology* (Academic Press 1987); *Sequence Analysis Primer* (M. Gribskov and J. Devereux, eds., M. Stockton Press 1991); and Carillo *et al.*, 1988, *SIAM J. Applied Math.*, 48:1073.

**[0090]** Preferred methods to determine sequence identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are described in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux *et al.*, 1984, *Nucleic Acids Res.* 12:387; Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (Altschul *et al.*, *BLAST Manual* (NCB NLM NIH, Bethesda, MD); Altschul *et al.*, 1990, *supra*). The well-known Smith Waterman algorithm may also be used to determine identity.

**[0091]** For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span," as determined by the algorithm). A gap opening penalty (which is calculated as 3X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 0.1X the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix is also used by the algorithm (*see* Dayhoff *et al.*, 5 *Atlas of Protein Sequence and Structure* (Supp. 3 1978)(PAM250 comparison matrix); Henikoff *et al.*, 1992, *Proc. Natl. Acad. Sci USA* 89:10915-19 (BLOSUM 62 comparison matrix)). The particular choices to be made with regard to algorithms, gap opening penalties, gap extension penalties, comparison matrices, and thresholds of similarity will be readily apparent to those of skill in the art and will depend on the specific comparison to be made.

**[0092]** Related peptides also include derivatives of the CD-NP peptides defined and/or disclosed herein, wherein the variant retains some of or all of at least one CD-NP activity of the parent peptide. The derivative may also exhibit an increase in at least one CD-NP activity of the parent peptide. Chemical alterations of CD-NP peptide derivatives

include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, biotinylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, T. E. Creighton, Proteins, Structure and Molecular Properties, 2nd ed., W.H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B. C. Johnson, ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth. Enzymol 182:626-46 (1990); Rattan et al., Ann. N.Y. Acad. Sci. 663:48-62, 1992).

**[0093]** CD-NP peptide derivatives also include molecules formed by the deletion of one or more chemical groups from the parent peptide. Methods for preparing chemically modified derivatives of the CD-NP peptides defined and/or disclosed herein are known to one of skill in the art.

**[0094]** In some embodiments of the invention, the CD-NP peptides may be modified with one or more methyl or other lower alkyl groups at one or more positions of the CD-NP peptide sequence. Examples of such groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, etc. In certain preferred embodiments, arginine, lysine, and histidine residues of the CD-NP peptides are modified with methyl or other lower alkyl groups.

**[0095]** In other embodiments of the invention, the CD-NP peptides may be modified with one or more glycoside moieties relative to the parent peptide. Although any glycoside can be used, in certain preferred embodiments the CD-NP peptide is modified by introduction of a monosaccharide, a disaccharide, or a trisaccharide or it may contain a glycosylation sequence found in natural peptides or proteins in any mammal. The saccharide may be introduced at any position, and more than one glycoside may be introduced. Glycosylation may occur on a naturally occurring amino acid residue in the CD-NP peptide, or alternatively, an amino acid may be substituted with another for modification with the saccharide.

**[0096]** Glycosylated CD-NP peptides may be prepared using conventional Fmoc chemistry and solid phase peptide synthesis techniques, *e.g.*, on resin, where the desired protected glycoamino acids are prepared prior to peptide synthesis and then introduced into

the peptide chain at the desired position during peptide synthesis. Thus, the CD-NP peptide polymer conjugates may be conjugated *in vitro*. The glycosylation may occur before deprotection. Preparation of aminoacid glycosides is described in U.S. Patent No. 5,767,254, WO 2005/097158, and Doores, K., *et al., Chem. Commun.,* 1401-1403, 2006, which are incorporated herein by reference in their entireties. For example, alpha and beta selective glycosylations of serine and threonine residues are carried out using the Koenigs-Knorr reaction and Lemieux's *in situ* anomerization methodology with Schiff base intermediates. Deprotection of the Schiff base glycoside is then carried out using mildly acidic conditions or hydrogenolysis. A composition, comprising a glycosylated CD-NP peptide conjugate made by stepwise solid phase peptide synthesis involving contacting a growing peptide chain with protected amino acids in a stepwise manner, wherein at least one of the protected amino acids is glycosylated, followed by water-soluble polymer conjugation, may have a purity of at least 95%, such as at least 97%, or at least 98%, of a single species of the glycosylated and conjugated CD-NP peptide.

**[0097]** Monosaccharides that may be used for introduction at one or more amino acid residues of the CD-NP peptides defined and/or disclosed herein include glucose (dextrose), fructose, galactose, and ribose. Additional monosaccharides suitable for use include glyceraldehydes, dihydroxyacetone, erythrose, threose, erythrulose, arabinose, lyxose, xylose, ribulose, xylulose, allose, altrose, mannose, N-Acetylneuraminic acid, fucose, N-Acetylgalactosamine, and N-Acetylglucosamine, as well as others. Glycosides, such as mono-, di-, and trisaccharides for use in modifying a CD-NP peptide, may be naturally occurring or may be synthetic. Disaccharides that may be used for introduction at one or more amino acid residues of the CD-NP peptides defined and/or disclosed herein include sucrose, lactose, maltose, trehalose, melibiose, and cellobiose, among others. Trisaccharides include acarbose, raffinose, and melezitose.

**[0098]** In further embodiments of the invention, the CD-NP peptides defined and/or disclosed herein may be chemically coupled to biotin. The biotin/thereapeutic peptide molecules can then to bind to avidin.

**[0099]** As previously noted, modifications may be made to the CD-NP peptides defined and/or disclosed herein that do not alter, or only partially abrogate, the properties and activities of these CD-NP peptides. In some instances, modifications may be made that result in an increase in CD-NP activity. Thus, included in the scope of the invention are modifications to the CD-NP peptides disclosed herein, including in Table 1, that retain at

least 1%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, and any range derivable therein, such as, for example, at least 70% to at least 80%, and more preferably at least 81% to at least 90%; or even more preferably, between at least 91% and at least 99% of the CD-NP activity relative to the unmodified CD-NP peptide. Also included in the scope of the invention are modification to the CD-NP peptides disclosed herein, including in Table 1, that have greater than 100%, greater than 110%, greater than 125%, greater than 150%, greater than 200%, or greater than 300%, or greater than 10-fold or greater than 100-fold, and any range derivable therein, of the CD-NP activity relative to the unmodified CD-NP peptide.

**[00100]** The level of CD-NP activity of a given CD-NP peptide, or a modified CD-NP peptide, may be determined by any suitable *in vivo* or *in vitro* assay. For example, CD-NP activity may be assayed in cell culture, or by clinical evaluation, $EC_{50}$ assays, $IC_{50}$ assays, or dose response curves. *In vitro* or cell culture assays, for example, are commonly available and known to one of skill in the art for many CD-NP peptides as disclosed herein, including in Table 1. It will be understood by one of skill in the art that the percent activity of a modified CD-NP peptide relative to its unmodified parent can be readily ascertained through a comparison of the activity of each as determined through the assays disclosed herein or as known to one of skill in the art.

**[00101]** One of skill in the art will be able to determine appropriate modifications to the CD-NP peptides defined and/or disclosed herein, including those disclosed herein, including in Table 1. For identifying suitable areas of the CD-NP peptides that may be changed without abrogating their CD-NP activities, one of skill in the art may target areas not believed to be essential for activity. For example, when similar peptides with comparable activities exist from the same species or across other species, one of skill in the art may compare those amino acid sequences to identify residues that are conserved among similar peptides. It will be understood that changes in areas of a CD-NP peptide that are not conserved relative to similar peptides would be less likely to adversely affect the thereapeutic activity. One skilled in the art would also know that, even in relatively conserved regions, one may substitute chemically similar amino acids while retaining CD-NP activity. Therefore, even areas that may be important for biological activity and/or for structure may

be subject to amino acid substitutions without destroying the CD-NP activity or without adversely affecting the peptide structure.

**[00102]** Additionally, as appropriate, one of skill in the art can review structure-function studies identifying residues in similar peptides that are important for activity or structure. In view of such a comparison, one can predict the importance of an amino acid residue in a CD-NP peptide that corresponds to an amino acid residue that is important for activity or structure in similar peptides. One of skill in the art may opt for amino acid substitutions within the same class of amino acids for such predicted important amino acid residues of the CD-NP peptides.

**[00103]** Also, as appropriate, one of skill in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar peptides. In view of such information, one of skill in the art may predict the alignment of amino acid residues of a CD-NP peptide with respect to its three dimensional structure. One of skill in the art may choose not to make significant changes to amino acid residues predicted to be on the surface of the peptide, since such residues may be involved in important interactions with other molecules. Moreover, one of skill in the art may generate variants containing a single amino acid substitution at each amino acid residue for test purposes. The variants could be screened using CD-NP activity assays known to those with skill in the art. Such variants could be used to gather information about suitable modifications. For example, where a change to a particular amino acid residue resulted in abrogated, undesirably reduced, or unsuitable activity, variants with such a modification would be avoided. In other words, based on information gathered from routine experimentation, one of skill in the art can readily determine the amino acids where further modifications should be avoided either alone or in combination with other modifications.

**[00104]** One of skill in the art may also select suitable modifications based on secondary structure predication. A number of scientific publications have been devoted to the prediction of secondary structure. *See* Moult, 1996, *Curr. Opin. Biotechnol.* 7:422-27; Chou *et al.*, 1974, *Biochemistry* 13:222-45; Chou *et al.*, 1974, *Biochemistry* 113:211-22; Chou *et al.*, 1978, *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:45-48; Chou *et al.*, 1978, *Ann. Rev. Biochem.* 47:251-276; and Chou *et al.*, 1979, *Biophys. J.* 26:367-84. Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two peptides or proteins which have a sequence identity of greater than 30%, or similarity greater than 40%, often have similar structural topologies. Recent growth of the protein

structural database (PDB, http://www.rcsb.org/pdb/home/home.do) has provided enhanced predictability of secondary, tertiary, and quarternary structure, including the potential number of folds within the structure of a peptide or protein. *See* Holm *et al.*, 1999, *Nucleic Acids Res.* 27:244-47. It has been suggested that there are a limited number of folds in a given peptide or protein and that once a critical number of structures have been resolved, structural prediction will become dramatically more accurate (Brenner *et al.*, 1997, *Curr. Opin. Struct. Biol.* 7:369-76).

**[00105]** Additional methods of predicting secondary structure include "threading" (Jones, 1997, *Curr. Opin. Struct. Biol.* 7:377-87; Sippl *et al.*, 1996, *Structure* 4:15-19), "profile analysis" (Bowie *et al.*, 1991, *Science*, 253:164-70; Gribskov *et al.*, 1990, *Methods Enzymol.* 183:146-59; Gribskov *et al.*, 1987, *Proc. Nat. Acad. Sci. U.S.A.* 84:4355-58), and "evolutionary linkage" (*See* Holm *et al.*, *supra*, and Brenner *et al.*, *supra*).

**CD-NP Peptide Conjugates**

**[00106]** As described above, a conjugate of the invention comprises a water-soluble polymer covalently attached (either directly or through a spacer moiety or linker) to a CD-NP peptide. Typically, for any given conjugate, there will be about one to five water-soluble polymers covalently attached to a CD-NP peptide (wherein for each water-soluble polymer, the water-soluble polymer can be attached either directly to the CD-NP peptide or through a spacer moiety).

**[00107]** To elaborate, a CD-NP peptide conjugate of the invention typically has about 1, 2, 3, or 4 water-soluble polymers individually attached to a CD-NP peptide. That is to say, in certain embodiments, a conjugate of the invention will possess about 4 water-soluble polymers individually attached to a CD-NP peptide, or about 3 water-soluble polymers individually attached to a CD-NP peptide, or about 2 water-soluble polymers individually attached to a CD-NP peptide, or about 1 water-soluble polymer attached to a CD-NP peptide. The structure of each of the water-soluble polymers attached to the CD-NP peptide may be the same or different. One CD-NP peptide conjugate in accordance with the invention is one having a water-soluble polymer releasably attached to the CD-NP peptide, particularly at the *N*-terminus of the CD-NP peptide. Another CD-NP peptide conjugate in accordance with the invention is one having a water-soluble polymer stably attached to the CD-NP peptide, particularly at the *N*-terminus of the CD-NP peptide. Another CD-NP peptide conjugate is one having a water-soluble polymer releasably attached to the CD-NP peptide, particularly at the *C*-terminus of the CD-NP peptide. Another CD-NP peptide conjugate in accordance with

the invention is one having a water-soluble polymer stably attached to the CD-NP peptide, particularly at the *C*-terminus of the CD-NP peptide. Other CD-NP peptide conjugates in accordance with the invention are those having a water-soluble polymer releasably or stably attached to an amino acid within the CD-NP peptide. Additional water-soluble polymers may be releasably or stably attached to other sites on the CD-NP peptide, *e.g.*, such as one or more additional sites. For example, a CD-NP peptide conjugate having a water-soluble polymer releasably attached to the *N*-terminus may additionally possess a water-soluble polymer stably attached to a lysine residue. In one embodiment, one or more amino acids may be inserted, at the *N*- or *C*-terminus, or within the peptide to releasably or stably attach a water soluble polymer. One preferred embodiment of the present invention is a mono-CD-NP peptide polymer conjugate, *i.e.*, a CD-NP peptide having one water-soluble polymer covalently attached thereto. In an even more preferred embodiment, the water-soluble polymer is one that is attached to the CD-NP peptide at its *N*-terminus.

**[00108]** In another embodiment of the invention, a CD-NP peptide polymer conjugate of the invention is absent a metal ion, *i.e.*, the CD-NP peptide is not chelated to a metal ion.

**[00109]** For the CD-NP peptide polymer conjugates described herein, the CD-NP peptide may optionally possess one or more *N*-methyl substituents. Alternatively, for the CD-NP peptide polymer conjugates described herein, the CD-NP peptide may be glycosylated, *e.g.*, having a mono- or disaccharide, or naturally-occuring amino acid glycosylation covalently attached to one or more sites thereof.

**[00110]** As discussed herein, the compounds of the present invention may be made by various methods and techniques known and available to those skilled in the art.

**The Water-Soluble Polymer**

**[00111]** A conjugate of the invention comprises a CD-NP peptide attached, stably or releasably, to a water-soluble polymer. The water-soluble polymer is typically hydrophilic, nonpeptidic, and biocompatible. A substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (*e.g.*, an active agent such a CD-NP peptide) in connection with living tissues (*e.g.*, administration to a patient) outweighs any deleterious effects as evaluated by a clinician, *e.g.*, a physician. A substance is considered nonimmunogenic if the intended use of the substance *in vivo* does not produce an undesired immune response (*e.g.*, the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as

evaluated by a clinician. Typically, the water-soluble polymer is hydrophilic, biocompatible and nonimmunogenic.

**[00112]** Further the water-soluble polymer is typically characterized as having from 2 to about 300 termini, preferably from 2 to 100 termini, and more preferably from about 2 to 50 termini. Examples of such polymers include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol (PEG), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), and combinations of any of the foregoing, including copolymers and terpolymers thereof.

**[00113]** The water-soluble polymer is not limited to a particular structure and may possess a linear architecture (*e.g.*, alkoxy PEG or bifunctional PEG), or a non-linear architecture, such as branched, forked, multi-armed (*e.g.*, PEGs attached to a polyol core), or dendritic (*i.e.* having a densely branched structure with numerous end groups). Moreover, the polymer subunits can be organized in any number of different patterns and can be selected, *e.g.*, from homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer.

**[00114]** One particularly preferred type of water-soluble polymer is a polyalkylene oxide, and in particular, polyethylene glycol (or PEG). Generally, a PEG used to prepare a CD-NP peptide polymer conjugate of the invention is "activated" or reactive. That is to say, the activated PEG (and other activated water-soluble polymers collectively referred to herein as "polymeric reagents") used to form a CD-NP peptide conjugate comprises an activated functional group suitable for coupling to a desired site or sites on the CD-NP peptide. Thus, a polymeric reagent for use in preparing a CD-NP peptide conjugate includes a functional group for reaction with the CD-NP peptide.

**[00115]** Representative polymeric reagents and methods for conjugating such polymers to an active moiety are known in the art, and are, *e.g.*, described in Harris, J.M. and Zalipsky, S., Eds, *Poly(ethylene glycol), Chemistry and Biological Applications*, ACS, Washington, 1997; Veronese, F., and J.M Harris, eds., *Peptide and Protein PEGylation*, Advanced Drug Delivery Reviews, 54(4); 453-609 (2002); Zalipsky, S., *et al.*, "*Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides*" in *Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications*, J. M. Harris, ed., Plenus Press, New York (1992);

Zalipsky (1995) *Advanced Drug Reviews*16:157-182, and in Roberts, *et al., Adv. Drug Delivery Reviews,* 54, 459-476 (2002).

**[00116]** Additional PEG reagents suitable for use in forming a conjugate of the invention, and methods of conjugation are described in the Pasut. G., et al., *Expert Opin. Ther. Patents* (2004), 14(5). PEG reagents suitable for use in the present invention also include those available from NOF Corporation, as described generally on the NOF website (http://nofamerica.net/store/). Products listed therein and their chemical structures are expressly incorporated herein by reference. Additional PEGs for use in forming a CD-NP peptide conjugate of the invention include those available from Polypure (Norway) and from QuantaBioDesign LTD (Ohio), where the contents of their catalogs with respect to available PEG reagents are expressly incorporated herein by reference. In addition, water soluble polymer reagents useful for preparing peptide conjugates of the invention can be prepared synthetically. Descriptions of the water soluble polymer reagent synthesis can be found in, for example, U.S. Patent Nos. 5,252,714, 5,650,234, 5,739,208, 5,932,462, 5,629,384, 5,672,662, 5,990,237, 6,448,369, 6,362,254, 6,495,659, 6,413,507, 6,376,604, 6,348,558, 6,602,498, and 7,026,440.

**[00117]** Typically, the weight-average molecular weight of the water-soluble polymer in the conjugate is from about 100 Daltons to about 150,000 Daltons. Exemplary ranges include weight-average molecular weights in the range of from about 250 Daltons to about 80,000 Daltons, from 500 Daltons to about 80,000 Daltons, from about 500 Daltons to about 65,000 Daltons, from about 500 Daltons to about 40,000 Daltons, from about 750 Daltons to about 40,000 Daltons, from about 1000 Daltons to about 30,000 Daltons. In a preferred embodiment, the weight average molecular weight of the water-soluble polymer in the conjugate ranges from about 1000 Daltons to about 10,000 Daltons. In certain other preferred embodiments, the range is from about 1000 Daltons to about 5000 Daltons, from about 5000 Daltons to about 10,000 Daltons, from about 2500 Daltons to about 7500 Daltons, from about 1000 Daltons to about 3000 Daltons, from about 3000 Daltons to about 7000 Daltons, or from about 7000 Daltons to about 10,000 Daltons. In a further preferred embodiment, the weight average molecular weight of the water-soluble polymer in the conjugate ranges from about 20,000 Daltons to about 40,000 Daltons. In other preferred embodiments, the range is from about 20,000 Daltons to about 30,000 Daltons, from about 30,000 Daltons to about 40,000 Daltons, from about 25,000 Daltons to about 35,000 Daltons, from about 20,000 Daltons to about 26,000 Daltons, from about 26,000 Daltons to about 34,000 Daltons, or from about 34,000 Daltons to about 40,000 Daltons.

**[00118]** For any given water-soluble polymer, a molecular weight in one or more of these ranges is typical. Generally, a CD-NP peptide conjugate in accordance with the invention, when intended for subcutaneous or intravenous administration, will comprise a PEG or other suitable water-soluble polymer having a weight average molecular weight of about 20,000 Daltons or greater, while a CD-NP peptide conjugate intended for pulmonary administration will generally, although not necessarily, comprise a PEG polymer having a weight average molecular weight of about 20,000 Daltons or less.

**[00119]** Exemplary weight-average molecular weights for the water-soluble polymer include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1,000 Daltons, about 1,500 Daltons, about 2,000 Daltons, about 2,200 Daltons, about 2,500 Daltons, about 3,000 Daltons, about 4,000 Daltons, about 4,400 Daltons, about 4,500 Daltons, about 5,000 Daltons, about 5,500 Daltons, about 6,000 Daltons, about 7,000 Daltons, about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 35,000 Daltons, about 40,000 Daltons, about 45,000 Daltons, about 50,000 Daltons, about 55,000 Daltons, about 60,000 Daltons, about 65,000 Daltons, about 70,000 Daltons, and about 75,000 Daltons.

**[00120]** Branched versions of the water-soluble polymer (*e.g.*, a branched 40,000 Dalton water-soluble polymer comprised of two 20,000 Dalton polymers or the like) having a total molecular weight of any of the foregoing can also be used. In one or more particular embodiments, depending upon the other features of the subject CD-NP peptide polymer conjugate, the conjugate is one that does not have one or more attached PEG moieties having a weight-average molecular weight of less than about 6,000 Daltons.

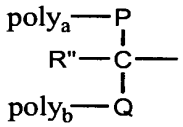
**[00121]** In instances in which the water-soluble polymer is a PEG, the PEG will typically comprise a number of ($OCH_2CH_2$) monomers. As used herein, the number of repeat units is typically identified by the subscript "*n*" in, for example, "$(OCH_2CH_2)_n$." Thus, the value of (n) typically falls within one or more of the following ranges: from 2 to about 3400, from about 100 to about 2300, from about 100 to about 2270, from about 136 to about 2050, from about 225 to about 1930, from about 450 to about 1930, from about 1200 to about 1930, from about 568 to about 2727, from about 660 to about 2730, from about 795 to about 2730, from about 795 to about 2730, from about 909 to about 2730, and from about 1,200 to about 1,900. Preferred ranges of n include from about 10 to about 700, and from about 10 to about

1800. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (i.e., "n") by dividing the total weight-average molecular weight of the polymer by the molecular weight of the repeating monomer.

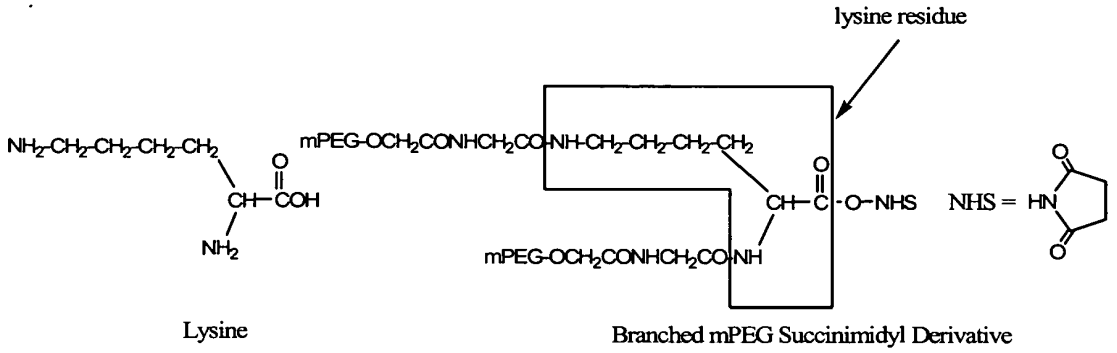
**[00122]** With regard to the molecular weight of the water-soluble polymer, in one or more embodiments of the invention, depending upon the other features of the particular CD-NP peptide conjugate, the conjugate comprises a CD-NP peptide covalently attached to a water-soluble polymer having a molecular weight greater than about 2,000 Daltons.

**[00123]** A polymer for use in the invention may be end-capped, that is, a polymer having at least one terminus capped with a relatively inert group, such as a lower alkoxy group (*i.e.*, a $C_{1-6}$ alkoxy group) or a hydroxyl group. One frequently employed end-capped polymer is methoxy-PEG (commonly referred to as mPEG), wherein one terminus of the polymer is a methoxy ($-OCH_3$) group. The -PEG- symbol used in the foregoing generally represents the following structural unit: $-CH_2CH_2O-(CH_2CH_2O)_n-CH_2CH_2-$, where (n) generally ranges from about zero to about 4,000.

**[00124]** Multi-armed or branched PEG molecules, such as those described in U.S. Patent No. 5,932,462, are also suitable for use in the present invention. For example, the PEG may be described generally according to the structure:

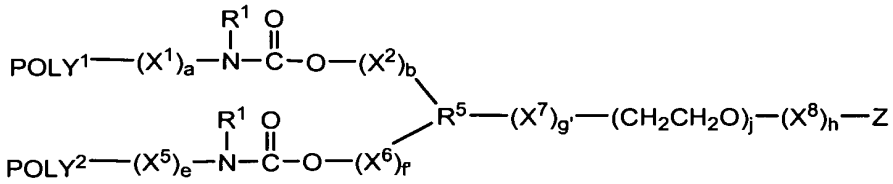



where $poly_a$ and $poly_b$ are PEG backbones (either the same or different), such as methoxy poly(ethylene glycol); R" is a non-reactive moiety, such as H, methyl or a PEG backbone; and P and Q are non-reactive linkages. In one embodiment, the branched PEG molecule is one that includes a lysine residue, such as the following reactive PEG suitable for use in forming a CD-NP peptide conjugate. Although the branched PEG below is shown with a reactive succinimidyl group, this represents only one of a myriad of reactive functional groups suitable for reacting with a CD-NP peptide.




**[00125]** In some instances, the polymeric reagent (as well as the corresponding conjugate prepared from the polymeric reagent) may lack a lysine residue in which the polymeric portions are connected to amine groups of the lysine via a "-$OCH_2CONHCH_2CO$-" group. In still other instances, the polymeric reagent (as well as the corresponding conjugate prepared from the polymeric reagent) may lack a branched water-soluble polymer that includes a lysine residue (wherein the lysine residue is used to effect branching).

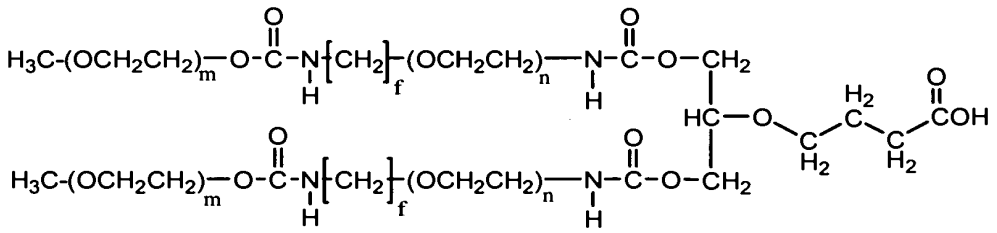
**[00126]** Additional branched-PEGs for use in forming a CD-NP peptide conjugate of the present invention include those described in co-owned U.S. Patent Application Publication No. 2005/0009988. Representative branched polymers described therein include those having the following generalized structure:




where $POLY^1$ is a water-soluble polymer; $POLY^2$ is a water-soluble polymer; (a) is 0, 1, 2 or 3; (b) is 0, 1, 2 or 3; (e) is 0, 1, 2 or 3; (f') is 0, 1, 2 or 3; (g') is 0, 1, 2 or 3; (h) is 0, 1, 2 or 3; (j) is 0 to 20; each $R^1$ is independently H or an organic radical selected from alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl and substituted aryl; $X^1$, when present, is a spacer moiety; $X^2$, when present, is a spacer moiety; $X^5$, when present, is a spacer moiety; $X^6$, when present, is a spacer moiety; $X^7$, when present, is a spacer moiety; $X^8$, when present, is a spacer moiety; $R^5$ is a branching moiety; and Z is a reactive group for coupling to a CD-NP peptide, optionally via an intervening spacer. $POLY^1$

and $POLY^2$ in the preceding branched polymer structure may be different or identical, *i.e.*, are of the same polymer type (structure) and molecular weight.

**[00127]** A preferred branched polymer falling into the above classification suitable for use in the present invention is:



where (m) is 2 to 4000, and (f) is 0 to 6 and (n) is 0 to 20.

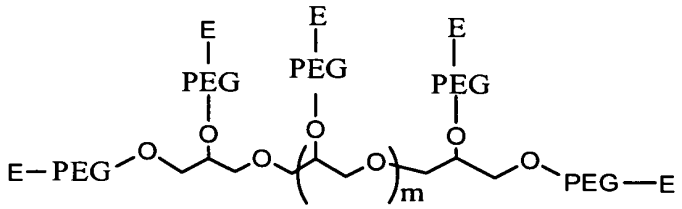
**[00128]** Branched polymers suitable for preparing a conjugate of the invention also include those represented more generally by the formula $R(POLY)_y$, where R is a central or core molecule from which extends 2 or more POLY arms such as PEG. The variable y represents the number of POLY arms, where each of the polymer arms can independently be end-capped or alternatively, possess a reactive functional group at its terminus. A more explicit structure in accordance with this embodiment of the invention possesses the structure, $R(POLY\text{-}Z)_y$, where each Z is independently an end-capping group or a reactive group, *e.g.*, suitable for reaction with a CD-NP peptide. In yet a further embodiment when Z is a reactive group, upon reaction with a CD-NP peptide, the resulting linkage can be hydrolytically stable, or alternatively, may be degradable, *i.e.*, hydrolyzable. Typically, at least one polymer arm possesses a terminal functional group suitable for reaction with, *e.g.*, a CD-NP peptide. Branched PEGs such as those represented generally by the formula, $R(PEG)_y$ above possess 2 polymer arms to about 300 polymer arms (*i.e.*, n ranges from 2 to about 300). Preferably, such branched PEGs typically possess from 2 to about 25 polymer arms, such as from 2 to about 20 polymer arms, from 2 to about 15 polymer arms, or from 3 to about 15 polymer arms. Multi-armed polymers include those having 3, 4, 5, 6, 7 or 8 arms.

**[00129]** Core molecules in branched PEGs as described above include polyols, which are then further functionalized. Such polyols include aliphatic polyols having from 1 to 10 carbon atoms and from 1 to 10 hydroxyl groups, including ethylene glycol, alkane diols, alkyl glycols, alkylidene alkyl diols, alkyl cycloalkane diols, 1,5-decalindiol, 4,8-bis(hydroxymethyl)tricyclodecane, cycloalkylidene diols, dihydroxyalkanes,

trihydroxyalkanes, and the like. Cycloaliphatic polyols may also be employed, including straight chained or closed-ring sugars and sugar alcohols, such as mannitol, sorbitol, inositol, xylitol, quebrachitol, threitol, arabitol, erythritol, adonitol, ducitol, facose, ribose, arabinose, xylose, lyxose, rhamnose, galactose, glucose, fructose, sorbose, mannose, pyranose, altrose, talose, tagitose, pyranosides, sucrose, lactose, maltose, and the like. Additional aliphatic polyols include derivatives of glyceraldehyde, glucose, ribose, mannose, galactose, and related stereoisomers. Other core polyols that may be used include crown ether, cyclodextrins, dextrins and other carbohydrates such as starches and amylose. Typical polyols include glycerol, pentaerythritol, sorbitol, and trimethylolpropane.

**[00130]** As will be described in more detail in the linker section below, although any of a number of linkages can be used to covalently attach a polymer to a CD-NP peptide, in certain instances, the linkage is degradable, designated herein as $L_D$, that is to say, contains at least one bond or moiety that hydrolyzes under physiological conditions, e.g., an ester, hydrolyzable carbamate, carbonate, or other such group. In other instances, the linkage is hydrolytically stable.

**[00131]** Illustrative multi-armed PEGs having 3 arms, 4 arms, and 8 arms are known and are available commercially and/or can be prepared following techniques known to those skilled in the art. Multi-armed activated polymers for use in the method of the invention include those corresponding to the following structure, where E represents a reactive group suitable for reaction with a reactive group on the CD-NP peptide. In one or more embodiments, E is an -OH (for reaction with a CD-NP peptide carboxy group or equivalent), a carboxylic acid or equivalaent (such as an active ester), a carbonic acid (for reaction with CD-NP peptide -OH groups), or an amino group.




**[00132]** In the structure above, PEG is $-(CH_2CH_2O)_nCH_2CH_2-$, and m is selected from 3, 4, 5, 6, 7, and 8. In certain embodiments, typical linkages are ester, carboxyl and hydrolyzable carbamate, such that the polymer-portion of the conjugate is hydrolyzed *in vivo*

to release the CD-NP peptide from the intact polymer conjugate. In such instances, the linker L is designated as $L_D$.

**[00133]** Alternatively, the polymer may possess an overall forked structure as described in U.S. Patent No. 6,362,254. This type of polymer segment is useful for reaction with two CD-NP peptide moieties, where the two CD-NP peptide moieties are positioned a precise or predetermined distance apart.

**[00134]** In any of the representative structures provided herein, one or more degradable linkages may additionally be contained in the polymer segment, POLY, to allow generation *in vivo* of a conjugate having a smaller PEG chain than in the initially administered conjugate. Appropriate physiologically cleavable (*i.e.*, releasable) linkages include but are not limited to ester, carbonate ester, carbamate, sulfate, phosphate, acyloxyalkyl ether, acetal, and ketal. Such linkages when contained in a given polymer segment will often be stable upon storage and upon initial administration.

**[00135]** The PEG polymer used to prepare a CD-NP peptide polymer conjugate may comprise a pendant PEG molecule having reactive groups, such as carboxyl or amino, covalently attached along the length of the PEG rather than at the end of the PEG chain(s). The pendant reactive groups can be attached to the PEG directly or through a spacer moiety, such as an alkylene group.

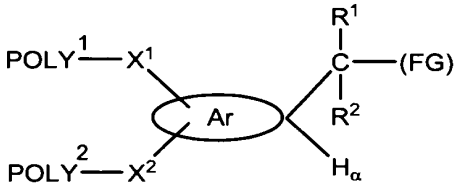
**[00136]** In certain embodiments, a CD-NP peptide polymer conjugate according to one aspect of the invention is one comprising a CD-NP peptide releasably attached, preferably at its N-terminus, to a water-soluble polymer. Hydrolytically degradable linkages, useful not only as a degradable linkage within a polymer backbone, but also, in the case of certain embodiments of the invention, for covalently attaching a water-soluble polymer to a CD-NP peptide, include: carbonate; imine resulting, for example, from reaction of an amine and an aldehyde (see, *e.g.*, Ouchi *et al.* (1997) *Polymer Preprints* 38(1):582-3); phosphate ester, formed, for example, by reacting an alcohol with a phosphate group; hydrazone, *e.g.*, formed by reaction of a hydrazide and an aldehyde; acetal, *e.g.*, formed by reaction of an aldehyde and an alcohol; orthoester, formed, for example, by reaction between a formate and an alcohol; and esters, and certain urethane (carbamate) linkages.

**[00137]** Illustrative PEG reagents for use in preparing a releasable CD-NP peptide conjugate in accordance with the invention are described in U.S. Patent Nos. 6,348,558, 5,612,460, 5,840,900, 5,880,131, and 6,376,470.

**[00138]** Additional PEG reagents for use in the invention include hydrolyzable and/or releasable PEGs and linkers such as those described in U.S. Patent Application Publication

No. 2006-0293499. In the resulting conjugate, the CD-NP peptide and the polymer are each covalently attached to different positions of the aromatic scaffold, *e.g.*, Fmoc or FMS structure, and are releasable under physiological conditions. Generalized structures corresponding to the polymers described therein are provided below.

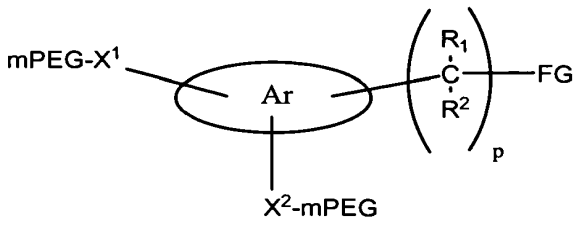
**[00139]** For example, one such polymeric reagent comprises the following structure:




where $POLY^1$ is a first water-soluble polymer; $POLY^2$ is a second water-soluble polymer; $X^1$ is a first spacer moiety; $X^2$ is a second spacer moiety; is an aromatic-containing moiety bearing an ionizable hydrogen atom, $H_\alpha$; $R^1$ is H or an organic radical; $R^2$ is H or an organic radical; and (FG) is a functional group capable of reacting with an amino group of an active agent to form a releasable linkage, such as a carbamate linkage (such as N-succinimidyloxy, 1-benzotriazolyloxy, oxycarbonylimidazole, -O-C(O)-Cl, O-C(O)-Br, unsubstituted aromatic carbonate radicals and substituted aromatic carbonate radicals). The polymeric reagent can include one, two, three, four or more electron altering groups attached to the aromatic-containing moiety.

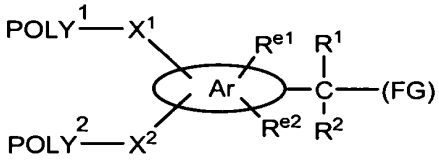
**[00140]** Preferred aromatic-containing moieties are bicyclic and tricyclic aromatic hydrocarbons. Fused bicyclic and tricyclic aromatics include pentalene, indene, naphthalene, azulene, heptalene, biphenylene, as-indacene, s-indacene, acenaphthylene, fluorene, phenalene, phenanthrene, anthracene, and fluoranthene.

**[00141]** A preferred polymer reagent possesses the following structure,



where mPEG corresponds to $CH_3O$-$(CH_2CH_2O)_nCH_2CH_2$-, $X^1$ and $X^2$ are each independently a spacer moiety having an atom length of from about 1 to about 18 atoms, n ranges from 10 to 1800, p is an integer ranging from 1 to 8, $R^1$ is H or lower alkyl, $R^2$ is H or lower alkyl, and Ar is an aromatic hydrodrocarbon, preferably a bicyclic or tricyclic aromatic hydrocarbon. FG is as defined above. Preferably, FG corresponds to an activated carbonate ester suitable for reaction with an amino group on CD-NP peptide. Preferred spacer moieties, $X^1$ and $X^2$, include –NH-C(O)-$CH_2$-O-, –NH-C(O)-$(CH_2)_q$-O-, –NH-C(O)-$(CH_2)_q$-C(O)-NH-, -NH-C(O)-$(CH_2)_q$-, and –C(O)-NH-, where q is selected from 2, 3, 4, and 5. Preferably, although not necessarily, the nitrogen in the preceding spacers is proximal to the PEG rather than to the aromatic moiety.

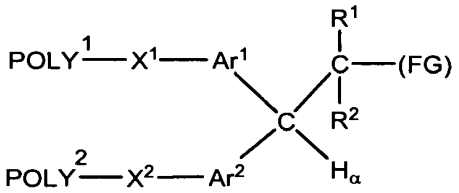
**[00142]** Another such branched (2-armed) polymeric reagent comprised of two electron altering groups comprises the following structure:




wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $R^1$, $R^2$, , and (FG) is as defined immediately above, and $R^{e1}$ is a first electron altering group; and $R^{e2}$ is a second electron altering group. An electron altering group is a group that is either electron donating (and therefore referred to as an "electron donating group"), or electron withdrawing (and therefore referred to as an "electron withdrawing group"). When attached to the aromatic-containing moiety bearing an ionizable hydrogen atom, an electron donating group is a group having the ability to position electrons away from itself and closer to or within the aromatic-containing moiety. When attached to the aromatic-containing moiety bearing an ionizable hydrogen atom, an electron withdrawing group is a group having the ability to position electrons toward itself and away from the aromatic-containing moiety. Hydrogen is used as the standard for comparison in the determination of whether a given group positions electrons away or toward itself. Preferred electron altering groups include, but are not limited to, -$CF_3$, -$CH_2CF_3$, -$CH_2C_6F_5$, -CN, -$NO_2$, -S(O)R, -S(O)Aryl, -$S(O_2)$R, -$S(O_2)$Aryl, -$S(O_2)$OR, -

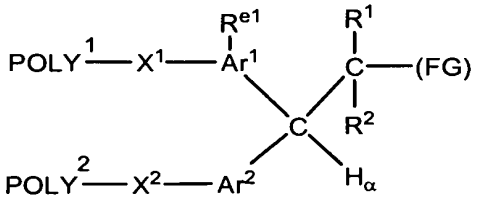
$S(O_2)OAryl$, , $-S(O_2)NHR$, $-S(O_2)NHAryl$, -C(O)R, -C(O)Aryl, -C(O)OR, -C(O)NHR, and the like, wherein R is H or an organic radical.

**[00143]** An additional branched polymeric reagent suitable for use in the present invention comprises the following structure:



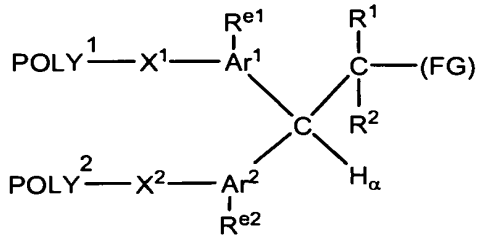

where $POLY^1$ is a first water-soluble polymer; $POLY^2$ is a second water-soluble polymer; $X^1$ is a first spacer moiety; $X^2$ is a second spacer moiety; $Ar^1$ is a first aromatic moiety; $Ar^2$ is a second aromatic moiety; $H_\alpha$ is an ionizable hydrogen atom; $R^1$ is H or an organic radical; $R^2$ is H or an organic radical; and (FG) is a functional group capable of reacting with an amino group of CD-NP peptide to form a releasable linkage, such as carbamate linkage.

**[00144]** Another exemplary polymeric reagent comprises the following structure:



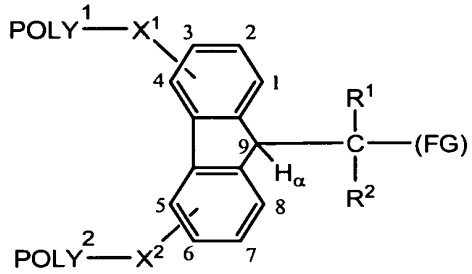

wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $Ar^1$, $Ar^2$, $H_\alpha$, $R^1$, $R^2$, and (FG) is as previously defined, and $R^{e1}$ is a first electron altering group. While stereochemistry is not specifically shown in any structure provided herein, the provided structures contemplate both enantiomers, as well as compositions comprising mixtures of each enantiomer in equal amounts (*i.e.*, a racemic mixture) and unequal amounts.

**[00145]** Yet an additional polymeric reagent for use in preparing a CD-NP peptide conjugate possesses the following structure:



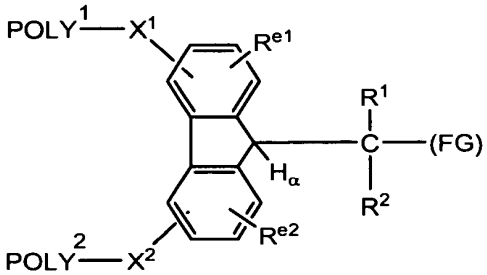
wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $Ar^1$, $Ar^2$, $H_\alpha$, $R^1$, $R^2$, and (FG) is as previously defined, and $R^{e1}$ is a first electron altering group; and $R^{e2}$ is a second electron altering group.

**[00146]** A preferred polymeric reagent comprises the following structure:



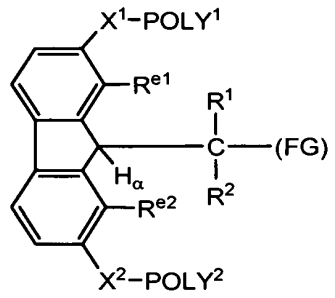
wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $R^1$, $R^2$, $H_\alpha$ and (FG) is as previously defined, and, as can be seen from the structure above, the aromatic moiety is a fluorene. The POLY arms substituted on the fluorene can be in any position in each of their respective phenyl rings, *i.e.*, $POLY^1$-$X^1$- can be positioned at any one of carbons 1, 2, 3, and 4, and $POLY^2$-$X^2$- can be in any one of positions 5, 6, 7, and 8.

**[00147]** Yet another preferred fluorene-based polymeric reagent comprises the following structure:



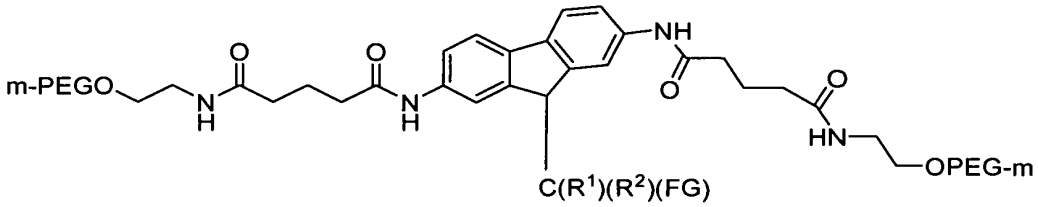
wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $R^1$, $R^2$, $H_\alpha$ and (FG) is as previously defined, and $R^{e1}$ is a first electron altering group; and $R^{e2}$ is a second electron altering group as described above.

**[00148]** Yet another exemplary polymeric reagent for conjugating to a CD-NP peptide comprises the following fluorene-based structure:




wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $R^1$, $R^2$, $H_\alpha$ and (FG) is as previously defined, and $R^{e1}$ is a first electron altering group; and $R^{e2}$ is a second electron altering group.

**[00149]** Particular fluorene-based polymeric reagents for forming a releasable CD-NP peptide polymer conjugate in accordance with the invention include the following:

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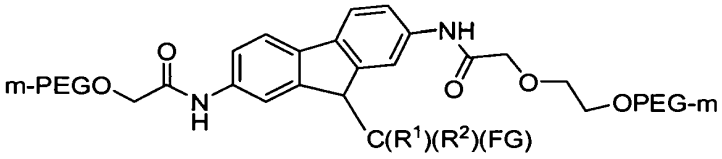
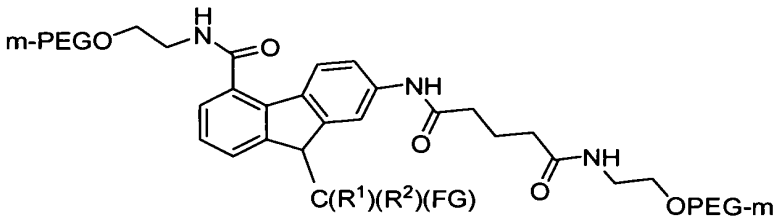


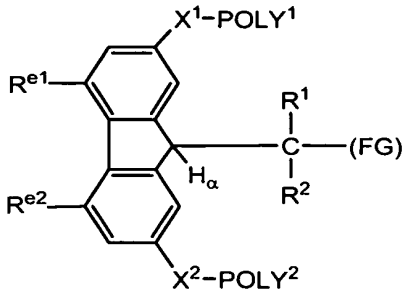

,

and




.

**[00150]** Still another exemplary polymeric reagent comprises the following structure:




wherein each of $POLY^1$, $POLY^2$, $X^1$, $X^2$, $R^1$, $R^2$, $H_\alpha$ and (FG) is as previously defined, and $R^{e1}$ is a first electron altering group; and $R^{e2}$ is a second electron altering group. Branched reagents suitable for preparing a releasable CD-NP peptide conjugate include N-{di(mPEG(20,000)oxymethylcarbonylamino)fluoren-9-ylmethoxycarbonyloxy}succinimide, N-[2,7 di(4mPEG(10,000)aminocarbonylbutyrylamino)fluoren-9 ylmethoxycarbonyloxy]-succinimide ("G2PEG2Fmoc$_{20k}$-NHS"), and PEG2-CAC-Fmoc$_{4k}$-BTC. Of course, PEGs of any molecular weight as set forth herein may be employed in the above structures, and the particular activating groups described above are not meant to be limiting in any respect, and may be substituted by any other suitable activating group suitable for reaction with a reactive group present on the CD-NP peptide.

**[00151]** Those of ordinary skill in the art will recognize that the foregoing discussion describing water-soluble polymers for use in forming a CD-NP peptide conjugate is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated. As used herein, the term "polymeric reagent" generally refers to an entire molecule, which can comprise a water-soluble polymer segment, as well as additional spacers and functional groups.

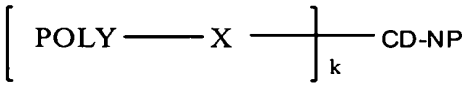
**The Linkage**

**[00152]** The particular linkage between the CD-NP peptide and the water-soluble polymer depends on a number of factors. Such factors include, for example, the particular linkage chemistry employed, the particular spacer moieties utilized, if any, the particular CD-NP peptide, the available functional groups within the CD-NP peptide (either for attachment to a polymer or conversion to a suitable attachment site), and the possible presence of additional reactive functional groups or absence of functional groups within the CD-NP

peptide due to modifications made to the peptide such as methylation and/or glycosylation, and the like.

**[00153]** In one or more embodiments of the invention, the linkage between the CD-NP peptide and the water-soluble polymer is a releasable linkage. That is, the water-soluble polymer is cleaved (either through hydrolysis, an enzymatic processes, or otherwise), thereby resulting in an unconjugated CD-NP peptide. Preferably, the releasable linkage is a hydrolytically degradable linkage, where upon hydrolysis, the CD-NP peptide, or a slightly modified version thereof, is released. The releasable linkage may result in the water-soluble polymer (and any spacer moiety) detaching from the CD-NP peptide *in vivo* (and *in vitro*) without leaving any fragment of the water-soluble polymer (and/or any spacer moiety or linker) attached to the CD-NP peptide. Exemplary releasable linkages include carbonate, carboxylate ester, phosphate ester, thiolester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, carbamates, and orthoesters. Such linkages can be readily formed by reaction of the CD-NP peptide and/or the polymeric reagent using coupling methods commonly employed in the art. Hydrolyzable linkages are often readily formed by reaction of a suitably activated polymer with a non-modified functional group contained within the CD-NP peptide. Preferred positions for covalent attachment of a water-soluble polymer incluce the N-terminal, the C-terminal, as well as the internal lysines. Preferred releasable linkages include carbamate and ester.

**[00154]** Generally speaking, a preferred CD-NP peptide conjugate of the invention will possess the following generalized structure:




where POLY is a water-soluble polymer such as any of the illustrative polymeric reagents provided in Tables 2-4 herein, X is a linker, and in some embodiments a hydrolyzable linkage ($L_D$), and k is an integer selected from 1, 2, and 3, and in some instances 4, 5, 6, 7, 8, 9 and 10. In the generalized structure above, where X is $L_D$, $L_D$ refers to the hydrolyzable linkage per se (*e.g.*, a carbamate or an ester linkage), while "POLY" is meant to include the polymer repeat units, e.g., $CH_3(OCH_2CH_2)_n$-. In a preferred embodiment of the invention, at least one of the water-soluble polymer molecules is covalently attached to the N-terminus of CD-NP peptide. In one embodiment of the invention, k equals 1 and X is –O-C(O)-NH-, where the –NH- is part of the CD-NP peptide residue and represents an amino group thereof.

**[00155]** Although releasable linkages are exemplary, the linkage between the CD-NP peptide and the water-soluble polymer (or the linker moiety that is attached to the polymer) may be a hydrolytically stable linkage, such as an amide, a urethane (also known as carbamate), amine, thioether (also known as sulfide), or urea (also known as carbamide). One such embodiment of the invention comprises a CD-NP peptide having a water-soluble polymer such as PEG covalently attached at the N-terminus of CD-NP peptide. In such instances, alkylation of the N-terminal residue permits retention of the charge on the N-terminal nitrogen.

**[00156]** With regard to linkages, in one or more embodiments of the invention, a conjugate is provided that comprises a CD-NP peptide covalently attached at an amino acid residue, either directly or through a linker comprised of one or more atoms, to a water-soluble polymer.

**[00157]** The conjugates (as opposed to an unconjugated CD-NP peptide) may or may not possess a measurable degree of CD-NP peptide activity. That is to say, a conjugate in accordance with the invention will typically possess anywhere from about 0% to about 100% or more of the CD-NP activity of the unmodified parent CD-NP peptide. Typically, compounds possessing little or no CD-NP activity contain a releasable linkage connecting the polymer to the CD-NP peptide, so that regardless of the lack of CD-NP activity in the conjugate, the active parent molecule (or a derivative thereof having CD-NP activity) is released by cleavage of the linkage (*e.g.*, hydrolysis upon aqueous-induced cleavage of the linkage). Such activity may be determined using a suitable *in vivo* or *in vitro* model, depending upon the known activity of the particular moiety having CD-NP peptide activity employed.

**[00158]** Optimally, cleavage of a linkage is facilitated through the use of hydrolytically cleavable and/or enzymatically cleavable linkages such as urethane, amide, certain carbamate, carbonate or ester-containing linkages. In this way, clearance of the conjugate via cleavage of individual water-soluble polymer(s) can be modulated by selecting the polymer molecular size and the type of functional group for providing the desired clearance properties. In certain instances, a mixture of polymer conjugates is employed where the polymers possess structural or other differences effective to alter the release (*e.g.*, hydrolysis rate) of the CD-NP peptide, such that one can achieve a desired sustained delivery profile.

**[00159]** One of ordinary skill in the art can determine the proper molecular size of the polymer as well as the cleavable functional group, depending upon several factors including the mode of administration. For example, one of ordinary skill in the art, using routine

experimentation, can determine a proper molecular size and cleavable functional group by first preparing a variety of polymer-CD-NP peptide conjugates with different weight-average molecular weights, degradable functional groups, and chemical structures, and then obtaining the clearance profile for each conjugate by administering the conjugate to a patient and taking periodic blood and/or urine samples. Once a series of clearance profiles has been obtained for each tested conjugate, a conjugate or mixture of conjugates having the desired clearance profile(s) can be determined.

**[00160]** For conjugates possessing a hydrolytically stable linkage that couples the CD-NP peptide to the water-soluble polymer, the conjugate will typically possess a measurable degree of CD-NP activity. For instance, such conjugates are typically characterized as having a CD-NP activity satisfying one or more of the following percentages relative to that of the unconjugated CD-NP peptide: at least 2%, at least 5%, at least 10%, at least 15%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 100%, more than 105%, more than 10-fold, or more than 100-fold (when measured in a suitable model, such as those presented here and/or known in the art). Often, conjugates having a hydrolytically stable linkage (*e.g.*, an amide linkage) will possess at least some degree of the CD-NP activity of the unmodified parent CD-NP peptide.

**[00161]** Exemplary conjugates in accordance with the invention will now be described. Amino groups on a CD-NP peptide provide a point of attachment between the CD-NP peptide and the water-soluble polymer. For example, a CD-NP peptide may comprise one or more lysine residues, each lysine residue containing an ε-amino group that may be available for conjugation, as well as the amino terminus.

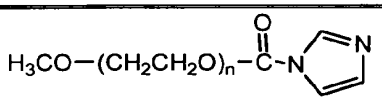
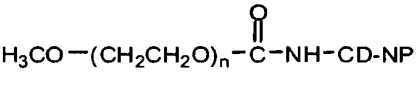
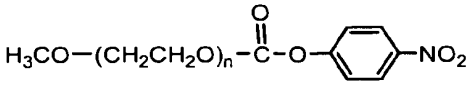
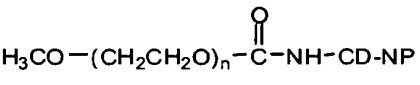
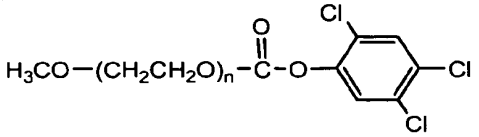
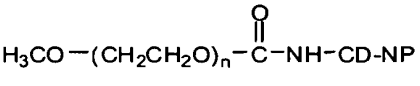
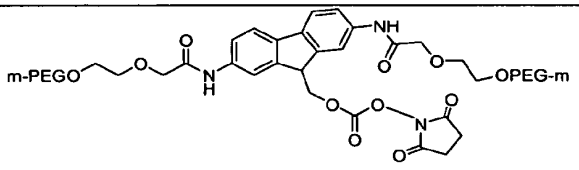
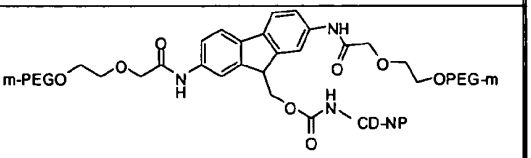
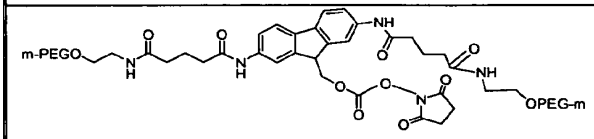
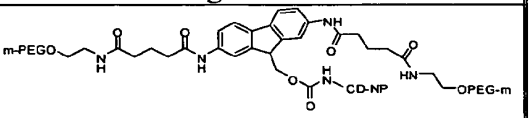
**[00162]** There are a number of examples of suitable water-soluble polymeric reagents useful for forming covalent linkages with available amines of a CD-NP peptide. Certain specific examples, along with the corresponding conjugates, are provided in Table 2 below. In the table, the variable (n) represents the number of repeating monomeric units and "CD-NP" represents a CD-NP peptide following conjugation to the water-soluble polymer. While each polymeric portion [*e.g.*, $(OCH_2CH_2)_n$ or $(CH_2CH_2O)_n$] presented in Table 2 terminates in a "$CH_3$" group, other groups (*e.g.*, H or benzyl) can be substituted therefore.

**[00163]** As will be clearly understood by one skilled in the art, for conjugates such as those set forth below resulting from reaction with a CD-NP peptide amino group, the amino group extending from the CD-NP peptide designation "~NH-CD-NP" represents the residue of the CD-NP peptide itself in which the ~NH- is an amino group of the CD-NP peptide. One

preferred site of attachment for the polymeric reagents shown below is the *N*-terminus. Further, although the conjugates in Tables 2-4 herein illustrate a single water-soluble polymer covalently attached to a CD-NP peptide, it will be understood that the conjugate structures on the right are meant to also encompass conjugates having more than one of such water-soluble polymer molecules covalently attached to CD-NP peptide, *e.g.*, 2, 3, or 4 water-soluble polymer molecules.

**Table 2**

Amine-Specific Polymeric Reagents and the CD-NP Peptide Conjugates Formed Therefrom

| | |
|---|---|
|  mPEG-Oxycarbonylimidazole Reagent |  Carbamate Linkage |
|  mPEG Nitrophenyl Reagent |  Carbamate Linkage |
|  mPEG-Trichlorophenyl Carbonate Reagent |  Carbamate Linkage |
|  Fmoc-NHS Reagent |  Carbamate Linkage |
|  Fmoc-NHS Reagent |  Carbamate Linkage |

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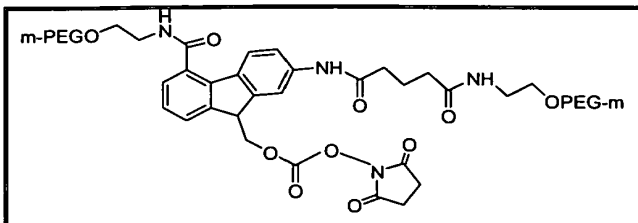
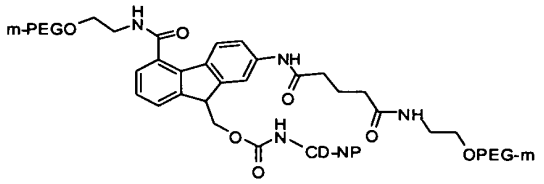
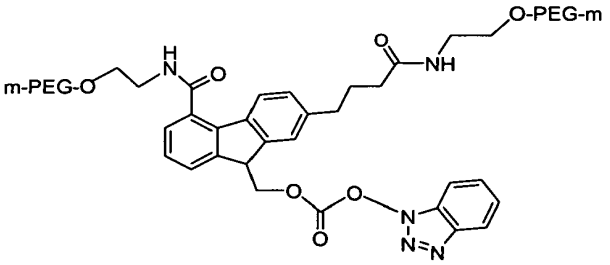
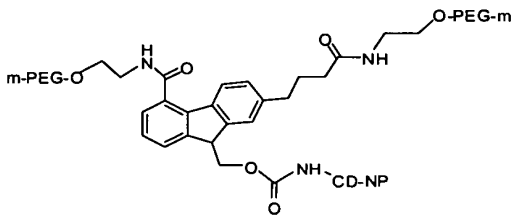
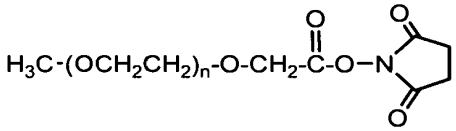
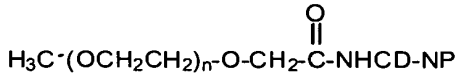
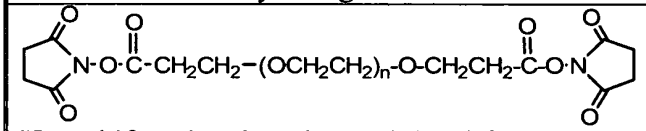
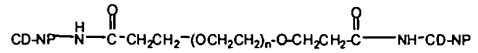
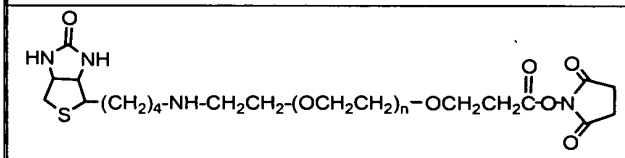
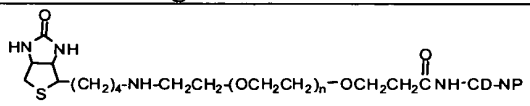
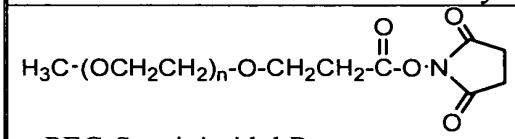
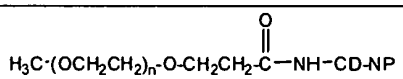
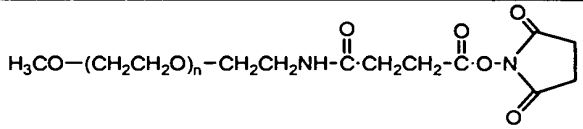
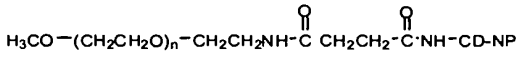
|  Fmoc-NHS Reagent |  Carbamate Linkage |
|  Fmoc-BTC Reagent |  Carbamate Linkage |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  Homobifunctional PEG-Succinimidyl Reagent |  Amide Linkages |
|  Heterobifunctional PEG-Succinimidyl Reagent |  Amide Linkage |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  mPEG-Succinimdyl Reagent |  Amide Linkage |

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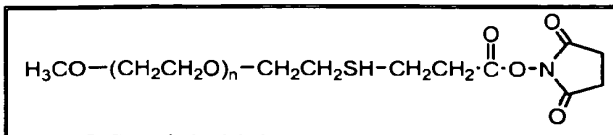
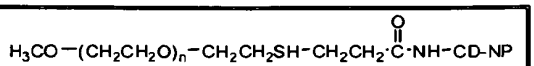
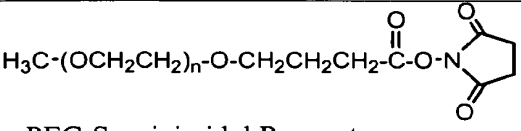
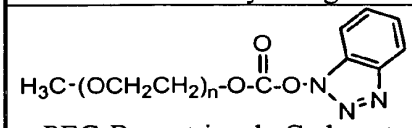
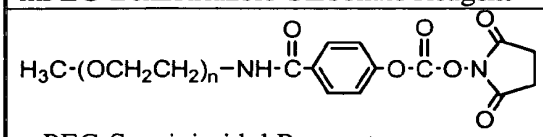
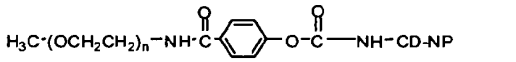
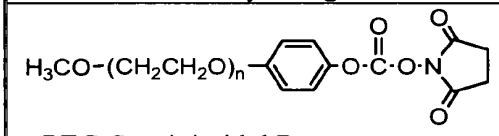
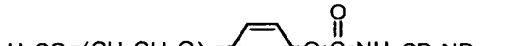
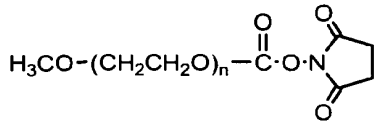
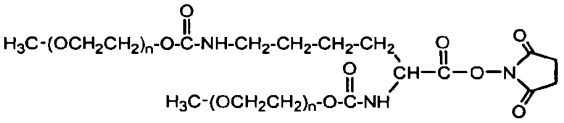
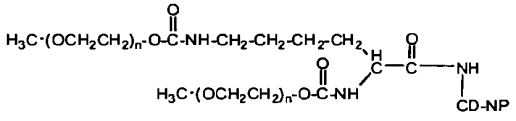
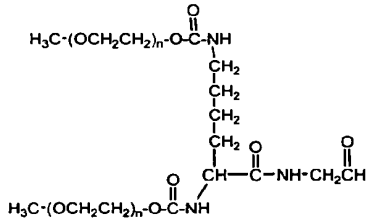
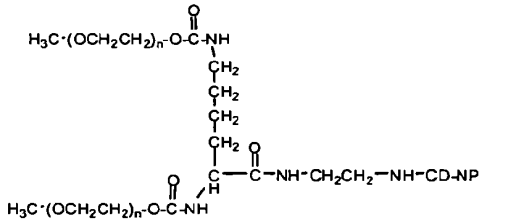
|  mPEG Succinimidyl Reagent |  Amide Linkage |
|  mPEG-Succinimidyl Reagent | Amide Linkage |
|  mPEG-Benzotriazole Carbonate Reagent | Carbamate Linkage |
|  mPEG-Succinimidyl Reagent |  Carbamate Linkage |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  mPEG Succinimidyl Reagent | Amide Linkage |
|  Branched mPEG2-N-Hydroxysuccinimide Reagent |  Amide Linkage |
|  Branched mPEG2-Aldehyde Reagent |  Secondary Amine Linkage |

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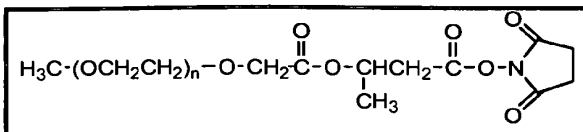
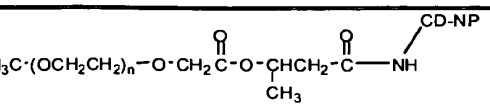
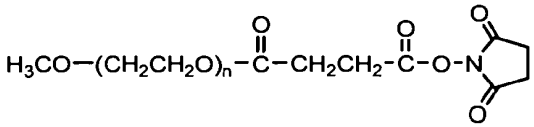
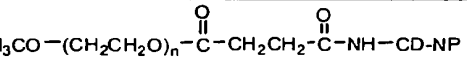
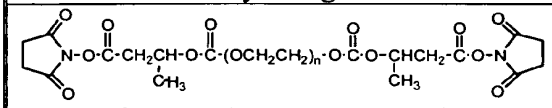
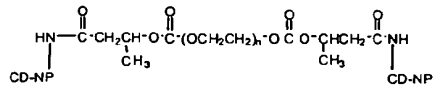
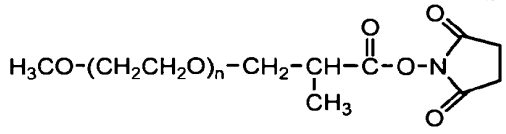
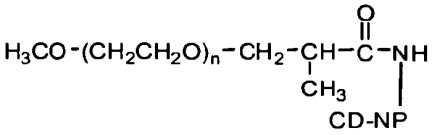
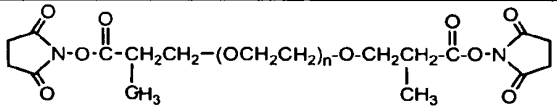
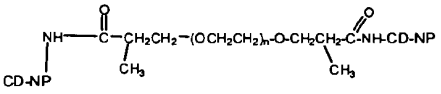
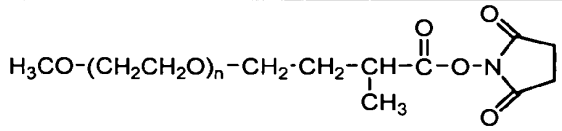
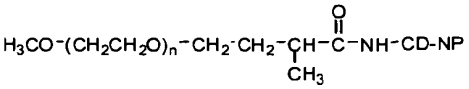
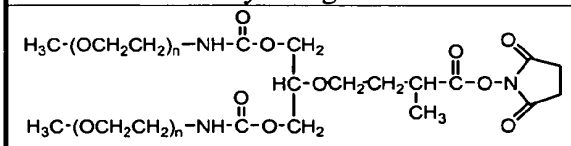
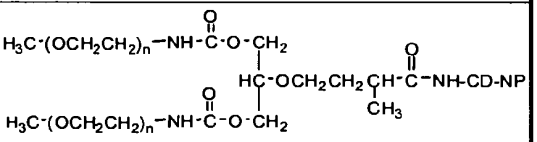
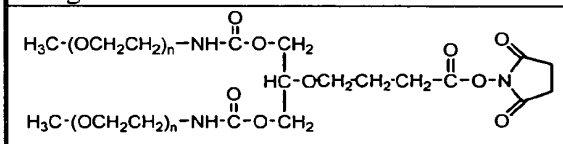
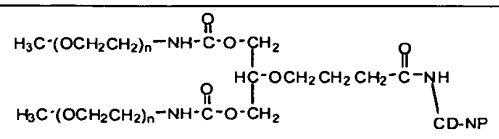
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  Homobifunctional PEG-Succinimidyl Reagent |  Amide Linkages |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  Homobifunctional PEG-Succinimidyl Propionate Reagent |  Amide Linkages |
|  mPEG-Succinimidyl Reagent |  Amide Linkage |
|  Branched mPEG2-N-Hydroxysuccinimide Reagent |  Amide Linkage |
|  Branched mPEG2-N-Hydroxysuccinimide Reagent |  Amide Linkage |

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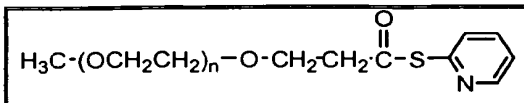
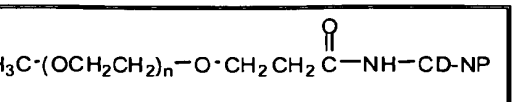
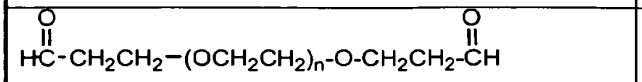
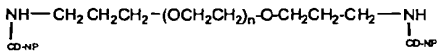
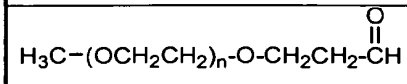
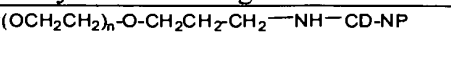
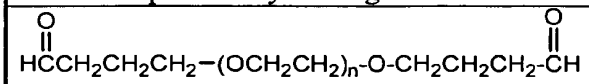
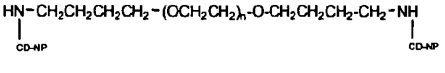
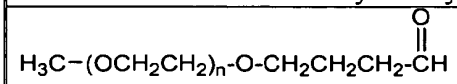
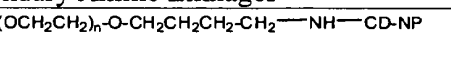
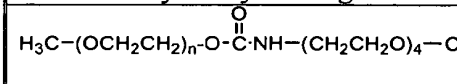
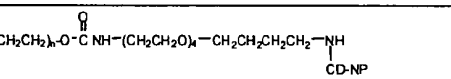
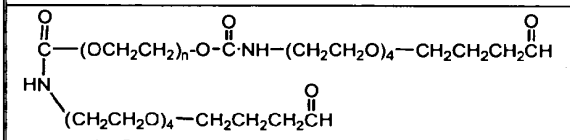
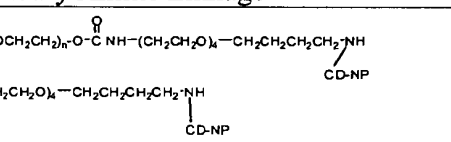
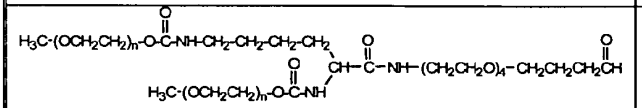
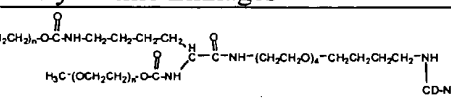
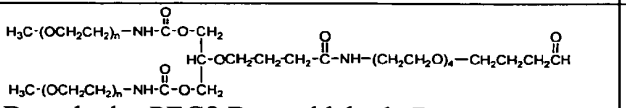
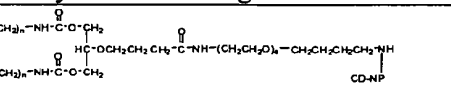
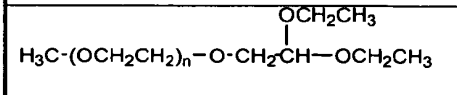
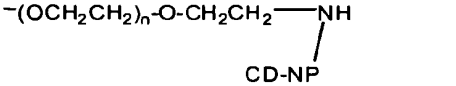
|  <br>mPEG-Thioester Reagent |  <br>Amide Linkage (typically to CD-NP moiety having an N-terminal cysteine or histidine) |
|  <br>Homobifunctional PEG Propionaldehyde Reagent |  <br>Secondary Amine Linkages |
|  <br>mPEG Propionaldehyde Reagent |  <br>Secondary Amine Linkage |
|  <br>Homobifunctional PEG Butyraldehyde Reagent |  <br>Secondary Amine Linkages |
|  <br>mPEG Butyraldehyde Reagent |  <br>Secondary Amine Linkage |
|  <br>mPEG Butyraldehyde Reagent |  <br>Secondary Amine Linkage |
|  <br>Homobifunctional PEG Butyraldehyde Reagent |  <br>Secondary Amine Linkages |
|  <br>Branched mPEG2 Butyraldehyde Reagent |  <br>Secondary Amine Linkage |
|  <br>Branched mPEG2 Butyraldehyde Reagent |  <br>Secondary Amine Linkage |
|  <br>mPEG Acetal Reagent |  <br>Secondary Amine Linkage |

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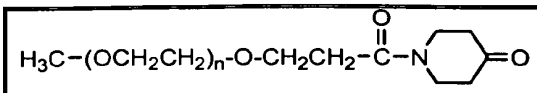
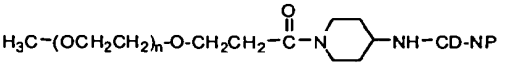
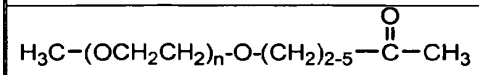
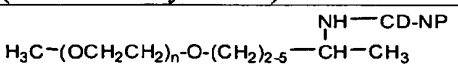
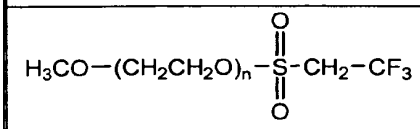
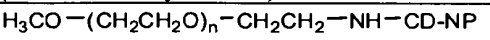
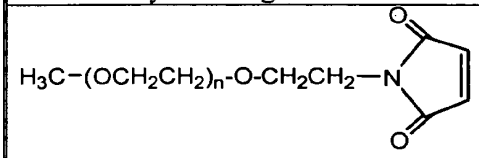
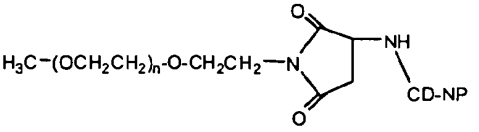
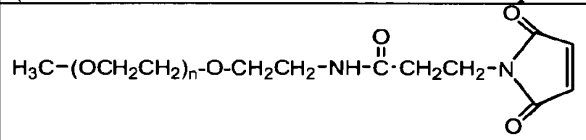
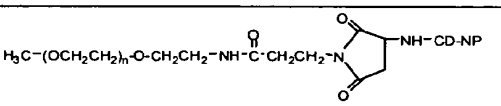
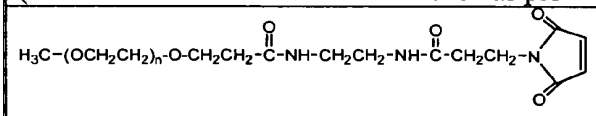
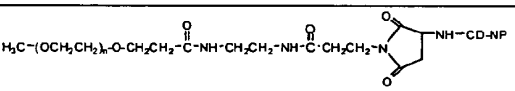
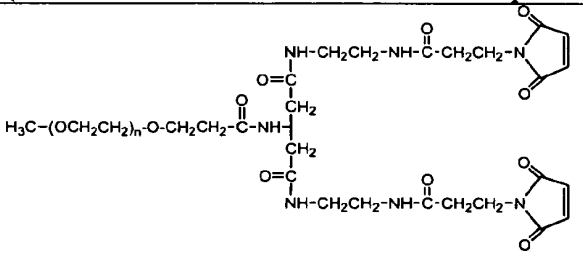
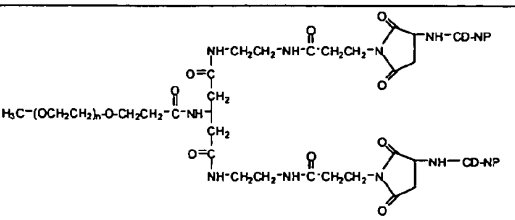
|  <br>mPEG Piperidone Reagent |  <br>Secondary Amine Linkage<br>(to a secondary carbon) |
|  <br>mPEG Methylketone Reagent |  <br>secondary amine linkage<br>(to a secondary carbon) |
|  <br>mPEG tresylate Reagent |  <br>Secondary Amine Linkage |
|  <br>mPEG Maleimide Reagent<br>(under certain reaction conditions such as pH > 8) |  <br>Secondary Amine Linkage |
|  <br>mPEG Maleimide Reagent<br>(under certain reaction conditions such as pH > 8) |  <br>Secondary Amine Linkage |
|  <br>mPEG Maleimide Reagent<br>(under certain reaction conditions such as pH > 8) |  <br>Secondary Amine Linkage |
|  <br>mPEG Forked Maleimide Reagent<br>(under certain reaction conditions such as pH > 8) |  <br>Secondary Amine Linkages |

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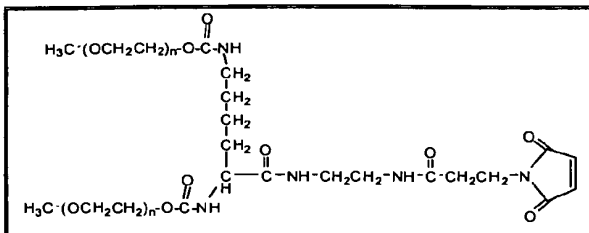
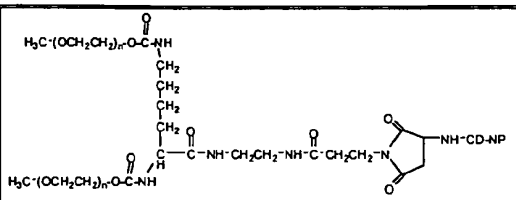
|  branched mPEG2 Maleimide Reagent (under certain reaction conditions such as pH > 8) |  Secondary Amine Linkage |

Amine Conjugation and Resulting Conjugates

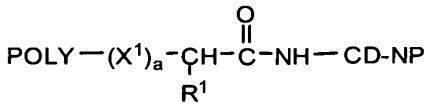
**[00164]** Conjugation of a polymeric reagent to an amine group of a CD-NP peptide can be accomplished by a variety of techniques. In one approach, a CD-NP peptide is conjugated to a polymeric reagent functionalized with an active ester such as a succinimidyl derivative (*e.g.*, an N-hydroxysuccinimide ester). In this approach, the polymeric reagent bearing the reactive ester is reacted with the CD-NP peptide in aqueous media under appropriate pH conditions, *e.g.*, from pHs ranging from about 3 to about 8, about 3 to about 7, or about 4 to about 6.5. Most polymer active esters can couple to a target peptide such as CD-NP peptide at physiological pH, *e.g.*, at 7.0. However, less reactive derivatives may require a different pH. Typically, activated PEGs can be attached to a peptide such as CD-NP peptide at pHs from about 7.0 to about 10.0 for covalent attachment to an internal lysine. Typically, lower pHs are used, *e.g.*, 4 to about 5.75, for preferential covalent attachment to the N-terminus. Thus, different reaction conditions (*e.g.*, different pHs or different temperatures) can result in the attachment of a water-soluble polymer such as PEG to different locations on the CD-NP peptide (*e.g.*, internal lysines versus the N-terminus). Coupling reactions can often be carried out at room temperature, although lower temperatures may be required for particularly labile CD-NP peptide moieties. Reaction times are typically on the order of minutes, *e.g.*, 30 minutes, to hours, *e.g.*, from about 1 to about 36 hours), depending upon the pH and temperature of the reaction. N-terminal PEGylation, *e.g.*, with a PEG reagent bearing an aldehyde group, is typically conducted under mild conditions, pHs from about 5-10, for about 6 to 36 hours. Varying ratios of polymeric reagent to CD-NP peptide may be employed, *e.g.*, from an equimolar ratio up to a 10-fold molar excess of polymer reagent. Typically, up to a 5-fold molar excess of polymer reagent will suffice.

**[00165]** In certain instances, it may be preferable to protect certain amino acids from reaction with a particular polymeric reagent if site specific or site selective covalent

attachment is desired using commonly employed protection/deprotection methodologies such as those well known in the art.

**[00166]** In an alternative approach to direct coupling reactions, the PEG reagent may be incorporated at a desired position of the CD-NP peptide during peptide synthesis. In this way, site-selective introduction of one or more PEGs can be achieved. See, *e.g.*, International Patent Publication No. WO 95/00162, which describes the site selective synthesis of conjugated peptides.

**[00167]** Exemplary conjugates that can be prepared using, for example, polymeric reagents containing a reactive ester for coupling to an amino group of CD-NP peptide, comprise the following alpha-branched structure:



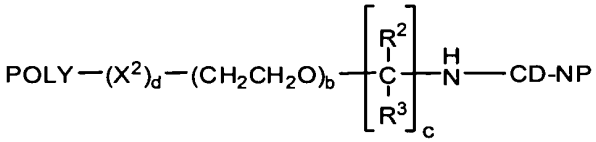
where POLY is a water-soluble polymer, (a) is either zero or one; $X^1$, when present, is a spacer moiety comprised of one or more atoms; $R^1$ is hydrogen an organic radical; and "~NH-CD-NP" represents a residue of a CD-NP peptide, where the underlined amino group represents an amino group of the CD-NP peptide.

**[00168]** With respect to the structure corresponding to that referred to in the immediately preceding paragraph, any of the water-soluble polymers provided herein can be defined as POLY, any of the spacer moieties provided herein can be defined as $X^1$ (when present), any of the organic radicals provided herein can be defined as $R^1$ (in instances where $R^1$ is not hydrogen), and any of the CD-NP peptides provided herein can be employed. In one or more embodiments corresponding to the structure referred to in the immediately preceding paragraph, POLY is a poly(ethylene glycol) such as $H_3CO(CH_2CH_2O)_n$-, wherein (n) is an integer having a value of from 3 to 4000, more preferably from 10 to about 1800; (a) is one; $X^1$ is a $C_{1-6}$ alkylene, such as one selected from methylene (*i.e.*, $-CH_2-$), ethylene (*i.e.*, $-CH_2-CH_2-$) and propylene (*i.e.*, $-CH_2-CH_2-CH_2-$); $R^1$ is H or lower alkyl such as methyl or ethyl; and CD-NP corresponds to any CD-NP peptide disclosed herein, including in Table 1.

**[00169]** Typical of another approach for conjugating a CD-NP peptide to a polymeric reagent is reductive amination. Typically, reductive amination is employed to conjugate a primary amine of a CD-NP peptide with a polymeric reagent functionalized with a ketone, aldehyde or a hydrated form thereof (*e.g.*, ketone hydrate and aldehyde hydrate). In this

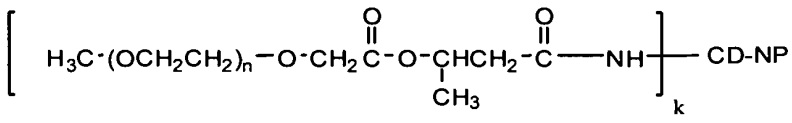
approach, the primary amine from the CD-NP peptide (*e.g.*, the N-terminus) reacts with the carbonyl group of the aldehyde or ketone (or the corresponding hydroxy-containing group of a hydrated aldehyde or ketone), thereby forming a Schiff base. The Schiff base, in turn, is then reductively converted to a stable conjugate through use of a reducing agent such as sodium borohydride or any other suitable reducing agent. Selective reactions (*e.g.*, at the N-terminus) are possible, particularly with a polymer functionalized with a ketone or an alpha-methyl branched aldehyde and/or under specific reaction conditions (*e.g.*, reduced pH).

**[00170]** Exemplary conjugates that can be prepared using, for example, polymeric reagents containing an aldehyde (or aldehyde hydrate) or ketone or (ketone hydrate) possess the following structure:



where POLY is a water-soluble polymer; (d) is either zero or one; $X^2$, when present, is a spacer moiety comprised of one or more atoms; (b) is an integer having a value of one through ten; (c) is an integer having a value of one through ten; $R^2$, in each occurrence, is independently H or an organic radical; $R^3$, in each occurrence, is independently H or an organic radical; and "~NH-CD-NP" represents a residue of a CD-NP peptide, where the underlined amino group represents an amino group of the CD-NP peptide.

**[00171]** Yet another illustrative conjugate of the invention possesses the structure:

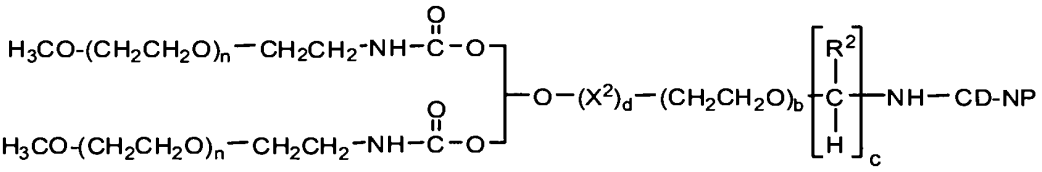


where k ranges from 1 to 3, and n ranges from 10 to about 1800.

**[00172]** With respect to the structure corresponding to that referred to in immediately preceding paragraph, any of the water-soluble polymers provided herein can be defined as POLY, any of the spacer moieties provided herein can be defined as $X^2$ (when present), any of the organic radicals provided herein can be independently defined as $R^2$ and $R^3$ (in instances where $R^2$ and $R^3$ are independently not hydrogen), and any of the CD-NP moieties provided herein can be defined as a CD-NP peptide. In one or more embodiments of the

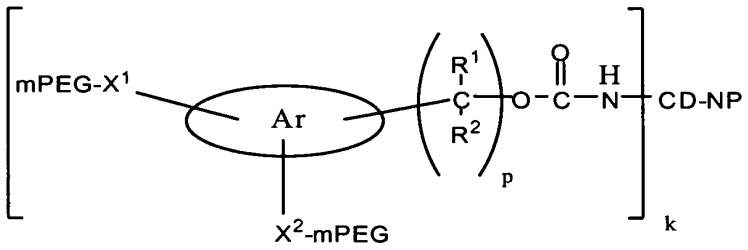
structure referred to in the immediately preceding paragraph, POLY is a poly(ethylene glycol) such as $H_3CO(CH_2CH_2O)_n$-, wherein (n) is an integer having a value of from 3 to 4000, more preferably from 10 to about 1800; (d) is one; $X^1$ is amide [*e.g.*, -C(O)NH-]; (b) is 2 through 6, such as 4; (c) is 2 through 6, such as 4; each of $R^2$ and $R^3$ are independently H or lower alkyl, such as methyl when lower alkyl; and CD-NP is CD-NP peptide.

**[00173]** Another example of a CD-NP peptide conjugate in accordance with the invention has the following structure:



wherein each (n) is independently an integer having a value of from 3 to 4000, preferably from 10 to 1800; $X^2$ is as previously defined; (b) is 2 through 6; (c) is 2 through 6; $R^2$, in each occurrence, is independently H or lower alkyl; and "~NH-CD-NP" represents a residue of a CD-NP peptide, where the underlined amino group represents an amino group of the CD-NP peptide.

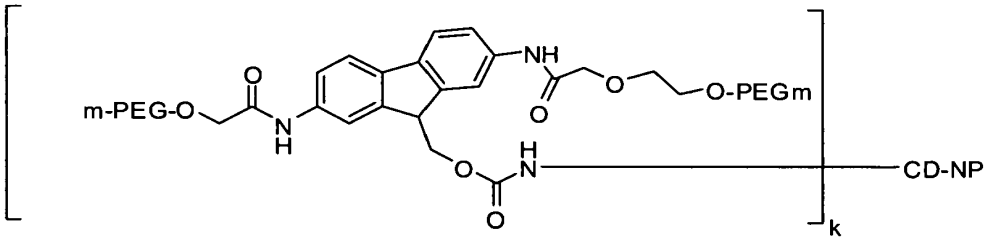
**[00174]** Additional CD-NP peptide polymer conjugates resulting from reaction of a water-soluble polymer with an amino group of CD-NP peptide are provided below. The following conjugate structures are releasable. One such structure corresponds to:

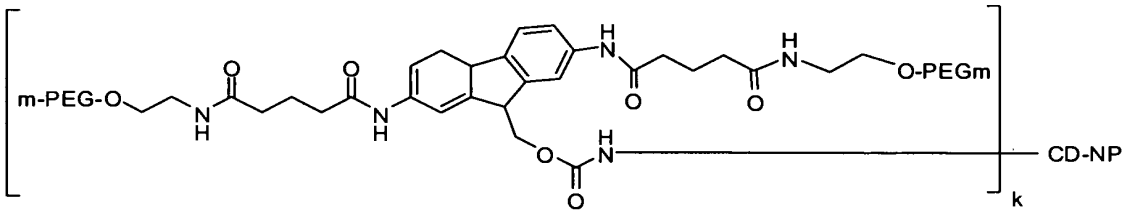


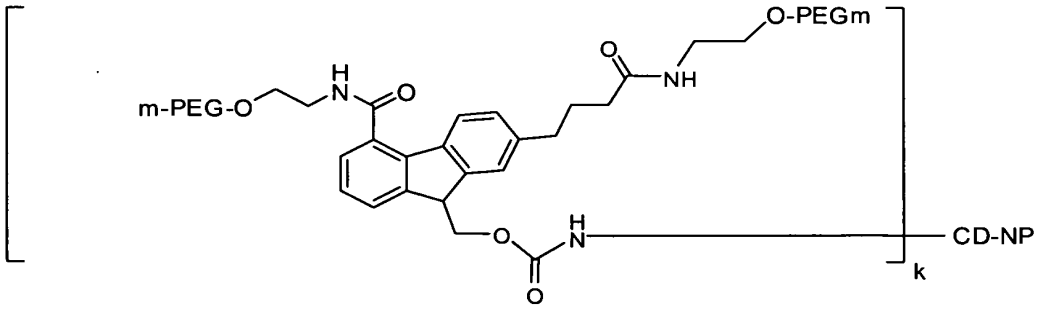
where mPEG is $CH_3O$-$(CH_2CH_2O)_nCH_2CH_2$-, n ranges from 10 to 1800, p is an integer ranging from 1 to 8, $R^1$ is H or lower alkyl, $R^2$ is H or lower alkyl, Ar is an aromatic hydrocarbon, such as a fused bicyclic or tricyclic aromatic hydrocarbon, $X^1$ and $X^2$ are each independently a spacer moiety having an atom length of from about 1 to about 18 atoms, ~NH-CD-NPis as previously described, and k is an integer selected from 1, 2, and 3. The value of k indicates the number of water-soluble polymer molecules attached to different sites on the CD-NP peptide. In a preferred embodiment, $R^1$ and $R^2$ are both H. The spacer

moieties, $X^1$ and $X^2$, preferably each contain one amide bond. In a preferred embodiment, $X^1$ and $X^2$ are the same. Preferred spacers, *i.e.*, $X^1$ and $X^2$, include –NH-C(O)-$CH_2$-O-, –NH-C(O)-$(CH_2)_q$-O-, –NH-C(O)-$(CH_2)_q$-C(O)-NH-, -NH-C(O)-$(CH_2)_q$-, and –C(O)-NH-, where q is selected from 2, 3, 4, and 5. Although the spacers can be in either orientation, preferably, the nitrogen is proximal to the PEG rather than to the aromatic moiety. Illustrative aromatic moieties include pentalene, indene, naphthalene, indacene, acenaphthylene, and fluorene.

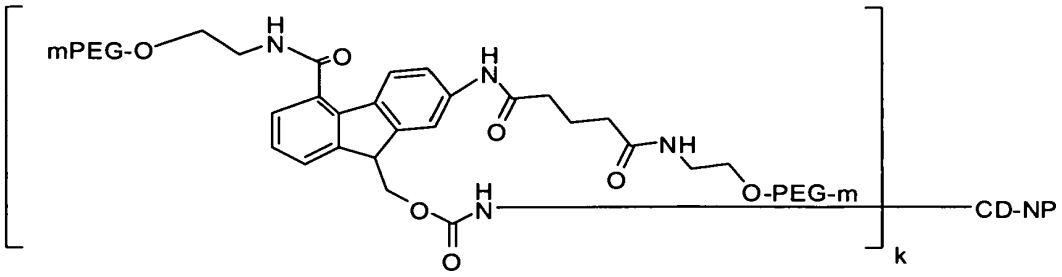
**[00175]** Particularly preferred conjugates of this type are provided below.



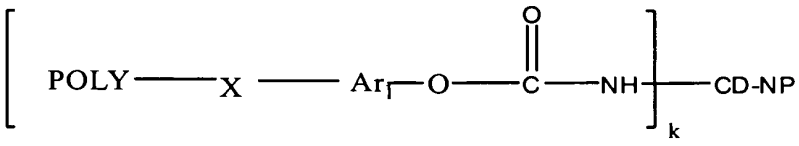



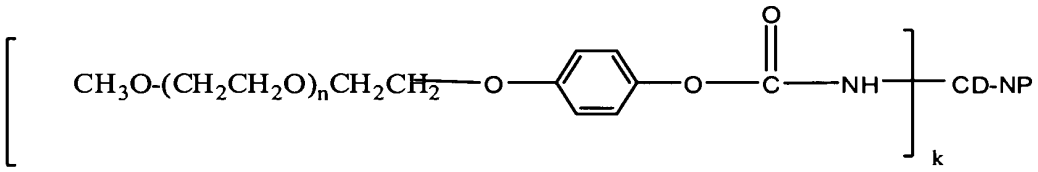

and

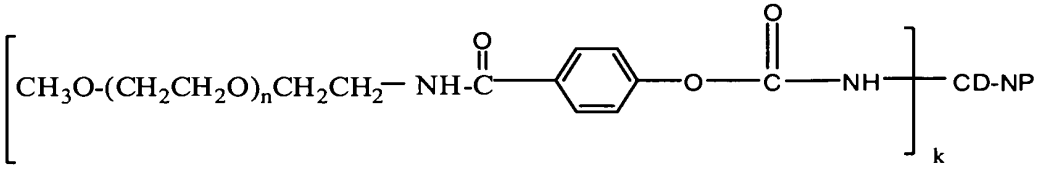



**[00176]** Additional CD-NP peptide conjugates resulting from covalent attachment to amino groups of CD-NP peptide that are also releasable include the following:




where X is either –O- or –NH-C(O)-, $Ar_1$ is an aromatic group, *e.g.*, *ortho, meta,* or *para*-substituted phenyl, and k is an integer selected from 1, 2, and 3. Particular conjugates of this type include:




where n ranges from about 10 to about 1800.

**[00177]** Additional releasable conjugates in accordance with the invention are prepared using water-soluble polymer reagents such as those described in U.S. Patent No. 6,214,966. Such water-soluble polymers result in a releasable linkage following conjugation, and possess at least one releasable ester linkage close to the covalent attachment to the active agent. The polymers generally possess the following structure, PEG-W-$CO_2$-NHS or an equivalent activated ester, where

| | | |
|---|---|---|
| W = | $-O_2C-(CH_2)_b-O-$ | b = 1-5 |
| | $-O-(CH_2)_bCO_2-(CH_2)_c-$ | b = 1-5, c = 2-5 |
| | $-O-(CH_2)_b-CO_2-(CH_2)_c-O-$ | b = 1-5, c = 2-5 |

and NHS is N-hydroxysuccinimidyl. Upon hydrolysis, the resulting released active agent, *e.g.*, CD-NP peptide, will possess a short tag resulting from hydrolysis of the ester functionality of the polymer reagent. Illustrative releasable conjugates of this type include: mPEG-O-$(CH_2)_b$-$COOCH_2C(O)$-NH-CD-NP peptide, and mPEG-O-$(CH_2)_b$-COO-$CH(CH_3)$-$CH_2$-C(O)-NH-CD-NP peptide, where the number of water-soluble polymers attached to CD-NP peptide can be anywhere from 1 to 4, or more preferably, from 1 to 3.

Carboxyl Coupling and Resulting Conjugates

**[00178]** Carboxyl groups represent another functional group that can serve as a point of attachment to the CD-NP peptide. The conjugate will have the following structure:

CD-NP-C(O) -X-POLY

where CD-NP-C(O) ~ corresponds to a residue of a CD-NP peptide where the carbonyl is a carbonyl (derived from the carboxy group) of the CD-NP peptide, X is a spacer moiety, such as a heteroatom selected from O, N(H), and S, and POLY is a water-soluble polymer such as PEG, optionally terminating in an end-capping moiety.

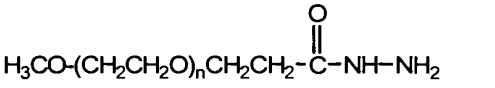
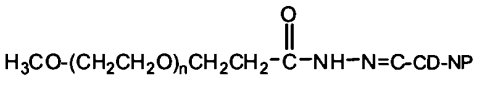
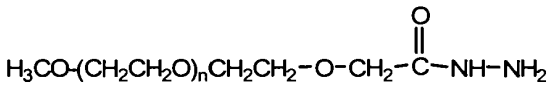
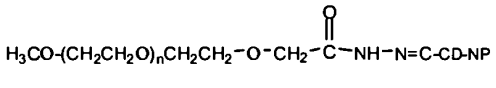
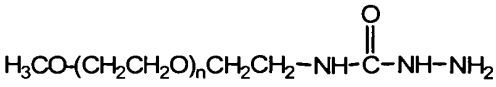
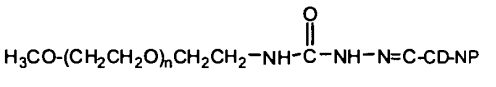
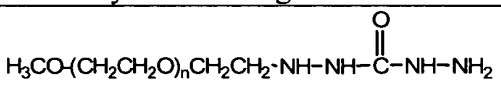
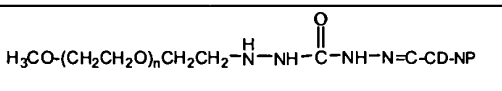
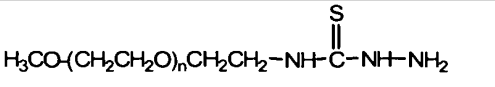
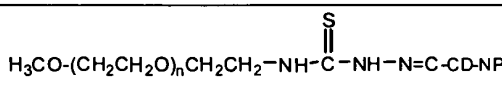
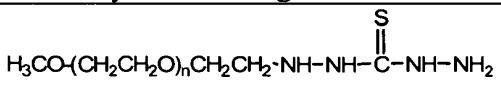
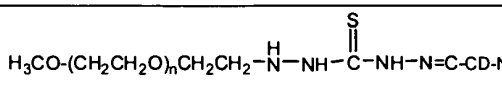
**[00179]** The C(O)-X linkage results from the reaction between a polymeric derivative bearing a terminal functional group and a carboxyl-containing CD-NP peptide. As discussed above, the specific linkage will depend on the type of functional group utilized. If the polymer is end-functionalized or "activated" with a hydroxyl group, the resulting linkage will be a carboxylic acid ester and X will be O. If the polymer backbone is functionalized with a thiol group, the resulting linkage will be a thioester and X will be S. When certain multi-arm, branched or forked polymers are employed, the C(O)X moiety, and in particular the X moiety, may be relatively more complex and may include a longer linker structure.

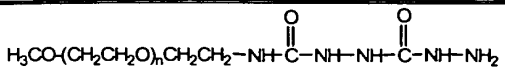
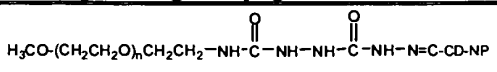
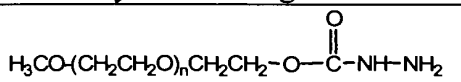
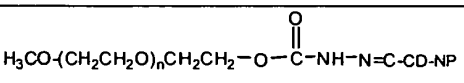
**[00180]** Polymeric reagents containing a hydrazide moiety are also suitable for conjugation at a carbonyl. To the extent that the CD-NP peptide does not contain a carbonyl moiety, a carbonyl moiety can be introduced by reducing any carboxylic acid functionality (*e.g.*, the C-terminal carboxylic acid). Specific examples of polymeric reagents comprising a hydrazide moiety, along with the corresponding conjugates, are provided in Table 3, below. In addition, any polymeric reagent comprising an activated ester (*e.g.*, a succinimidyl group) can be converted to contain a hydrazide moiety by reacting the polymer activated ester with hydrazine ($NH_2$-$NH_2$) or tert-butyl carbamate [$NH_2NHCO_2C(CH_3)_3$]. In the table, the variable (n) represents the number of repeating monomeric units and "=C-CD-NP" represents

a residue of a CD-NP peptide following conjugation to the polymeric reagent were the underlined C is part of the CD-NP peptide. Optionally, the hydrazone linkage can be reduced using a suitable reducing agent. While each polymeric portion [e.g., $(OCH_2CH_2)_n$ or $(CH_2CH_2O)_n$] presented in Table 3 terminates in a "$CH_3$" group, other groups (such as H and benzyl) can be substituted therefor.

**Table 3**

Carboxyl-Specific Polymeric Reagents and the CD-NP Peptide Conjugates Formed Therefrom

| Polymeric Reagent | Corresponding Conjugate |
|---|---|
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |

| Polymeric Reagent | Corresponding Conjugate |
|---|---|
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |
|  mPEG-Hydrazine Reagent |  Hydrazone Linkage |

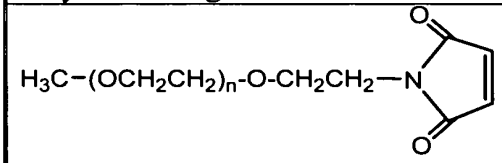
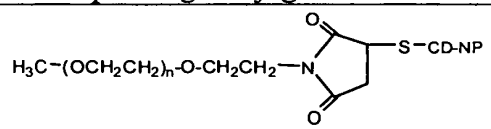
Thiol Coupling and Resulting Conjugates

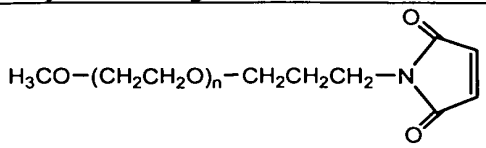
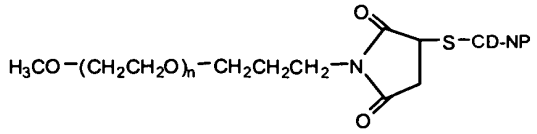
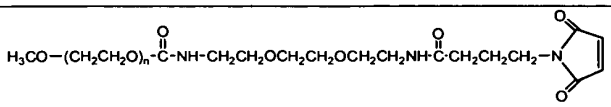
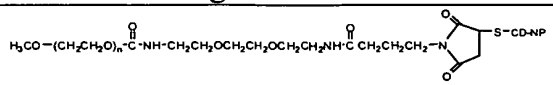
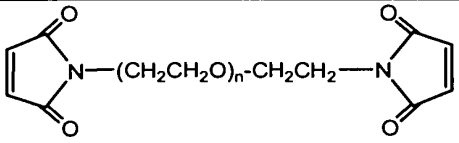
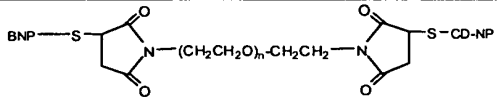
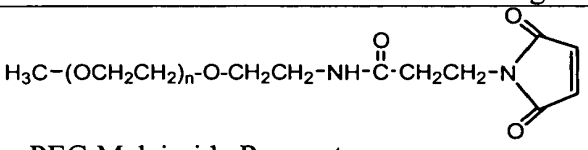
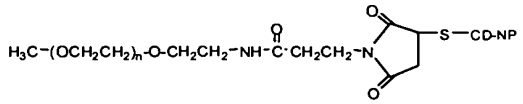
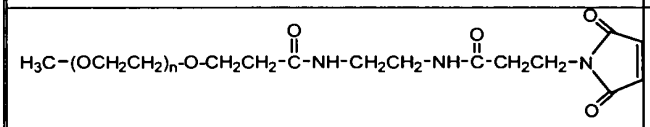
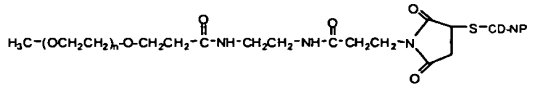
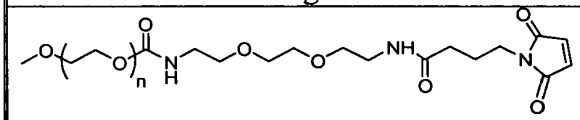
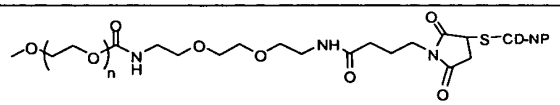
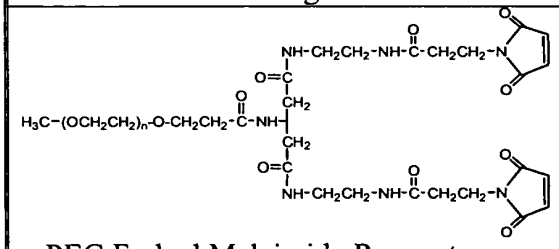
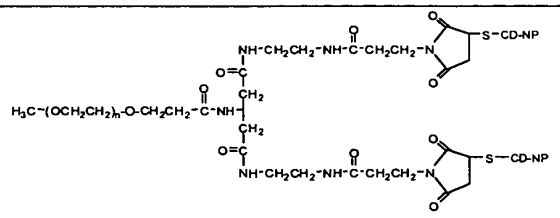
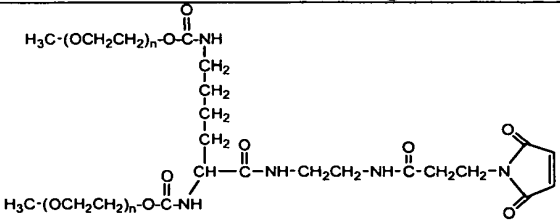
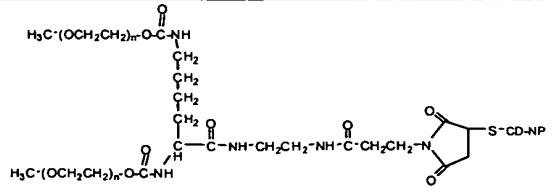
**[00181]** Thiol groups contained within the CD-NP peptide can serve as effective sites of attachment for the water-soluble polymer. The thiol groups contained in cysteine residues of the CD-NP peptide can be reacted with an activated PEG that is specific for reaction with thiol groups, *e.g.*, an N-maleimidyl polymer or other derivative, as described in, for example, U.S. Patent No. 5,739,208, WO 01/62827, and in Table 4 below. In certain embodiments, cysteine residues may be introduced in the CD-NP peptide and may be used to attach a water-soluble polymer.

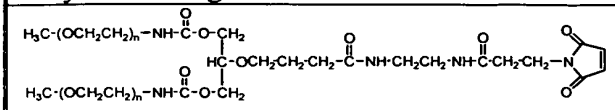
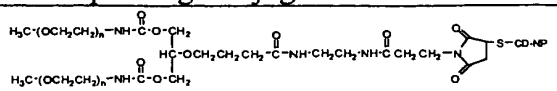
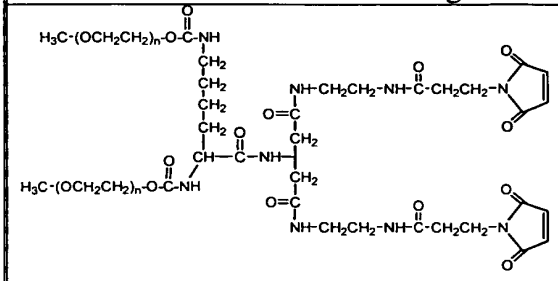
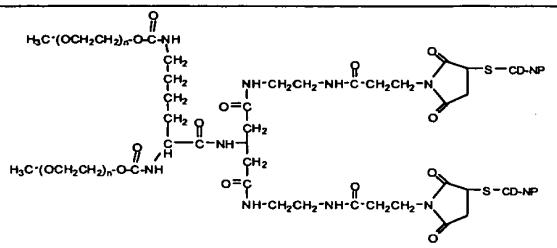
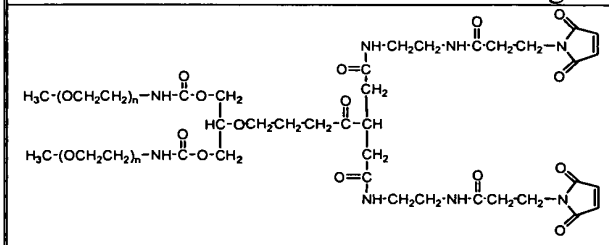
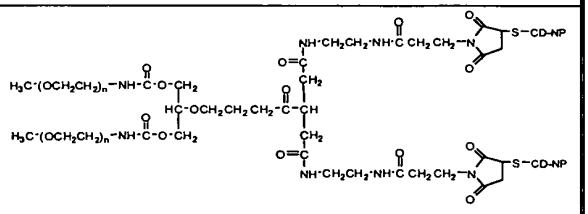
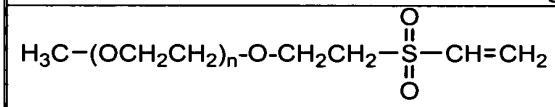
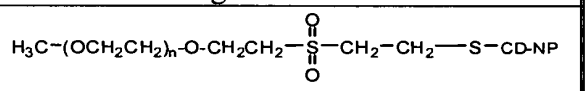
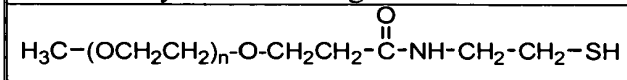
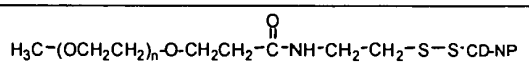
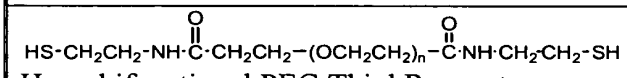
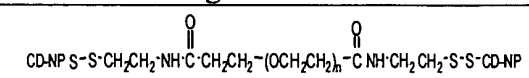
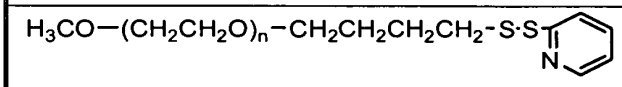
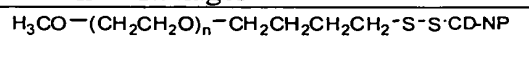
**[00182]** Specific examples of the reagents themselves, along with the corresponding conjugates, are provided in Table 4 below. In the table, the variable (n) represents the number of repeating monomeric units and "~S-CD-NP" represents a residue of a CD-NP peptide following conjugation to the water-soluble polymer, where the S represents the residue of a CD-NP peptide thiol group. While each polymeric portion [*e.g.*, $(OCH_2CH_2)_n$ or $(CH_2CH_2O)_n$] presented in Table 4 terminates in a "$CH_3$" group, other end-capping groups (such as H and benzyl) or reactive groups may be used as well.

**Table 4**

Thiol-Specific Polymeric Reagents and the CD-NP peptide Conjugates Formed Therefrom

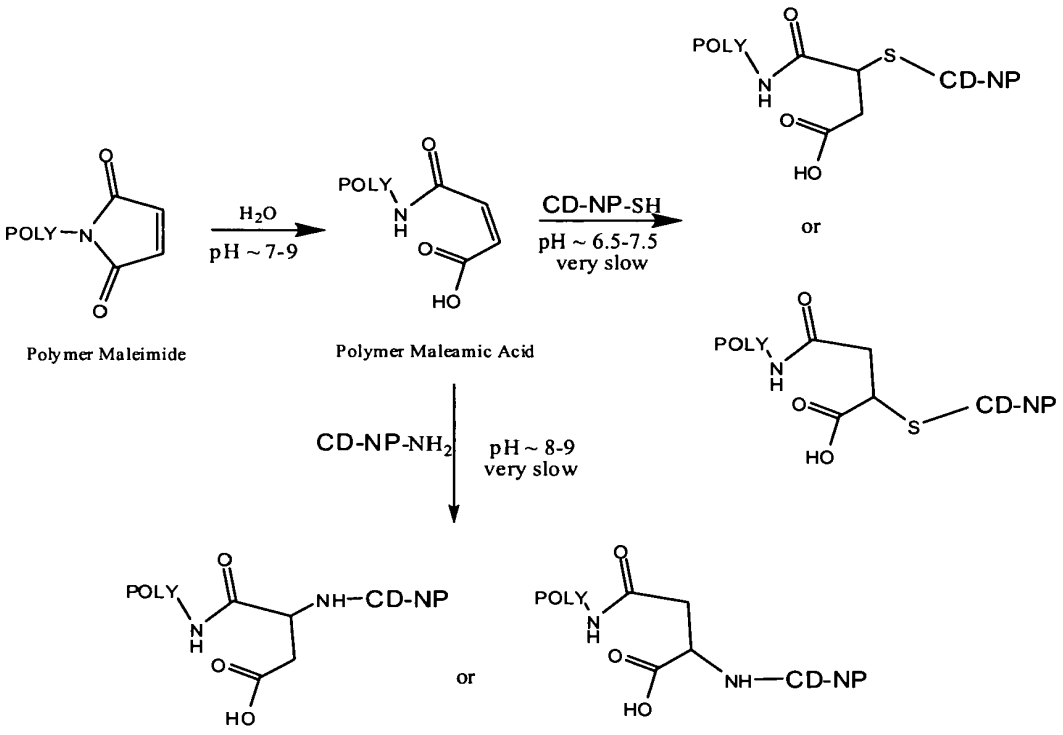
| Polymeric Reagent | Corresponding Conjugate |
|---|---|
|  mPEG Maleimide Reagent |  Thioether Linkage |

| Polymeric Reagent | Corresponding Conjugate |
|---|---|
|  mPEG Maleimide Reagent |  Thioether Linkage |
|  mPEG Maleimide Reagent |  Thioether Linkage |
|  Homobifunctional mPEG Maleimide Reagent |  Thioether Linkages |
|  mPEG Maleimide Reagent |  Thioether Linkage |
|  mPEG Maleimide Reagent |  Thioether Linkage |
|  mPEG Maleimide Reagent |  Thioether Linkage |
|  mPEG Forked Maleimide Reagent |  Thioether Linkage |
|  branched mPEG2 Maleimide Reagent |  Thioether Linkage |

| Polymeric Reagent | Corresponding Conjugate |
|---|---|
|  branched mPEG2 Maleimide Reagent |  Thioether Linkage |
|  Branched mPEG2 Forked Maleimide Reagent |  Thioether Linkages |
|  Branched mPEG2 Forked Maleimide Reagent |  Thioether Linkages |
|  mPEG Vinyl Sulfone Reagent |  Thioether Linkage |
|  mPEG Thiol Reagent |  Disulfide Linkage |
|  Homobifunctional PEG Thiol Reagent |  Disulfide Linkages |
|  mPEG Disulfide Reagent |  Disulfide Linkage |

**[00183]** With respect to conjugates formed from water-soluble polymers bearing one or more maleimide functional groups (regardless of whether the maleimide reacts with an amine or thiol group on the CD-NP peptide), the corresponding maleamic acid form(s) of the water-soluble polymer can also react with the CD-NP peptide. Under certain conditions (*e.g.*,

a pH of about 7-9 and in the presence of water), the maleimide ring will "open" to form the corresponding maleamic acid. The maleamic acid, in turn, can react with an amine or thiol group of a CD-NP peptide. Exemplary maleamic acid-based reactions are schematically shown below. POLY represents the water-soluble polymer, and ~S-CD-NP represents a residue of a CD-NP peptide, where the S is derived from a thiol group of the CD-NP peptide.




**[00184]** Thiol PEGylation is specific for free thiol groups on the CD-NP peptide. Tyically, a polymer maleimide is conjugated to a sulfhydryl-containing CD-NP peptide at pHs ranging from about 6-9 (*e.g.*, at 6, 6.5, 7, 7.5, 8, 8.5, or 9), more preferably at pHs from about 7-9, and even more preferably at pHs from about 7 to 8. Generally, a slight molar excess of polymer maleimide is employed, for example, a 1.5 to 15-fold molar excess, preferably a 2-fold to 10 fold molar excess. Reaction times generally range from about 15 minutes to several hours, *e.g.*, 8 or more hours, at room temperature. For sterically hindered sulfhydryl groups, required reaction times may be significantly longer. Thiol-selective conjugation is preferably conducted at pHs around 7. Temperatures for conjugation reactions are typically, although not necessarily, in the range of from about 0 °C to about 40 °C;

conjugation is often carried out at room temperature or less. Conjugation reactions are often carried out in a buffer such as a phosphate or acetate buffer or similar system.

**[00185]** With respect to reagent concentration, an excess of the polymeric reagent is typically combined with the CD-NP peptide. The conjugation reaction is allowed to proceed until substantially no further conjugation occurs, which can generally be determined by monitoring the progress of the reaction over time.

**[00186]** Progress of the reaction can be monitored by withdrawing aliquots from the reaction mixture at various time points and analyzing the reaction mixture by SDS-PAGE or MALDI-TOF mass spectrometry or any other suitable analytical method. Once a plateau is reached with respect to the amount of conjugate formed or the amount of unconjugated polymer remaining, the reaction is assumed to be complete. Typically, the conjugation reaction takes anywhere from minutes to several hours (*e.g.*, from 5 minutes to 24 hours or more). The resulting product mixture is preferably, but not necessarily purified, to separate out excess reagents, unconjugated reactants (*e.g.*, CD-NP peptide) undesired multi-conjugated species, and free or unreacted polymer. The resulting conjugates can then be further characterized using analytical methods such as MALDI, capillary electrophoresis, gel electrophoresis, and/or chromatography.

**[00187]** An illustrative CD-NP peptide conjugate formed by reaction with one or more CD-NP peptide thiol groups may possess the following structure:

$$POLY\text{-}X_{0,1}\text{-}C(O)Z\text{-}Y\text{-}S\text{-}S\text{-}CD\text{-}NP$$

where POLY is a water-soluble polymer, X is an optional linker, Z is a heteroatom selected from the group consisting of O, NH, and S, and Y is selected from the group consisting of $C_{2\text{-}10}$ alkyl, $C_{2\text{-}10}$ substituted alkyl, aryl, and substituted aryl, and ~S-CD-NP is a residue of a CD-NP peptide, where the S represents the residue of a CD-NP peptide thiol group. Such polymeric reagents suitable for reaction with a CD-NP peptide to result in this type of conjugate are described in U.S. Patent Application Publication No. 2005/0014903, which is incorporated herein by reference.

**[00188]** With respect to polymeric reagents suitable for reacting with a CD-NP peptide thiol group, those described here and elsewhere can be obtained from commercial sources. In addition, methods for preparing polymeric reagents are described in the literature.

Additional Conjugates and Features Thereof

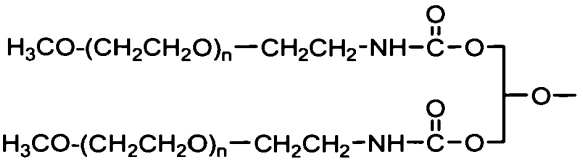
**[00189]** As is the case for any CD-NP peptide polymer conjugate of the invention, the attachment between the CD-NP peptide and water-soluble polymer can be direct, wherein no intervening atoms are located between the CD-NP peptide and the polymer, or indirect, wherein one or more atoms are located between the CD-NP peptide and polymer. With respect to the indirect attachment, a "spacer moiety or linker" serves as a link between the CD-NP peptide and the water-soluble polymer. The one or more atoms making up the spacer moiety can include one or more of carbon atoms, nitrogen atoms, sulfur atoms, oxygen atoms, and combinations thereof. The spacer moiety can comprise an amide, secondary amine, carbamate, thioether, and/or disulfide group. Nonlimiting examples of specific spacer moieties (including "X", $X^1$ , $X^2$, and $X^3$) include those selected from the group consisting of -O-, -S-, -S-S-, -C(O)-, -C(O)O-, -OC(O)-, $-CH_2$-C(O)O-, $-CH_2$-OC(O)-, -C(O)O-$CH_2$-, -OC(O)-$CH_2$-, -C(O)-NH-, -NH-C(O)-NH-, -O-C(O)-NH-, -C(S)-, $-CH_2$-, $-CH_2-CH_2$-, $-CH_2-CH_2-CH_2$-, $-CH_2-CH_2-CH_2-CH_2$-, -O-$CH_2$-, $-CH_2$-O-, -O-$CH_2-CH_2$-, $-CH_2$-O-$CH_2$-, $-CH_2-CH_2$-O-, -O-$CH_2-CH_2-CH_2$-, $-CH_2$-O-$CH_2-CH_2$-, $-CH_2-CH_2$-O-$CH_2$-, $-CH_2-CH_2-CH_2$-O-, -O-$CH_2-CH_2-CH_2-CH_2$-, $-CH_2$-O-$CH_2-CH_2-CH_2$-, $-CH_2-CH_2$-O-$CH_2-CH_2$-, $-CH_2-CH_2-CH_2$-O-$CH_2$-, $-CH_2-CH_2-CH_2-CH_2$-O-, -C(O)-NH-$CH_2$-, -C(O)-NH-$CH_2-CH_2$-, $-CH_2$-C(O)-NH-$CH_2$-, $-CH_2-CH_2$-C(O)-NH-, -C(O)-NH-$CH_2-CH_2-CH_2$-, $-CH_2$-C(O)-NH-$CH_2-CH_2$-, $-CH_2-CH_2$-C(O)-NH-$CH_2$-, $-CH_2-CH_2-CH_2$-C(O)-NH-, -C(O)-NH-$CH_2-CH_2-CH_2-CH_2$-, $-CH_2$-C(O)-NH-$CH_2-CH_2-CH_2$-, $-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2$-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-, $-CH_2-CH_2-CH_2-CH_2$-C(O)-NH-, -C(O)-O-$CH_2$-, $-CH_2$-C(O)-O-$CH_2$-, $-CH_2-CH_2$-C(O)-O-$CH_2$-, -C(O)-O-$CH_2-CH_2$-, -NH-C(O)-$CH_2$-, $-CH_2$-NH-C(O)-$CH_2$-, $-CH_2-CH_2$-NH-C(O)-$CH_2$-, -NH-C(O)-$CH_2-CH_2$-, $-CH_2$-NH-C(O)-$CH_2-CH_2$-, $-CH_2-CH_2$-NH-C(O)-$CH_2-CH_2$-, -C(O)-NH-$CH_2$-, -C(O)-NH-$CH_2-CH_2$-, -O-C(O)-NH-$CH_2$-, -O-C(O)-NH-$CH_2-CH_2$-, -NH-$CH_2$-, -NH-$CH_2-CH_2$-, $-CH_2$-NH-$CH_2$-, $-CH_2-CH_2$-NH-$CH_2$-, -C(O)-$CH_2$-, -C(O)-$CH_2-CH_2$-, $-CH_2$-C(O)-$CH_2$-, $-CH_2-CH_2$-C(O)-$CH_2$-, $-CH_2-CH_2$-C(O)-$CH_2-CH_2$-, $-CH_2-CH_2$-C(O)-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-NH-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-NH-C(O)-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-NH-C(O)-$CH_2$-, $-CH_2-CH_2-CH_2$-C(O)-NH-$CH_2-CH_2$-NH-C(O)-$CH_2-CH_2$-, -O-C(O)-NH-$[CH_2]_h$-$(OCH2CH2)_j$-, bivalent cycloalkyl group, -O-, -S-, an amino acid, -N($R^6$)-, and combinations of two or more of any of the foregoing, wherein $R^6$ is H or an organic radical selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl and substituted aryl, (h) is zero to six,

and (j) is zero to 20. Other specific spacer moieties have the following structures: -C(O)-NH-$(CH_2)_{1-6}$-NH-C(O)-, -NH-C(O)-NH-$(CH_2)_{1-6}$-NH-C(O)-, and -O-C(O)-NH-$(CH_2)_{1-6}$-NH-C(O)-, wherein the subscript values following each methylene indicate the number of methylenes contained in the structure, e.g., $(CH_2)_{1-6}$ means that the structure can contain 1, 2, 3, 4, 5 or 6 methylenes. Additionally, any of the above spacer moieties may further include an ethylene oxide oligomer chain comprising 1 to 20 ethylene oxide monomer units [*i.e.*, -$(CH_2CH_2O)_{1-20}$]. That is, the ethylene oxide oligomer chain can occur before or after the spacer moiety, and optionally in between any two atoms of a spacer moiety comprised of two or more atoms. Also, the oligomer chain would not be considered part of the spacer moiety if the oligomer is adjacent to a polymer segment and merely represent an extension of the polymer segment.

**[00190]** As indicated above, in some instances the water-soluble polymer-(CD-NP) conjugate will include a non-linear water-soluble polymer. Such a non-linear water-soluble polymer encompasses a branched water-soluble polymer (although other non linear water-soluble polymers are also contemplated). Thus, in one or more embodiments of the invention, the conjugate comprises a CD-NP peptide covalently attached, either directly or through a spacer moiety comprised of one or more atoms, to a branched water-soluble polymer, at in a non-limiting example, an internal or *N*-terminal amine. As used herein, an internal amine is an amine that is not part of the *N*-terminal amino acid (meaning not only the *N*-terminal amine, but any amine on the side chain of the *N*-terminal amino acid).

**[00191]** Although such conjugates include a branched water-soluble polymer attached (either directly or through a spacer moiety) to a CD-NP peptide at an internal amino acid of the CD-NP peptide, additional branched water-soluble polymers can also be attached to the same CD-NP peptide at other locations as well. Thus, for example, a conjugate including a branched water-soluble polymer attached (either directly or through a spacer moiety) to a CD-NP peptide at an internal amino acid of the CD-NP peptide, can further include an additional branched water-soluble polymer covalently attached, either directly or through a spacer moiety comprised of one or more atoms, to the *N*-terminal amino acid residue, such as at the *N*-terminal amine.

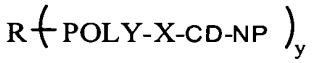
**[00192]** One preferred branched water-soluble polymer comprises the following structure:



wherein each (n) is independently an integer having a value of from 3 to 4000, or more preferably, from about 10 to 1800.

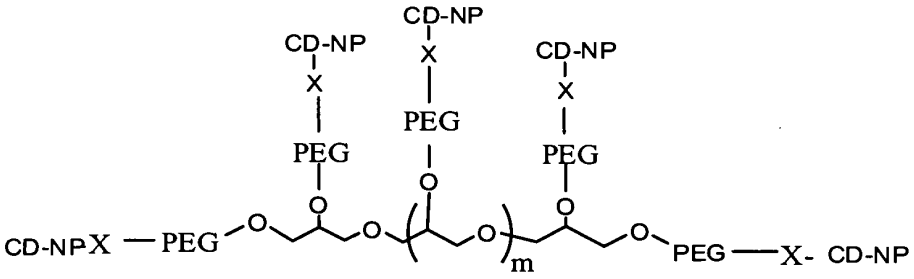
**[00193]** Also forming part of the invention are multi-armed polymer conjugates comprising a polymer scaffold having 3 or more polymer arms each suitable for capable of covalent attachment of a CD-NP peptide.

Exemplary conjugates in accordance with this embodiment of the invention will generally comprise the following structure:



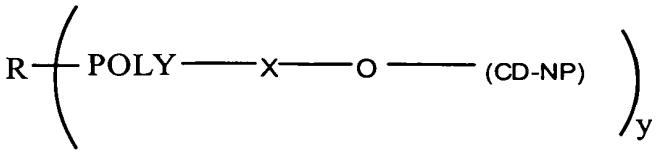
wherein R is a core molecule as previously described, POLY is a water-soluble polymer, X is a cleavable, *e.g.*, hydrolyzable linkage, and y ranges from about 3 to 15.

**[00194]** More particularly, such a conjugate may comprise the structure:



where m is selected from 3, 4, 5, 6, 7, and 8.

**[00195]** In yet a related embodiment, the CD-NP peptide conjugate may correspond to the structure:




where R is a core molecule as previously described, X is -NH-P-Z-C(O) P is a spacer, Z is –O-, -NH-, or $-CH_2-$, –O-CD-NP is a hydroxyl residue of a CD-NP peptide, and y is 3 to 15. Preferably, X is a residue of an amino acid.

Purification

**[00196]** The CD-NP peptide polymer conjugates described herein can be purified to obtain/isolate different conjugate species. Specifically, a product mixture can be purified to obtain an average of anywhere from one, two, or three or even more PEGs per CD-NP peptide. In one embodiment of the invention, preferred CD-NP peptide conjugates are mono-conjugates. The strategy for purification of the final conjugate reaction mixture will depend upon a number of factors, including, for example, the molecular weight of the polymeric reagent employed, the CD-NP peptide, and the desired characteristics of the product – *e.g.*, monomer, dimer, particular positional isomers, etc.

**[00197]** If desired, conjugates having different molecular weights can be isolated using gel filtration chromatography and/or ion exchange chromatography. Gel filtration chromatography may be used to fractionate different CD-NP peptide conjugates (*e.g.*, 1-mer, 2-mer, 3-mer, and so forth, wherein "1-mer" indicates one polymer molecule per CD-NP peptide, "2-mer" indicates two polymers attached to CD-NP peptide, and so on) on the basis of their differing molecular weights (where the difference corresponds essentially to the average molecular weight of the water-soluble polymer). While this approach can be used to separate PEG and other CD-NP peptide polymer conjugates having different molecular weights, this approach is generally ineffective for separating positional isomers having different polymer attachment sites within the CD-NP peptide. For example, gel filtration chromatography can be used to separate from each other mixtures of PEG 1-mers, 2-mers, 3-mers, and so forth, although each of the recovered PEG-mer compositions may contain PEGs attached to different reactive amino groups (*e.g.*, lysine residues) or other functional groups of the CD-NP peptide.

**[00198]** Gel filtration columns suitable for carrying out this type of separation include Superdex™ and Sephadex™ columns available from Amersham Biosciences (Piscataway,

NJ). Selection of a particular column will depend upon the desired fractionation range desired. Elution is generally carried out using a suitable buffer, such as phosphate, acetate, or the like. The collected fractions may be analyzed by a number of different methods, for example, (i) optical density (OD) at 280 nm for protein content, (ii) bovine serum albumin (BSA) protein analysis, (iii) iodine testing for PEG content (Sims *et al.* (1980) *Anal. Biochem*, 107:60-63), and (iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), followed by staining with barium iodide.

**[00199]** Separation of positional isomers is typically carried out by reverse phase chromatography using a reverse phase-high performance liquid chromatography (RP-HPLC) C18 column (Amersham Biosciences or Vydac) or by ion exchange chromatography using an ion exchange column, *e.g.*, a DEAE- or CM-Sepharose™ ion exchange column available from Amersham Biosciences. Either approach can be used to separate polymer-CD-NP peptide isomers having the same molecular weight (positional isomers).

**[00200]** The resulting purified compositions are preferably substantially free of the non-conjugated CD-NP peptide. In addition, the compositions preferably are substantially free of all other non-covalently attached water-soluble polymers.

**Compositions**

Compositions of conjugate isomers

**[00201]** Also provided herein are compositions comprising one or more of the CD-NP peptide polymer conjugates described herein. In certain instances, the composition will comprise a plurality of CD-NP peptide polymer conjugates. For instance, such a composition may comprise a mixture of CD-NP peptide polymer conjugates having one, two, three and/or even four water-soluble polymer molecules covalently attached to sites on the CD-NP peptide. That is to say, a composition of the invention may comprise a mixture of monomer, dimer, and possibly even trimer or 4-mer. Alternatively, the composition may possess only mono-conjugates, or only di-conjugates, etc. A mono-conjugate CD-NP peptide composition will typically comprise CD-NP peptide moieties having only a single polymer covalently attached thereto, e.g., preferably releasably attached. A mono-conjugate composition may comprise only a single positional isomer, or may comprise a mixture of different positional isomers having polymer covalently attached to different sites within the CD-NP peptide.

**[00202]** In yet another embodiment, a CD-NP peptide conjugate may possess multiple CD-NP peptides covalently attached to a single multi-armed polymer having 3 or more

polymer arms. Typically, the CD-NP peptide moieties are each attached at the same CD-NP peptide amino acid site, *e.g.*, the N-terminus.

**[00203]** With respect to the conjugates in the composition, the composition will typically satisfy one or more of the following characteristics: at least about 85% of the conjugates in the composition will have from one to four polymers attached to the CD-NP peptide; at least about 85% of the conjugates in the composition will have from one to three polymers attached to the CD-NP peptide; at least about 85% of the conjugates in the composition will have from one to two polymers attached to the CD-NP peptide; or at least about 85% of the conjugates in the composition will have one polymer attached to the CD-NP peptide (*e.g.*, be monoPEGylated); at least about 95% of the conjugates in the composition will have from one to four polymers attached to the CD-NP peptide; at least about 95% of the conjugates in the composition will have from one to three polymers attached to the CD-NP peptide; at least about 95% of the conjugates in the composition will have from one to two polymers attached to the CD-NP peptide; at least about 95% of the conjugates in the composition will have one polymers attached to the CD-NP peptide; at least about 99% of the conjugates in the composition will have from one to four polymers attached to the CD-NP peptide; at least about 99% of the conjugates in the composition will have from one to three polymers attached to the CD-NP peptide; at least about 99% of the conjugates in the composition will have from one to two polymers attached to the CD-NP peptide; and at least about 99% of the conjugates in the composition will have one polymer attached to the CD-NP peptide (*e.g.*, be monoPEGylated).

**[00204]** In one or more embodiments, the conjugate-containing composition is free or substantially free of albumin.

**[00205]** In one or more embodiments of the invention, a pharmaceutical composition is provided comprising a conjugate comprising a CD-NP peptide covalently attached, *e.g.*, releasably, to a water-soluble polymer, wherein the water-soluble polymer has a weight-average molecular weight of greater than about 2,000 Daltons; and a pharmaceutically acceptable excipient.

**[00206]** Control of the desired number of polymers for covalent attachment to CD-NP peptide is achieved by selecting the proper polymeric reagent, the ratio of polymeric reagent to the CD-NP peptide, temperature, pH conditions, and other aspects of the conjugation reaction. In addition, reduction or elimination of the undesired conjugates (*e.g.*, those conjugates having four or more attached polymers) can be achieved through purification mean as previously described.

[00207] For example, the water-soluble polymer-(CD-NP peptide) conjugates can be purified to obtain/isolate different conjugated species. Specifically, the product mixture can be purified to obtain an average of anywhere from one, two, three, or four PEGs per CD-NP peptide, typically one, two or three PEGs per CD-NP peptide. In one or more embodiments, the product comprises one PEG per CD-NP peptide, where PEG is releasably (via hydrolysis) attached to PEG polymer, *e.g.*, a branched or straight chain PEG polymer.

Pharmaceutical Compositions

[00208] Optionally, a CD-NP peptide conjugate composition of the invention will comprise, in addition to the CD-NP peptide conjugate, a pharmaceutically acceptable excipient. More specifically, the composition may further comprise excipients, solvents, stabilizers, membrane penetration enhancers, etc., depending upon the particular mode of administration and dosage form.

[00209] Pharmaceutical compositions of the invention encompass all types of formulations and in particular those that are suited for injection, *e.g.*, powders or lyophilates that can be reconstituted as well as liquids, as well as for inhalation. Examples of suitable diluents for reconstituting solid compositions prior to injection include bacteriostatic endotoxin-free water for injection, dextrose 5% in water, phosphate-buffered saline, Ringer's solution, saline, sterile water, deionized water, and combinations thereof. With respect to liquid pharmaceutical compositions, solutions and suspensions are envisioned.

[00210] Exemplary pharmaceutically acceptable excipients include, without limitation, carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof.

[00211] Representative carbohydrates for use in the compositions of the present invention include sugars, derivatized sugars such as alditols, aldonic acids, esterified sugars, and sugar polymers. Exemplary carbohydrate excipients suitable for use in the present invention include, for example, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol), pyranosyl sorbitol, myoinositol and the like. Preferred, in particular for formulations intended for inhalation, are non-reducing sugars, sugars that can form a substantially dry amorphous or glassy phase when combined with the composition of the

present invention, and sugars possessing relatively high glass transition temperatures, or Tgs (*e.g.*, Tgs greater than 40°C, or greater than 50°C, or greater than 60°C, or greater than 70°C, or having Tgs of 80°C and above). Such excipients may be considered glass-forming excipients.

**[00212]** Additional excipients include amino acids, peptides and particularly oligomers comprising 2-9 amino acids, or 2-5 mers, and polypeptides, all of which may be homo or hetero species.

**[00213]** Exemplary protein excipients include albumins such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, hemoglobin, and the like. The compositions may also include a buffer or a pH-adjusting agent, typically but not necessarily a salt prepared from an organic acid or base. Representative buffers include organic acid salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid. Other suitable buffers include Tris, tromethamine hydrochloride, borate, glycerol phosphate, and phosphate. Amino acids such as glycine are also suitable.

**[00214]** The compositions of the present invention may also include one or more additional polymeric excipients/additives, *e.g.*, polyvinylpyrrolidones, derivatized celluloses such as hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose, FICOLLs (a polymeric sugar), hydroxyethylstarch (HES), dextrates (*e.g.*, cyclodextrins, such as 2-hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin), polyethylene glycols, and pectin.

**[00215]** The compositions may further include flavoring agents, taste-masking agents, inorganic salts (*e.g.*, sodium chloride), antimicrobial agents (*e.g.*, benzalkonium chloride), sweeteners, antioxidants, antistatic agents, surfactants (*e.g.*, polysorbates such as "TWEEN 20" and "TWEEN 80," and pluronics such as F68 and F88, available from BASF), sorbitan esters, lipids (*e.g.*, phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines, although preferably not in liposomal form), fatty acids and fatty esters, steroids (*e.g.*, cholesterol), and chelating agents (*e.g.*, zinc and other such suitable cations). The use of certain di-substituted phosphatidylcholines for producing perforated microstructures (*i.e.*, hollow, porous microspheres) may also be employed.

**[00216]** Other pharmaceutical excipients and/or additives suitable for use in the compositions according to the present invention are listed in "Remington: The Science & Practice of Pharmacy," 21st ed., Williams & Williams, (2005), and in the "Physician's Desk Reference," 60th ed., Medical Economics, Montvale, N.J. (2006).

**[00217]** The amount of the CD-NP peptide conjugate (*i.e.*, the conjugate formed between the active agent and the polymeric reagent) in the composition will vary depending on a number of factors, but will optimally be a therapeutically effective amount when the composition is stored in a unit dose container (*e.g.*, a vial). In addition, a pharmaceutical preparation, if in solution form, can be housed in a syringe. A therapeutically effective amount can be determined experimentally by repeated administration of increasing amounts of the conjugate in order to determine which amount produces a clinically desired endpoint.

**[00218]** The amount of any individual excipient in the composition will vary depending on the activity of the excipient and particular needs of the composition. Typically, the optimal amount of any individual excipient is determined through routine experimentation, *i.e.*, by preparing compositions containing varying amounts of the excipient (ranging from low to high), examining the stability and other parameters, and then determining the range at which optimal performance is attained with no significant adverse effects.

**[00219]** Generally, however, the excipient or excipients will be present in the composition in an amount of about 1% to about 99% by weight, from about 5% to about 98% by weight, from about 15 to about 95% by weight of the excipient, or with concentrations less than 30% by weight. In general, a high concentration of the CD-NP peptide is desired in the final pharmaceutical formulation.

Combination of Actives

**[00220]** A composition of the invention may also comprise a mixture of water-soluble polymer-(CD-NP peptide) conjugates and unconjugated CD-NP peptide, to thereby provide a mixture of fast-acting and long-acting CD-NP peptide.

**[00221]** Additional pharmaceutical compositions in accordance with the invention include those comprising, in addition to an extended-action CD-NP peptide water-soluble polymer conjugate as described herein, a rapid acting CD-NP peptide polymer conjugate where the water-soluble polymer is releasably attached to a suitable location on the CD-NP peptide.

**Administration**

**[00222]** The CD-NP peptide conjugates of the invention can be administered by any of a number of routes including without limitation, oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal), intrathecal, and pulmonary. Preferred forms of administration include parenteral and pulmonary. Suitable formulation types for parenteral administration include ready-for-injection solutions, dry powders for combination with a solvent prior to use, suspensions ready for injection, dry insoluble compositions for combination with a vehicle prior to use, and emulsions and liquid concentrates for dilution prior to administration, among others.

**[00223]** In some embodiments of the invention, the compositions comprising the peptide-polymer conjugates may further be incorporated into a suitable delivery vehicle. Such delivery vehicles may provide controlled and/or continuous release of the conjugates and may also serve as a targeting moiety. Non-limiting examples of delivery vehicles include, adjuvants, synthetic adjuvants, microcapsules, microparticles, liposomes, and yeast cell wall particles. Yeast cells walls may be variously processed to selectively remove protein component, glucan, or mannan layers, and are referred to as whole glucan particles (WGP), yeast beta-glucan mannan particles (YGMP), yeast glucan particles (YGP), \Rhodotorula yeast cell particles (YCP). Yeast cells such as S.cerevisiae and Rhodotorula sp. are preferred; however, any yeast cell may be used. These yeast cells exhibit different properties in terms of hydrodynamic volume and also differ in the target organ where they may release their contents. The methods of manufacture and characterization of these particles are described in US Patent Nos. 5,741,495; 4,810,646; 4,992,540; 5,028,703; 5,607,677, and US Patent Applications Nos. 2005/0281781, and 2008/0044438.

**[00224]** In one or more embodiments of the invention, a method is provided, the method comprising delivering a conjugate to a patient, the method comprising the step of administering to the patient a pharmaceutical composition comprising a CD-NP peptide polymer conjugate as provided herein. Administration can be effected by any of the routes herein described. The method may be used to treat a patient suffering from a condition that is responsive to treatment with CD-NP peptide by administering a therapeutically effective amount of the pharmaceutical composition.

**[00225]** As previously stated, the method of delivering a CD-NP peptide polymer conjugate as provided herein may be used to treat a patient having a condition that can be remedied or prevented by administration of CD-NP peptide.

**[00226]** Certain conjugates of the invention, *e.g.*, releasable conjugates, include those effective to release the CD-NP peptide, *e.g.*, by hydrolysis, over a period of several hours or even days (*e.g.*, 2-7 days, 2-6 days, 3-6 days, 3-4 days) when evaluated in a suitable *in-vivo* model.

**[00227]** The actual dose of the CD-NP peptide conjugate to be administered will vary depending upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. Therapeutically effective amounts are known to those skilled in the art and/or are described in the pertinent reference texts and literature. Generally, a conjugate of the invention will be delivered such that plasma levels of a CD-NP peptide are within a range of about 0.5 picomoles/liter to about 500 picomoles/liter. In certain embodiments the conjugate of the invention will be delivered such that plasma leves of a CD-NP peptide are within a range of about 1 picomoles/liter to about 400 picomoles/liter, a range of about 2.5 picomoles/liter to about 250 picomoles/liter, a range of about 5 picomoles/liter to about 200 picomoles/liter, or a range of about 10 picomoles/liter to about 100 picomoles/liter.

**[00228]** On a weight basis, a therapeutically effective dosage amount of a CD-NP peptide conjugate as described herein will range from about 0.01 mg per day to about 1000 mg per day for an adult. For example, dosages may range from about 0.1 mg per day to about 100 mg per day, or from about 1.0 mg per day to about 10 mg/day. On an activity basis, corresponding doses based on international units of activity can be calculated by one of ordinary skill in the art.

**[00229]** The unit dosage of any given conjugate (again, such as provided as part of a pharmaceutical composition) can be administered in a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. Once the clinical endpoint has been achieved, dosing of the composition is halted.

**[00230]** It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the

invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

**[00231]** All articles, books, patents and other publications referenced herein are hereby incorporated by reference in their entireties.

## EXPERIMENTAL

**[00232]** The practice of the invention will employ, unless otherwise indicated, conventional techniques of organic synthesis and the like, which are within the skill of the art. Such techniques are fully explained in the literature. Reagents and materials are commercially available unless specifically stated to the contrary. See, for example, J. March, Advanced Organic Chemistry: Reactions Mechanisms and Structure, 4th Ed. (New York: Wiley-Interscience, 1992), *supra*.

**[00233]** In the following examples, efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.) but some experimental error and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees C and pressure is at or near atmospheric pressure at sea level.

**[00234]** Although other abbreviations known by one having ordinary skill in the art will be referenced, other reagents and materials will be used, and other methods known by one having ordinary skill in the art will be used, the following list and methods description is provided for the sake of convenience.

Abbreviations

mPEG-SPA mPEG-succinimidyl propionate

mPEG-SPC mPEG-succinimidyl phenyl carbonate

mPEG-SBA mPEG-succinimidyl butanoate

mPEG-OPSS mPEG-orthopyridyl-disulfide

mPEG-MAL mPEG-maleimide, $CH_3O$-$(CH_2CH_2O)_n$-$CH_2CH_2$-MAL

mPEG-SMB mPEG-succinimidyl α-methylbutanoate, $CH_3O$-$(CH_2CH_2O)_n$-$CH_2CH_2$-$CH(CH_3)$-C(O)-O-succinimide

mPEG-ButyrALD $H_3O$-$(CH_2CH_2O)_n$-$CH_2CH_2$-O-C(O)-NH-$(CH_2CH_2O)_4$-$CH_2CH_2CH_2C(O)H$

mPEG-PIP $CH_3O$-$(CH_2CH_2O)_n$-$CH_2CH_2$-C(O)-piperidin-4-one

mPEG-CM $CH_3O$-$(CH_2CH_2O)_n$-$CH_2CH_2$-O-$CH_2$-C(O)-OH)

| | |
|---|---|
| anh. | Anhydrous |
| CV | column volume |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| $NaCNBH_3$ | sodium cyanoborohydride |
| HCl | hydrochloric acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| NMR | nuclear magnetic resonance |
| DCC | 1,3-dicyclohexylcarbodiimide |
| DMF | dimethylformamide |
| DMSO | dimethyl sulfoxide |
| DI | deionized |
| MW | molecular weight |
| K or kDa | kilodaltons |
| SEC | Size exclusion chromatography |
| HPLC | high performance liquid chromatography |
| FPLC | fast protein liquid chromatography |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| MALDI-TOF | Matrix Assisted Laser Desorption Ionization Time-of-Flight |
| TLC | Thin Layer Chromatography |
| THF | Tetrahydrofuran |

Materials

**[00235]** All PEG reagents referred to in the appended examples are commercially available unless otherwise indicated.

mPEG reagent preparation

**[00236]** Typically, a water-soluble polymer reagent is used in the preparation of peptide conjugates of the invention. For purposes of the present invention, a water-soluble polymer reagent is a water-soluble polymer-containing compound having at least one functional group that can react with a functional group on a peptide (*e.g.*, the *N*-terminus, the *C*-terminus, a functional group associated with the side chain of an amino acid located within the peptide) to create a covalent bond. Taking into account the known reactivity of the functional group(s) associated with the water-soluble polymer reagent, it is possible for one

of ordinary skill in the art to determine whether a given water-soluble polymer reagent will form a covalent bond with the functional group(s) of a peptide.

**[00237]** Representative polymeric reagents and methods for conjugating such polymers to an active moiety are known in the art, and are, *e.g.*, described in Harris, J.M. and Zalipsky, S., eds, *Poly(ethylene glycol), Chemistry and Biological Applications*, ACS, Washington, 1997; Veronese, F., and J.M Harris, eds., *Peptide and Protein PEGylation*, Advanced Drug Delivery Reviews, 54(4); 453-609 (2002); Zalipsky, S., *et al.*, "*Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides*" in *Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications*, J. M. Harris, ed., Plenus Press, New York (1992); Zalipsky (1995) *Advanced Drug Reviews* 16:157-182, and in Roberts, *et al., Adv. Drug Delivery Reviews,* 54, 459-476 (2002).

**[00238]** Additional PEG reagents suitable for use in forming a conjugate of the invention, and methods of conjugation are described in Shearwater Corporation, Catalog 2001; Shearwater Polymers, Inc., Catalogs, 2000 and 1997-1998, and in Pasut. G., et al., *Expert Opin. Ther. Patents* (2004), 14(5). PEG reagents suitable for use in the present invention also include those available from NOF Corporation (Tokyo, Japan), as described generally on the NOF website (2006) under Products, High Purity PEGs and Activated PEGs. Products listed therein and their chemical structures are expressly incorporated herein by reference. Additional PEGs for use in forming a GLP-1 conjugate of the invention include those available from Polypure (Norway) and from QuantaBioDesign LTD (Powell, Ohio), where the contents of their online catalogs (2006) with respect to available PEG reagents are expressly incorporated herein by reference.

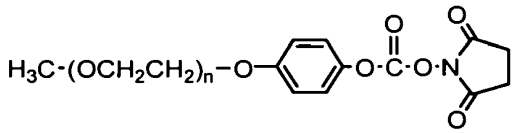
**[00239]** In addition, water-soluble polymer reagents useful for preparing peptide conjugates of the invention is prepared synthetically. Descriptions of the water-soluble polymer reagent synthesis can be found in, for example, U.S. Patent Nos. 5,252,714, 5,650,234, 5,739,208, 5,932,462, 5,629,384, 5,672,662, 5,990,237, 6,448,369, 6,362,254, 6,495,659, 6,413,507, 6,376,604, 6,348,558, 6,602,498, and 7,026,440.

**Example CD-NP1**

*CD-NP-mPEG conjugates*

*a) mPEG-$N^{ter}$-CD-NP via mPEG-SPC*

**[00240]** CD-NP is prepared and purified according to standard automated peptide synthesis or recombinant techniques known to those skilled in the art. An illustrative polymeric reagent, mPEG-SPC reagent,



'SPC' polymer reagent

is covalently attached to the N-terminus of CD-NP, to provide a $N^{ter}$-conjugate form of the peptide. mPEG-SPC 20 kDa, stored at -20 °C under argon, is warmed to ambient temperature. The reaction is performed at room temperature. About 3-5-fold molar excess of mPEG-SPC 20 kDa reagent is used based upon absolute peptide content. The mPEG-SPC reagent is weighed into a glass vial containing a magnetic stirrer bar. A solution of CD-NP prepared in phosphate buffered saline, PBS, pH 7.4 is added and the mixture is stirred using a magnetic stirrer until the mPEG-SPC is fully dissolved. The stirring speed is reduced and the reaction is allowed to proceed to formation of conjugate product. The reaction is optionally quenched to terminate the reaction. The pH of the conjugate solution at the end of the reaction is measured and further acidified by addition of 0.1M HCl, if necessary, to bring the pH of the final solution to about 5.5. The conjugate solution is then analyzed by SDS-PAGE and RP-HPLC (C18) to determine the extent of mPEG-$N^{ter}$-CD-NP conjugate formation.

**[00241]** Using this same approach, other conjugates are prepared using mPEG derivatives having other weight-average molecular weights that also bear an N-hydroxysuccinimide moiety.

*b)* CD-NP -$C^{ter}$-*mPEG*

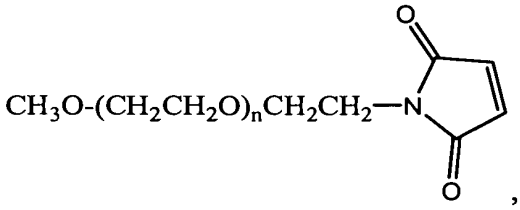
**[00242]** An illustrative polymeric reagent, mPEG-$NH_2$ reagent is covalently attached to the C-terminus of CD-NP, to provide a $C^{ter}$-conjugate form of the peptide. For coupling to the C-terminus, a protected CD-NP (Prot-CD-NP) is prepared and purified according to standard automated peptide synthesis techniques known to those skilled in the art. mPEG-$NH_2$ 20 kDa, stored at -20 °C under argon, is warmed to ambient temperature. The reaction is performed at room temperature. About 5-fold molar excess of mPEG-$NH_2$, PyBOP

(benzotriazol-1-yloxy)tripyrrolidinonophosphonium hexafluorophosphate), and 1-hydroxybenzotriazole (HOBt) are used, based upon absolute peptide content. The mPEG-$NH_2$, PyBOP, HOBt are weighed into a glass vial containing a magnetic stirrer bar. A solution of Prot-CD-NP is prepared in *N, N*-dimethylformamide is added and the mixture is stirred using a magnetic stirrer until the mPEG-$NH_2$ is fully dissolved. The stirring speed is reduced and the reaction is allowed to proceed to formation of conjugate product. The conjugate solution is then analyzed by SDS-PAGE and RP-HPLC (C18) to determine the extent of Prot-CD-NP-*$C^{ter}$-mPEG* conjugate formation. The remaining protecting groups are removed under standard deprotection conditions to yield the CD-NP-*$C^{ter}$-mPEG* conjugate.

**[00243]** Using this same approach, other conjugates are prepared using mPEG derivatives having other weight-average molecular weights that also bear an amino moiety.

*c)* CD-NP-*Cys(S-m*PEG)

**[00244]** mPEG-Maleimide is obtained having a molecular weight of 5 kDa and having the basic structure shown below:



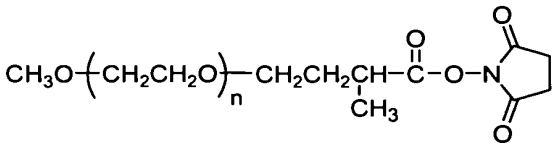
mPEG-MAL, 5kDa

**[00245]** CD-NP, which has a thiol-containing cysteine residue, is dissolved in buffer. To this peptide solution is added a 3-5 fold molar excess of mPEG-MAL, 5 kDa. The mixture is stirred at room temperature under an inert atmosphere for several hours. Analysis of the reaction mixture reveals successful conjugation of this peptide.

**[00246]** Using this same approach, other conjugates are prepared using mPEG-MAL having other weight average molecular weights.

*d) mPEG-$N^{ter}$-CD-NP via mPEG-SMB*

**[00247]** An mPEG-N-Hydroxysuccinimide is obtained having a molecular weight of 5 kDa and having the basic structure shown below:



(mPEG-Succinimidyl α-Methylbutanoate Derivative, 5kDa ("mPEG-SMB"))

**[00248]** mPEG-SMB, 5kDa, stored at -20 °C under argon, is warmed to ambient temperature. A five-fold excess (relative to the amount of the peptide) of the warmed mPEG-SMB is dissolved in buffer to form a 10% reagent solution. The 10% reagent solution is quickly added to the aliquot of a stock CD-NP solution and mixed well. After the addition of the mPEG-SMB, the pH of the reaction mixture is determined and adjusted to 6.7 to 6.8 using conventional techniques. To allow for coupling of the mPEG-SMB to the peptide via an amide linkage, the reaction solution is stirred for several hours (*e.g.,* 5 hours) at room temperature in the dark or stirred overnight at 3-8 °C in a cold room, thereby resulting in a conjugate solution. The reaction is quenched with a 20-fold molar excess (with respect to the peptide) of Tris buffer.

**[00249]** Using this same approach, other conjugates are prepared using mPEG derivatives having other weight-average molecular weights that also bear an N-hydroxysuccinimide moiety.

*e)* CD-NP-*Glu*(O-*m*PEG)

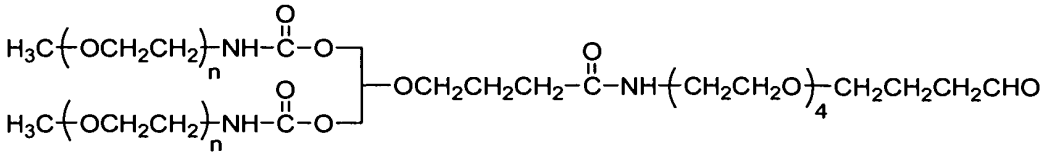
**[00250]** An illustrative polymeric reagent, mPEG-$NH_2$ reagent is covalently attached to the Glu residue of CD-NP, to provide a Glu-conjugate form of the peptide. For coupling to the Glu residue, a protected CD-NP (Prot-CD-NP) is prepared and purified according to standard automated peptide synthesis techniques known to those skilled in the art. Deprotection of the Glu(OBz) residue ($H_2$/Pd) yields the free-Glu carboxylate for subsequent coupling. mPEG-$NH_2$ 20 kDa, stored at -20 °C under argon, is warmed to ambient temperature. The reaction is performed at room temperature. A 5-fold molar excess of mPEG-$NH_2$, PyBOP (benzotriazol-1-yloxy)tripyrrolidinonophosphonium hexafluorophosphate), and 1-hydroxybenzotriazole (HOBt) are used, based upon absolute peptide content. The mPEG-$NH_2$, PyBOP, HOBt are weighed into a glass vial containing a magnetic stirrer bar. A solution of Prot-CD-NP is prepared in *N, N*-dimethylformamide is added and the mixture is stirred using a magnetic stirrer until the mPEG-$NH_2$ is fully dissolved. The stirring speed is reduced and the reaction is allowed to proceed to formation of conjugate product. The conjugate solution is then analyzed by SDS-PAGE and RP-HPLC

(C18) to determine the extent of Prot-CD-NP-(Glu-O-mPEG) conjugate formation. The remaining protecting groups are removed under standard deprotection conditions to yield the CD-NP-*Glu*(O-*m*PEG) conjugate.

**[00251]** Using this same approach, other conjugates are prepared using mPEG derivatives having other weight-average molecular weights that also bear an amino moiety.

**Example BNP2**

**PEGylation of BNP-32 with mPEG2-Butyr-ALD-40K**



**[00252]** A BNP-32 stock solution of 4 mg/mL peptide content was made in 20 mM Na-citrate buffer pH 4.5 in a sterile low-endotoxin polypropylene tube. This solution could be stored aseptically for at least 1 week at 4 °C. Immediately before a PEGylation reaction was performed, a 100 mg/mL stock solution of mPEG-Butyr-ALD-40K was made in the same buffer. A 50 mg/mL solution of sodium-cyanoborohydride (Na-CNHBr) reducing reagent in Milli-Q water was also made immediately before use. A typical PEGylation reaction was carried out as follows: Peptide stock solution (3 mL) was transferred to an appropriate tube containing a magnetic stir-bar and 5.208 mL of the same buffer was added. While stirring, 3.672 mL of a 100 mg/mL solution of mPEG-Butyr-ALD 40K was added dropwise within 1 minute. The reaction was allowed to stir for 15 min after which 0.12 mL of a 50 mg/mL Na-CNHBr solution was added, and the reaction mixture allowed to stir overnight (16 – 18h) at room temperature. The resultant reaction mixture contained 1 mg/mL peptide, 2.0 mol equivalents of PEG (with respect to peptide) and 10 mol equivalents of NaCNBr (with respect to PEG). The reaction rate analysis is shown in Fig. BNP2.1. The reaction yields were determined by reversed phase HPLC to be 80.4% mono-PEG conjugate (N-terminus directed), 8.9% di-PEG conjugate and 10.7% non-conjugated peptide.

**[00253]** The mono-PEGylated conjugate was purified from the reaction mixture by cation exchange chromatography using a Hi Trap SP Sepharose HP media (GE Healthcare). The linear flow rate of the column was 150 cm/h and the sample loading was 2.0 mg/mL of column bed volume (CV) with a column bed height of 10cm. The buffers used for purification were: Buffer A: 10 mM $NaPO_4$, pH 7.0 and Buffer B: Buffer A + 0.5 M NaCl.

**[00254]** The PEGylation reaction mixture was diluted with 4 volumes of buffer A and the pH adjusted to 8.0. The column was equilibrated in buffer A. The diluted reaction mixture was loaded onto the column and unbound substances washed off the column with 3 column volumes of buffer A. The conjugated peptide was eluted from the column using a linear gradient of 0 – 100% B over 10 CV. A typical chromatogram is shown in Fig. BNP2.2. The purity of the conjugate was 99.5% (by RP-HPLC analysis, Fig. BNP2.3) and the mass (as determined by MALDI-TOF, Fig. BNP2.4) was within the expected range. The detection wavelength for preparative and analytical chromatography was 225 nm.

**[00255]** Samples were analyzed using reversed-phase HPLC. The mobile phases were A, 0.1% TFA in water and B, 0.05% TFA in acetonitrile. An Agilent Poroshell 300-SB-C8 (P/N 660750-906) column was used with a flow of 0.5 ml/min and column temperature of 50 °C. The column was equilibrated in 10% B and conjugate separation was achieved using the gradient timetable shown in Table BNP2.1 below.

| Time (min) | %B |
|---|---|
| 0 | 10 |
| 2 | 10 |
| 5.5 | 45 |
| 10.5 | 65 |
| 10.6 | 95 |
| 13.6 | 95 |
| 13.7 | 10 |
| Post run | 5 min |

**[00256]** Fig. BNP2.1. PEGylation rate of BNP-32 with mPEG2-40kDa Butyr-ALD. The reaction yields were 80.4% mono-PEG conjugate, 8.9% di-PEG conjugate and 10.7% remaining non-PEGylated peptide after 18h reaction time. Yields were determined by RP-HPLC.

**[00257]** Fig. BNP2.2. Typical purification profile for the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32. The mono-PEGylated conjugate is indicated. The di-PEG conjugate eluted during the loading step.

**[00258]** Fig BNP2.3. HPLC analysis of the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32. The mono- and di-PEGylated forms of BNP-32 are indicated. The peak at 8 min retention time was instrument related and not any product of interest.

**[00259]** Fig. BNP2.4. MALDI-TOF analysis of the 40 kDa mPEG2-Butyr-ALD mono-PEG conjugate of BNP-32. The detected mass of the major peak was 45138 Da, which was within the expected range for the mono-conjugate.

**[00260]** Fig. BNP2.5. SDS-PAGE (4-12% Bis-Tris-Nu-PAGE, Invitrogen) analysis of BNP-32 and purified [mono]-[mPEG2-Butyr-ALD-40K]-[BNP-32] conjugate. . Lanes 1, 2 and 3 are 0.5, 1.0 and 2.0 μg of the non-PEGylated peptide respectively. Lanes 4, 5 and 6 are 0.5, 1.0 and 2.0 μg of the purified mono-PEG-conjugate, respectively.

## Example BNP3

**Site specific acetylation of brain natriuretic peptide (BNP-32)**

**[00261]** Specific amine sites can be blocked by acetylation leaving other sites open for PEGylation. BNP-32 is composed of 32 amino acids with a single disulfide bond. The peptide contains 3 lysine residues and an N-terminus containing a free amine group. Previous PEGylation studies with BNP-32 indicate that all four amine groups are sterically accessible for reaction with PEG reagents. (Miller *et al.*, Bioconjugate Chemistry 2006 Mar-Apr;17(2):267-74). In the current study, the pKa difference between the N-terminal amine and the epsilon amines of the lysine residues was used to specifically acetylate the N-terminus, leaving the lysine amines available for PEGylation.

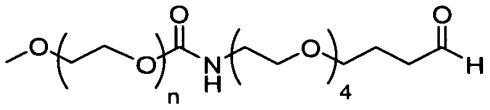
**[00262]** One milligram of BNP-32 was combined with 2 mol equivalents of acetic acid-NHS (previously dissolved in 2 mM HCl) in a total volume of 1 mL in 20 mM MES buffer at pH 6.0 and incubated at room temperature for 2 h. At this pH, one predominant acetylated product was formed based on RP-HPLC analysis. Based on accepted chemical principles known to those skilled in the art, at pH 6.0 the N-terminal amine group is more reactive than the epsilon amines and acetylation would occur predominantly at this position. Also, at lower pH, all amines are less reactive while at higher pH all amines are more reactive. The reaction above was also performed at other pH levels: At pH 4.5 (20 mM citrate buffer) there was significantly lower acetylation for all amine groups, while at pH 7.5 (20 mM HEPES buffer) and pH 9.0 (20 mM boric acid buffer), all amine groups were more reactive and significant acetylation occurred at all four sites as assed by RP-HPLC. Site specificity of the purified reaction products may also be confirmed using methods known to the art such as peptide mapping.

**[00263]** The predominant acetylated product from the reaction performed at pH 6.0 can be purified by standard chromatographic methods. The acetylated peptide can then be

PEGylated using any of the reagents that are specific for amine reactive groups and standard methods known to the art, again followed by standard chromatographic methods to purify the conjugate of interest.

## Example BNP4

**PEGylation of BNP-32 with [mPEG-Butyr-ALD-10K]**



**[00264]** A BNP-32 stock solution of 4 mg/mL peptide content was made in 20 mM sodium-citrate buffer pH 4.5 in a sterile low-endotoxin polypropylene tube. This solution could be stored aseptically for at least 1 week at 4 °C. Immediately before a PEGylation reaction was performed, a 100 mg/mL stock solution of [mPEG-Butyr-ALD-10K] was made in the same buffer used to dissolve the peptide. A 50 mg/mL solution of sodium-cyanoborohydride (Na-CNHBr) reducing reagent in Milli-Q water was also made immediately before use. A typical PEGylation reaction was carried out as follows: Peptide stock solution (3 mL, 12 mg) was transferred to an appropriate tube containing a magnetic stir-bar and 8.11 mL of 20 mM sodium-citrate buffer pH 4.5 was added. While stirring, 0.77 mL of a 100 mg/mL solution of mPEG-Butyr-ALD 10K was added drop wise within 1 minute. The reaction was allowed to stir for 15 min after which 0.12 mL of a 50 mg/mL Na-CNHBr solution was added, and the reaction mixture allowed to stir overnight (16 – 18h) at room temperature. The resultant reaction mixture contained 1 mg/mL peptide, 2.0 mol equivalents of PEG (with respect to peptide) and 10 mol equivalents of NaCNBr (with respect to PEG). The reaction yields were determined by reversed phase HPLC to be 76 % mono-PEG conjugate (N-terminus directed), 10.6 % di- and tri-PEG conjugate and 13.4 % non-conjugated peptide. This PEG reagent forms stable bonds with amine groups.

**[00265]** The mono-PEGylated conjugate was purified from the reaction mixture by cation exchange chromatography using Hi Trap SP Sepharose HP media (GE Healthcare). The linear flow rate of the column was 150 cm/h and the sample loading was 2.0 mg/mL of column bed volume (CV) with a column bed height of 10cm. The buffers used for purification were: Buffer A: 10 mM $NaPO_4$, pH 7.0 and Buffer B: Buffer A + 0.5 M NaCl. The PEGylation reaction mixture was diluted with 4 volumes of buffer A and the pH adjusted to 8.0 with 0.1 M sodium hydroxide. The column was equilibrated in buffer A. The diluted reaction mixture was loaded onto the column and unbound substances washed off the column with 3 column volumes of buffer A. The conjugated peptide was

eluted from the column using a linear gradient of 0 – 100% B over 10 CV. The detection wavelength for preparative and analytical chromatography was 225 nm.

**[00266]** Fractions collected during cation exchange chromatography were analyzed using reversed-phase HPLC. The mobile phases were: A, 0.1% TFA in water and B, 0.05% TFA in acetonitrile. An Agilent Poroshell 300-SB-C8 (P/N 660750-906) column was used with a flow of 0.5 ml/min and column temperature of 50 °C. The column was equilibrated in 10% B and conjugate separation was achieved using the gradient timetable shown in Table 2.1.

| Time (min) | %B |
|---|---|
| 0 | 10 |
| 2 | 10 |
| 5.5 | 45 |
| 10.5 | 65 |
| 10.6 | 95 |
| 13.6 | 95 |
| 13.7 | 10 |
| Post run | 5 min |

**[00267]** Fractions containing pure [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32] as determined by RP-HPLC were pooled and stored in aliquots at -80 °C as the purified conjugate.

**[00268]** A typical cation-exchange chromatogram is shown in Fig. BNP4.1. SDS-PAGE analysis of BNP-32 and purified [mono]-[mPEG2-Butyr-ALD-10K]-[BNP-32] conjugate is shown in Fig. BNP4.2. RP-HPLC analysis of the purified conjugate is shown in Fig. BNP4.3, and MALDI-TOF analysis of the purified conjugate is shown in Fig. BNP4.4. The purity of the mono-PEG-conjugate was 98% by SDS-PAGE analysis and 98.4% by RP-HPLC analysis with 1.6% of di-PEG-conjugate. The mass as determined by MALDI-TOF was within the expected range.

**[00269]** Fig. BNP4.1. Typical cation-exchange purification profile of [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32]. The PEGylated conjugates and the free peptide peaks are indicated.

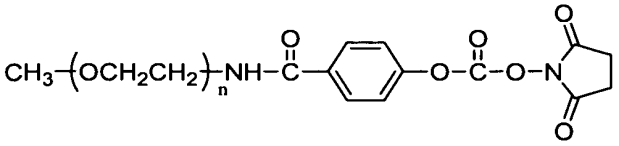
**[00270]** Fig. BNP4.2. SDS-PAGE (4-12% Bis-Tris-Nu-PAGE, Invitrogen) analysis of BNP-32 and the purified [mono]-[mPEG2-Butyr-ALD-40K]-[BNP-32] conjugate.. Lane 1: BNP-32 peptide only (1 μg); Lanes 2, 3 and 4 are 0.5, 1.0 and 2.0 μg of the purified mono-PEG-conjugate, respectively.

**[00271]** Fig BNP4.3. RP-HPLC analysis of the purified [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32] conjugate.. The peaks at 7.851 and 8.396 min contain the mono-PEG and di-PEG conjugates, respectively.

**[00272]** Fig. BNP4.4. MALDI-TOF analysis of the purified [mono]-[mPEG-Butyr-ALD-10K]-[BNP-32] conjugate. The detected mass of the major peak was 14568 Da, which was within the expected range for the mono-PEG conjugate. The peak at 7232 Da represents the doubly charged conjugate.

## Example BNP5

**PEGylation of BNP-32 with releasable [mPEG-SBC-30K]**



**[00273]** A BNP-32 stock solution of 4 mg/mL peptide content was made in 20 mM MES buffer pH 6.0 in a sterile low-endotoxin polypropylene tube. This solution could be stored aseptically for at least 1 week at 4 °C.

**[00274]** A typical PEGylation reaction was carried out as follows: [mPEG-SBC-30K] PEG reagent (1220 mg) was weighed-out in an appropriate tube and dissolved with stirring in 9 ml of the same buffer used to dissolve the peptide. After the PEG had dissolved and with stirring, 3.0 mL of the peptide solution was added. The reaction was allowed to stir for 10 min at room temperature. The resultant reaction mixture contained 1 mg/mL peptide and 8.0 mol equivalents of PEG. After the incubation period, 1/9 volume of a 1 M glycine solution (in the same buffer) was added to quench the reaction. After a further 60 min of stirring at room temperature, 1 volume of 0.2 M acetic acid was added to stabilize the conjugate and the reaction mixture was stored at -20 °C. The reaction yielded >80% mono-PEG conjugate. The mPEG SBC reagent forms hydrolysable bonds with amine groups and upon hydrolysis, leaves the peptide modified (tagged).

**[00275]** The mono-PEGylated conjugate was purified from the reaction mixture by cation exchange chromatography using Hi Trap SP Sepharose HP media (GE Healthcare). The linear flow rate of the column was 150 cm/h and the sample loading was 2.0 mg/mL of column bed volume (CV) with a column bed height of 10cm. The buffers used for purification were: Buffer A: 10 mM $NaPO_4$, pH 7.0 and Buffer B: Buffer A + 0.5 M NaCl. The PEGylation reaction mixture was diluted with 4 volumes of buffer A and the pH adjusted to 8.0 with 0.1 M sodium hydroxide. The column was equilibrated in buffer A. The diluted reaction mixture was loaded onto the column and unbound substances washed off the column with 3 column volumes of buffer A. The conjugated peptide was eluted from the column using a linear gradient of 0 – 100% B over 10 CV. The pooled mono-PEGylated fraction was diluted with 4 volumes of buffer A and the purification step repeated. The detection wavelength for preparative and analytical chromatography was 225 nm.

**[00276]** Fractions collected during cation exchange chromatography were analyzed using reversed-phase HPLC. The mobile phases were A, 0.1% TFA in water and B, 0.05% TFA in acetonitrile. An Agilent Zorbax 5 μm 300-SB-C18, 4.5 X 50 mm (P/N 860950-902) column was used with a flow of 1.0 ml/min and column temperature of 60 °C. The column was equilibrated in 10% B and conjugate separation was achieved using the gradient timetable shown in Table BNP5.1 below.

| Time (min) | %B |
|---|---|
| 0 | 10 |
| 2 | 10 |
| 4 | 30 |
| 8 | 34 |
| 10.2 | 56 |
| 16.2 | 62 |
| 16.3 | 90 |
| 17.0 | 90 |
| 17.01 | 10 |
| Post run | 5 min |

**[00277]** Fractions containing pure [mono]-[mPEG-SBC-30K]-[BNP-32] from the repeat cation-eschange chromatography as determined by RP-HPLC were pooled and stored in aliquots at -80 °C as the purified conjugate.

**[00278]** A typical cation-exchange purification chromatogram is shown in Fig. BNP5.1. SDS-PAGE analysis of purified [mono]-[mPEG-SBC-30K]-[BNP-32] is shown in Fig. BNP5.2. RP-HPLC analysis of the purified conjugate is shown in Fig. BNP5.3, and MALDI-TOF analysis of the purified product is shown in Fig. BNP5.4. The purity of the mono-PEG-conjugate was 95.8% by RP-HPLC analysis with 4.2% di-PEG conjugate also present. The mass as determined by MALDI-TOF was within the expected range.

**[00279]** Fig. BNP5.1. Typical first cation-exchange purification profile for [mono]-[mPEG-SBC-30K]-[BNP-32]. The mono- and di-PEGylated conjugates are indicated. The free peptide eluted in two peaks. On release, this PEG reagent leaves a modified (tagged) peptide. Peak 1 and peak 2 contain modified and unmodified peptide, respectively.

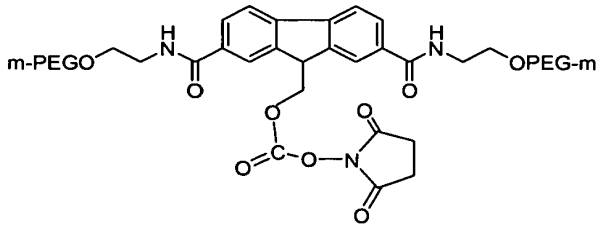
**[00280]** Fig. BNP5.2. SDS-PAGE (4-12% Bis-Tris-Nu-PAGE, Invitrogen) analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate. Lanes 1,2 and 3 are 0.7, 1.4 and 2.1 μg of the PEGylated peptide, respectively.

**[00281]** Fig BNP5.3. RP-HPLC analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate. The peaks at 12.041 min and 12.726 retention times contain the mono-PEG and di-PEG conjugates, respectively.

**[00282]** Fig. BNP5.4. MALDI-TOF analysis of the purified [mono]-[mPEG-SBC-30K]-[BNP-32] conjugate. The detected mass of the major peak was 32580 Da, which was within the expected range for the mono-PEG-conjugate. The peak at 17444 Da represents the doubly charged conjugate.

**Example BNP6**

**PEGylation of BNP-32 with [mPEG2-C2-fmoc-NHS-40K]**




**[00283]** A BNP-32 stock solution of 4 mg/mL peptide content was made in 20 mM MES buffer pH 5.8 in a sterile low-endotoxin polypropylene tube. This solution could be stored aseptically for at least 1 week at 4 °C.

**[00284]** Immediately before a PEGylation reaction was performed, a 100 mg/mL stock solution of [mPEG2-C2-fmoc-NHS-40K] PEG reagent was made in the same buffer used to dissolve the peptide. A typical PEGylation reaction was carried out as follows: Peptide stock solution (6 mL, 24 mg) was transferred to an appropriate tube containing a magnetic stir-bar and 10.16 mL of 20 mM MES buffer pH 5.8 was added. While stirring, 7.84 mL of a 100 mg/mL PEG reagent solution was added. The resultant reaction mixture contained 1 mg/mL peptide and 2 mol equivalents of PEG. The reaction was allowed to stir for 90 min at room temperature after which a 1/9 volume of 0.2 M glycine solution (in 20 mM MES buffer pH 5.8) was added and the reaction mixture stirred for another 60 min to quench the reaction. These reaction conditions yielded approximately 60% mono-PEGylated peptide. This PEG reagent forms hydrolysable bonds with amine groups and upon hydrolysis, an unmodified peptide is generated.. The reaction mixture was stored at 4 °C.

**[00285]** The mono-PEGylated conjugate was purified from the reaction mixture by cation exchange chromatography using Hi Trap SP Sepharose HP media (GE Healthcare). The linear flow rate of the column was 150 cm/h and the sample loading was 1.0 mg/mL of column bed volume (CV) with a column bed height of 11cm. The buffers used for purification were: Buffer A: 10 sodium-citrate, pH 4.0 and Buffer B: Buffer A + 0.8 M NaCl. The PEGylation reaction mixture was diluted with 4 volumes of buffer A. The column was equilibrated in buffer A. The diluted reaction mixture

was loaded onto the column and unbound substances washed off the column with 3 column volumes of buffer A. The conjugated peptides were eluted from the column using the following elution steps: (a) linear gradient of 0 – 4% B over 1 CV followed by a hold at 4% B for 4 CV; (b) linear gradient of 4 – 50% B over 5 CV followed by a hold at 50% B for 1 CV; (c) step gradient to 80% B followed by a hold at 80% B for 2 CV. The pooled mono-PEGylated fraction was diluted with 4 volumes of buffer A and the purification step repeated. The detection wavelength for preparative and analytical chromatography was 225 nm.

**[00286]** Fractions collected during cation exchange chromatography were analyzed using reversed-phase HPLC. The mobile phases were A, 0.1% TFA in water and B, 0.05% TFA in acetonitrile. An Agilent Zorbax XDB-C8, 5 μm, 4.5 X 150 mm (P/N 993967-906) column was used with a flow of 0.5 ml/min and column temperature of 60 °C. The column was equilibrated in 10% B and conjugate separation was achieved using the gradient timetable shown in Table BNP6.1 below.

| Time (min) | %B |
|---|---|
| 0 | 10 |
| 4 | 10 |
| 9 | 35 |
| 10.5 | 50 |
| 23 | 75 |
| 24 | 95 |
| 25 | 95 |
| 25.2 | 10 |
| Post run | 6 min |

**[00287]** Fractions containing pure [mono]-[mPEG2-C2-fmoc-NHS-40K]-[BNP-32] from the repeat cation-eschange chromatography as determined by RP-HPLC were pooled and stored in aliquots at -80 °C as the purified conjugate.

**[00288]** A typical first cation-exchange purification chromatogram is shown in Fig. BNP6.1. SDS-PAGE analysis of purified [mono]-[mPEG2-C2-fmoc-NHS-40K]-[BNP-32] is shown in Fig. BNP6.2. RP-HPLC analysis of the purified conjugate is shown in Fig. BNP6.3, and MALDI-TOF analysis of the purified conjugate is shown in Fig. BNP6.4. The purity of the mono-PEG-conjugate was 100% by RP-HPLC analysis and >95% (by SDS-PAGE). The mass as determined by MALDI-TOF was within the expected range.

**[00289]** Fig. BNP6.1. Typical first cation-exchange purification profile of [mPEG2-C2-fmoc-NHS-40K]. The mono-, di- and non-PEGylated (free peptide) elution peaks are indicated.

**[00290]** Fig. BNP6.2. SDS-PAGE (4-12% Bis-Tris-Nu-PAGE, Invitrogen) analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate. Lanes 1 and 2 are 1.0 and 2.0

μg of the PEGylated peptide, respectively. Low levels of hi-PEGylated forms are also visible.

**[00291]** Fig BNP6.3. RP-HPLC analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate.. Fig. BNP6.4. MALDI-TOF analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate.

**[00292]** Fig. BNP6.4. MALDI-TOF analysis of the purified [mPEG2-C2-fmoc-NHS-40K]-[BNP-32] conjugate. The detected mass of the major peak was 44725 Da, which was within the expected range for the mono-PEG-conjugate.

**Example BNP7**

**[00293]** Pharmacokinetic Studies

**[00294]** Thirty one (31) adult male Sprague-Dawley rats with indwelling jugular vein and carotid artery catheters (JVC/CAC) (Charles River Labs, Hollister, CA) were utilized for this study. The weight range of the animals was 315 - 358 grams. All animals were food fasted overnight. Prior to dosing, the rats were weighed, the tails and cage cards were labeled for identification and the doses were calculated. Anesthesia was induced and maintained with 3.0-5.0% isoflurane. The JVC and CAC were externalized and flushed with HEP/saline (10 IU/mL HEP/ mL saline). The predose sample was collected from the JVC and the catheters were plugged, and labeled to identify the jugular vein and carotid artery. When all of the animals had recovered from anesthesia and the predose samples were processed, the animals were dosed, intravenously (IV) via the JVC using a 1 mL syringe containing the appropriate test article and the dead volume of the catheter was flushed with 0.9% saline to ensure the animals received the correct dose.

**[00295]** Following a single IV dose, blood samples were collected from groups 1A, 2A, 3A and 4A, at 0 (pre-dose collected as described above), 0.03, 0.33, 2.0, 6.0, 12.0 and 72.0 hours and from Groups 1B, 2B, 3B and 4B at 0 (pre-dose collected as described above), 0.17, 1.0, 4.0, 8.0, 24.0 and 48.0 hours via the carotid artery catheter and processed as stated in the protocol. Following the last collection point, the animals were euthanized.

**[00296]** Pharmacokinetic Analyses: Noncompartmental PK data analysis and report preparation was completed by Research Biology at Nektar Therapeutics (India) Pvt. Ltd. Hyderabad, A.P., India. Individual plasma concentration data are listed and summarized in Appendix A1.1-1.3. PK analysis was performed using WinNonlin (Version 5.2, Mountain View, CA-94014). Concentrations in plasma that were below LLOQ were replaced with

zeros prior to generating Tables and PK analysis. In the event that more than half (>50%) of the data points were below zero, mean concentration will not be shown in the figures or used in PK parameters estimation. The following PK parameters were estimated using plasma concentration-time profile of each animal:

C0 Extrapolated concentration to time "zero"

Cmax Maximum (peak) concentration

AUCall Area under the concentration-time from zero to time of last concentration value

T1/2(Z) Terminal elimination half-life

AUCinf Area under the concentration-time from zero to time infinity

Tmax Time to reach maximum or peak concentration following administration

CL Total body clearance

Vz Volume of distribution based on terminal phase

Vss Volume of distribution at steady state

MRTlast Mean residence time to last observable concentration

**[00297]** Releasable-PEG:

**[00298]** Figure BNP7.1 shows the mean plasma concentration-time profiles of for C2-FMOC-PEG2-40K-BNP, its corresponding metabolite (free PEG) and released BNP. No measurable plasma concentrations observed after BNP administration and hence the data is not shown in Figure BNP7.1. At first time point collection which was at 0.03 hr, concentration was < 20 ng/mL in all the animals.

**[00299]** Table BNP7.1 summarizes the PK parameters of BNP following equivalent protein mass of 0.459 mg/kg administered intravenously into rats via C2-FMOC-PEG2-40K-BNP or BNP.

Table 1. Comparative PK Parameters of BNP Released from C2-FMOC-PEG2-40K-BNP in BNP Given as Non-Conjugated Native Protein

| Test Article | Cmax ( ng/mL) | T1/2 (hr) | AUCINF (ng.hr/mL) | Tmax (hr) | MRTlast (hr) |
|---|---|---|---|---|---|
| BNP | 0.00 | NC | NC | NC | NC |
| C2-FMOC-PEG2-40K-BNP | 55.4 | 1.25 | 162 | 0.33 | 1.84 |

NC- Cannot be calculated.

**[00300]** Figure BNP7.2 shows the non-released PEG-BNP levels after the administration of the two non-releasable PEG constructs (ButyrALD-40K-BNP, ButyrALD-10K-BNP). Table BNP7.2 summarizes the PK parameters of following equivalent protein mass of 0.459 mg/kg administered intravenously into rats.

**[00301]** Table BNP7.2. Comparative PK Parameters of Test Articles (Non-Releasable-PEG Conjugates) versus Native BNP Following Equivalent Protein Mass Intravenous Administration to Sprague Dawley rats (Mean ± SD)

| Test Compound | Cmax (ng/mL) | T1/2 (hr) | AUCINF (ng.hr/mL) | MRTlast (hr) | CL (mL/hr/kg) | Vss (mL/kg) |
|---|---|---|---|---|---|---|
| BNP | 0.00 | NC | NC | NC | NC | NC |
| ButyrALD-40K-BNP | 1410 | 26.1 | 41300 | 24.0 | 11.1 | 631 |
| ButyrALD-10K-BNP | 355 | 0.272 | 96.6 | 0.368 | 4750 | 2270 |

NC- Cannot be calculated, there were no measurable plasma concentrations.

**[00302]** BNP concentrations were <LLOQ (LLOQ: 20 ng/mL) and therefore, no PK Parameters were reported.

**[00303]** BNP released from C2-FMOC-PEG2-40K-BNP reached peak concentrations of 55.4 ng/mL at 0.3 h and stayed above 20 ng/mL for 8 hr following C2-FMOC-PEG2-40K-BNP dosing. Half-life value for released BNP is 1.25 h following C2-FMOC-PEG2-40K-BNP IV bolus administration. Peak concentrations of 1300 ng/mL, a half-life of 15.0 hr and with plasma C2-FMOC-PEG2-40K-BNP concentrations remained above 100 ng/mL up to 24 h supported the prolonged release of BNP in plasma. The observed release of BNP from releasable-PEG C2-FMOC-PEG2-40K-BNP is consistent with the appearance of free PEG-metabolite (PEG-fulvene) which was also released from the conjugate. Binding to cell surface clearance receptors with internalization and degradation, proteolytic cleavage and renal filtration are the possible route of elimination for releasable C2-FMOC-PEG2-40K-BNP.

**[00304]** For the non-releasable PEG-constructs, ButyrALD-40K-BNP was observed to have longer half-life, lower clearance and higher exposure than ButyrALD-10K-BNP,

probably due to increased PEG-length of the conjugate. No BNP was measurable in plasma following parent BNP administration.

**[00305]** Due to staggered sample collection, two very distinct concentration-time profiles were observed for two subgroups received ButyrALD-40K-BNP treatment. Therefore, the PK parameters estimated from the pooled data from the two subgroups to be interpreted with caution. ButyrALD-40K-BNP showed higher peak plasma concentration, approximately higher exposure and longer half-life than ButyrALD-10K-BNP when compared using pooled data.

**What is claimed is:**

1. A conjugate comprising a residue of a CD-NP moiety covalently attached, either directly or through a spacer moiety of one or more atoms, to a water-soluble, non-peptidic polymer.

2. A conjugate of claim 1, wherein the polymer is a linear polymer.

3. A conjugate of claim 1, wherein the polymer is a branched polymer.

4. The conjugate of any one of claims 1, 2 and 3, wherein the CD-NP moiety is recombinantly prepared.

5. The conjugate of any one of claims 1, 2 and 3, wherein the CD-NP moiety is prepared by chemical synthesis.

6. The conjugate of any one of claims 1 to 5, wherein the polymer is selected from the group consisting of poly(alkylene oxide), poly(vinyl pyrrolidone), poly(vinyl alcohol), polyoxazoline, and poly(acryloylmorpholine).

7. The conjugate of claim 6, wherein the polymer is a poly(alkylene oxide).

8. The conjugate of claim 7, wherein the poly(alkylene oxide) is a poly(ethylene glycol).

9. The conjugate of claim 8, wherein the poly(ethylene glycol) is terminally capped with an end-capping moiety selected from the group consisting of hydroxy, alkoxy, substituted alkoxy, alkenoxy, substituted alkenoxy, alkynoxy, substituted alkynoxy, aryloxy and substituted aryloxy.

10. The conjugate of claim 8 , wherein the the poly(ethylene glycol) has a weight-average molecular weight in a range of from about 500 Daltons to about 100,000 Daltons.

11. The conjugate of claim 10, wherein the the poly(ethylene glycol) has a weight-average molecular weight in a range of from about 2000 Daltons to about 50,000 Daltons.

12. The conjugate of claim 11, wherein the the poly(ethylene glycol) has a weight-average molecular weight in a range of from about 5000 Daltons to about 40,000 Daltons.

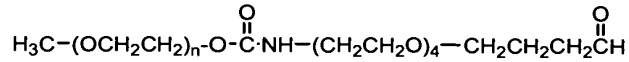
13. The conjugate of any of the preceding claims, wherein the water-soluble, non-peptidic polymer is conjugated at an amino-terminal amino acid of the CD-NP moiety.

14. The conjugate of any of the preceding claims, wherein the water-soluble, non-peptidic polymer is conjugated at a carboxy-terminal amino acid of the CD-NP moiety.

15. The conjugate of any of the preceding claims, wherein the water-soluble, non-peptidic polymer is conjugated at an internal cycteine amino acid of the CD-NP moiety.

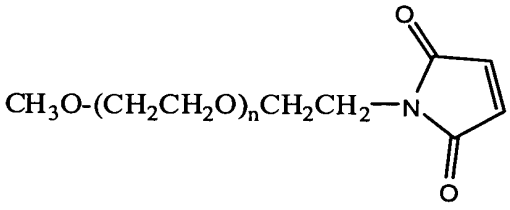
16. The conjugate of any of the preceding claims, wherein the water-soluble, non-peptidic polymer is conjugated at an epsilon amino group of an internal lysine amino acid of the CD-NP moiety.

17. The conjugate of claim 8, wherein the polymeric reagent has the following structure:



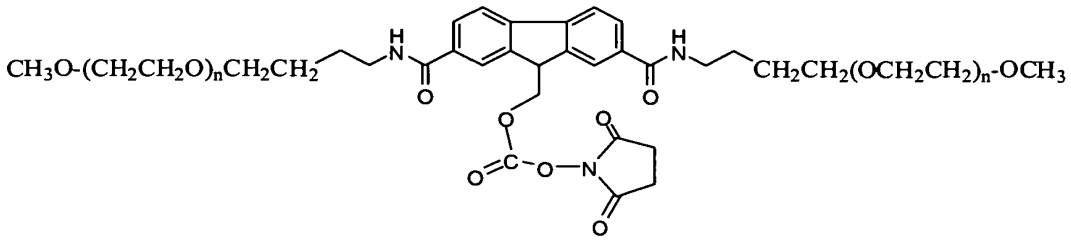
wherein, (n) is independently an integer having a value of from about 2 to about 4000.

18. The conjugate of claim 8, wherein the polymeric reagent has the following structure:



Wherein, (n) is independently an integer having a value of from about 2 to about 4000.

19. The conjugate of claim 8, wherein the polymeric reagent has the following structure:



wherein, (n) is independently an integer having a value of from about 2 to about 4000.

20. The conjugate of any one of the preceding claims, wherein the CD-NP residue is covalently attached through a spacer moiety of one or more atoms.

21. The conjugate of claim 20, wherein the spacer moiety includes an amine linkage.

22. The conjugate of claim 20, wherein the spacer moiety includes an amide linkage.

23. The conjugate of claim 20, wherein the spacer moiety includes a disulfide linkage.

24. The compound of any one of the preceding claims, wherein the CD-NP residue is covalently attached via a stable linkage.

25. The compound of any one of the preceding claims, wherein the CD-NP residue is covalently attached via a releasable linkage.

26. A pharmaceutical composition comprising a conjugate of any one of the preceding claims and a pharmaceutically acceptable excipient.

27. A method for making a conjugate comprising contacting, under conjugation conditions, a CD-NP moiety with a polymeric reagent bearing a functional group.

28. A method of treatment comprising administering a compound of any of the preceding claims to a subject in need thereof.

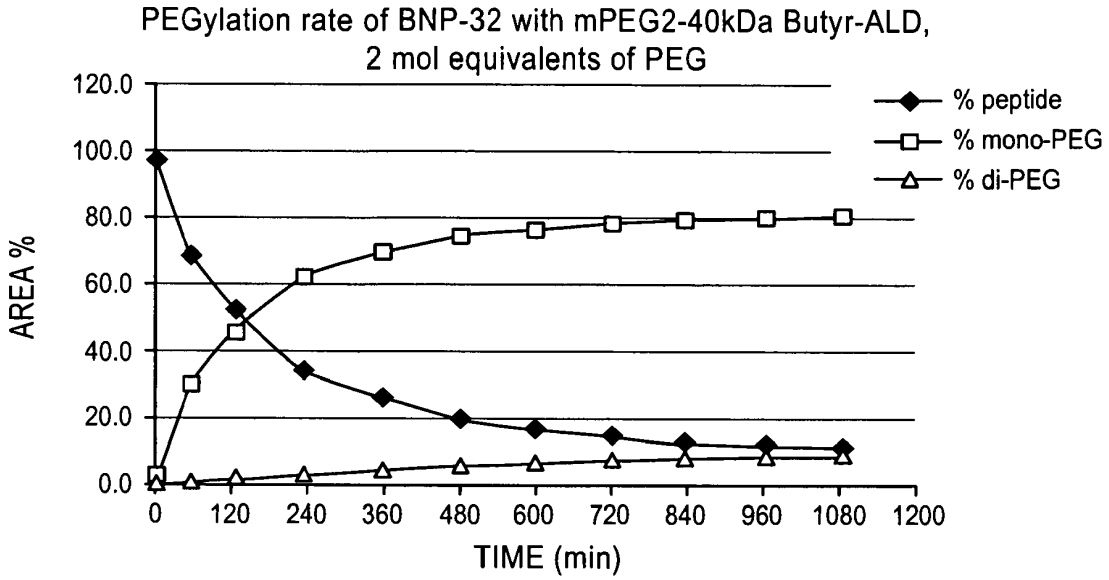
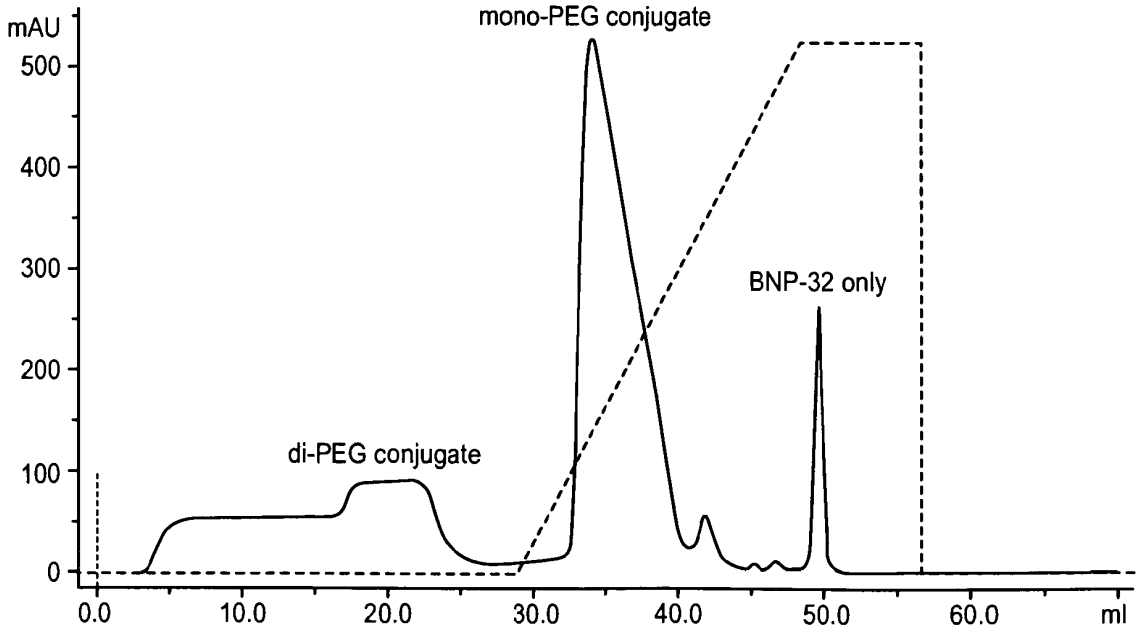



Figure BNP2.1

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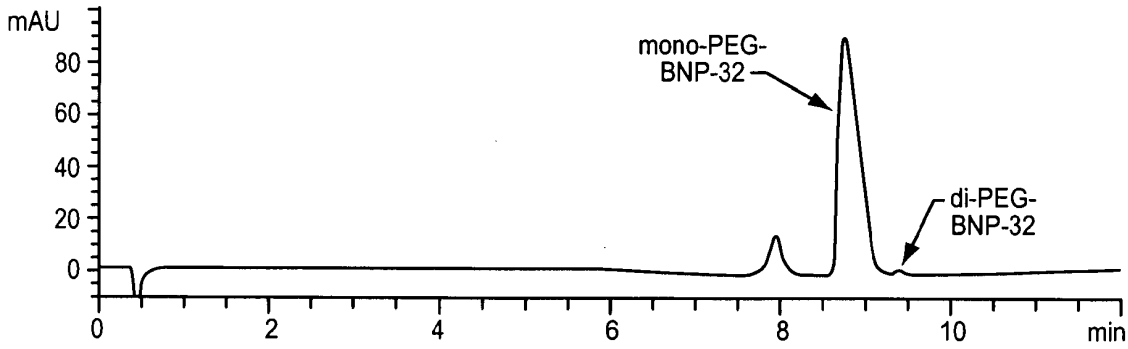


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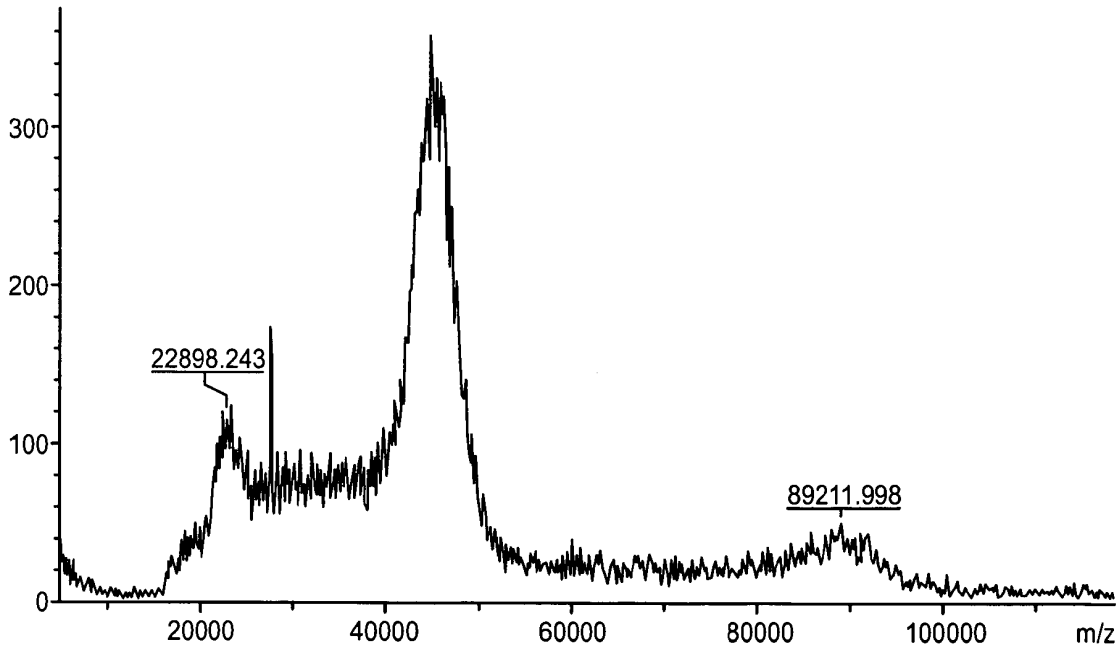


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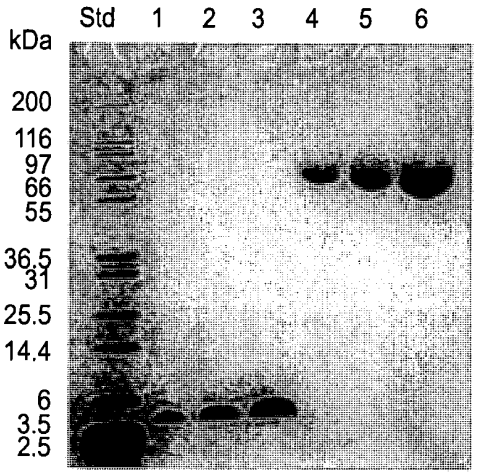


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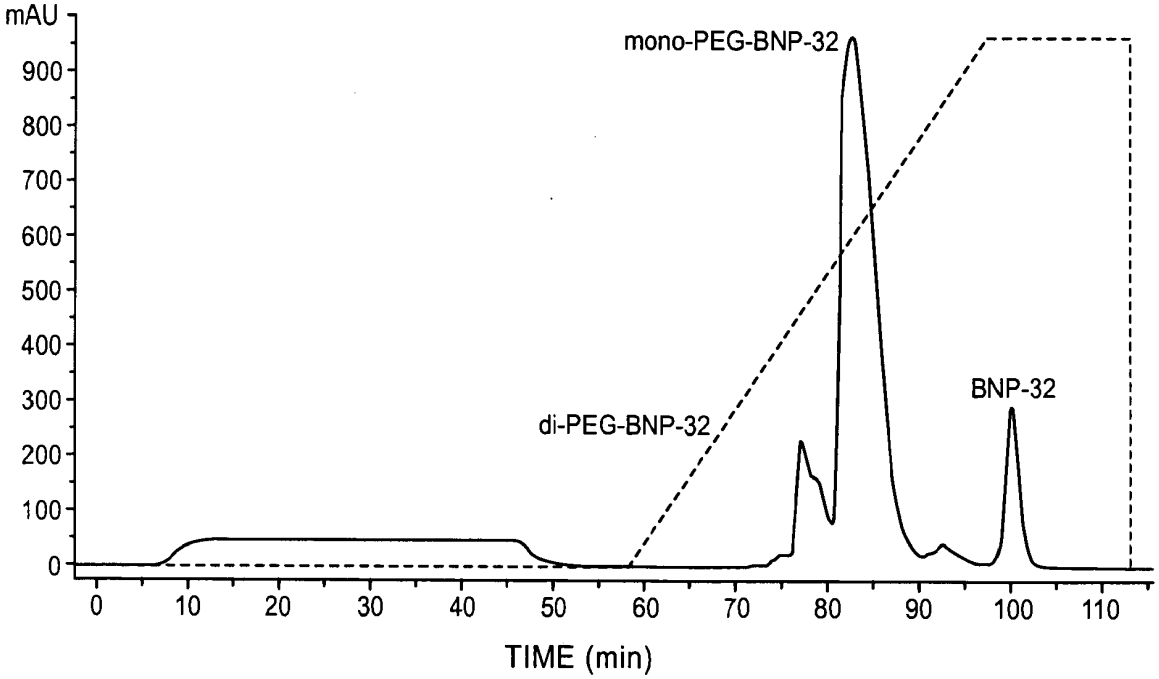


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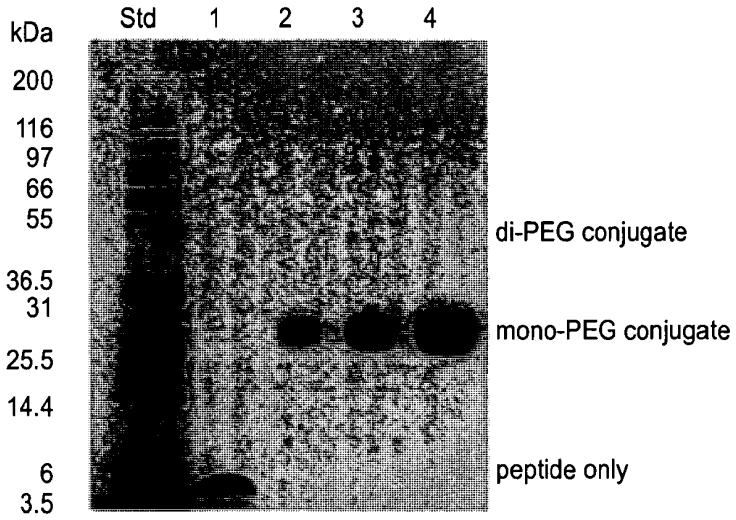


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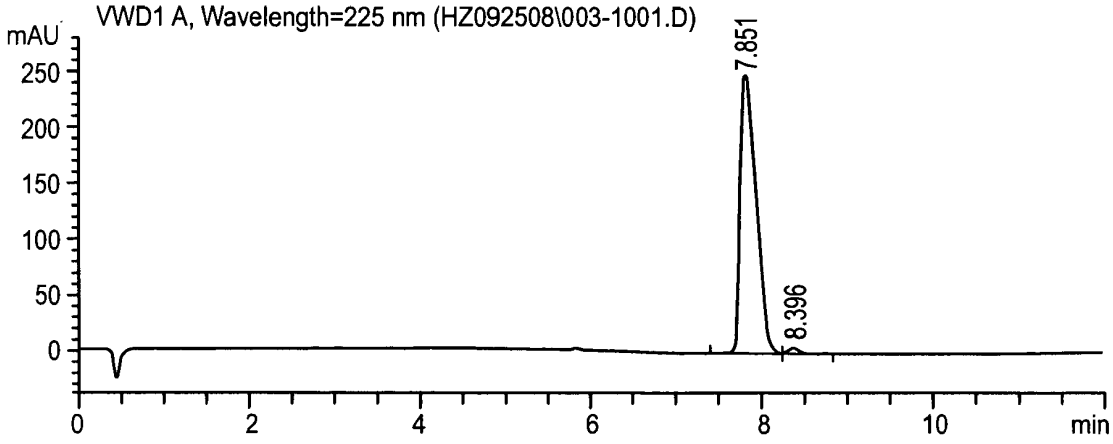


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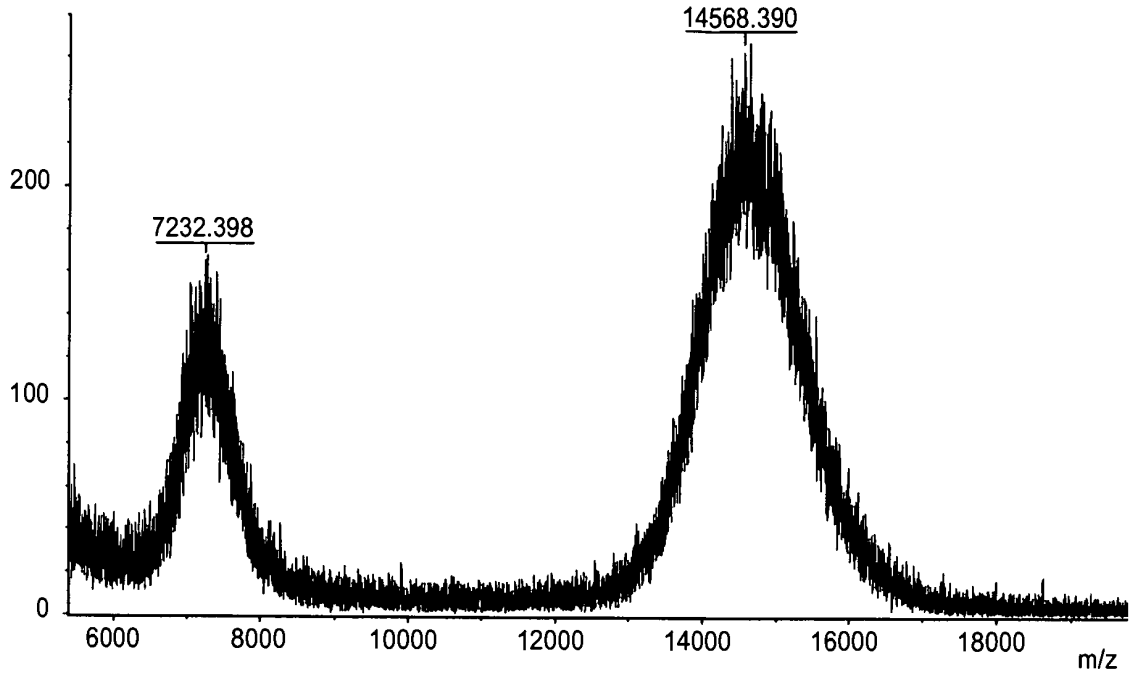
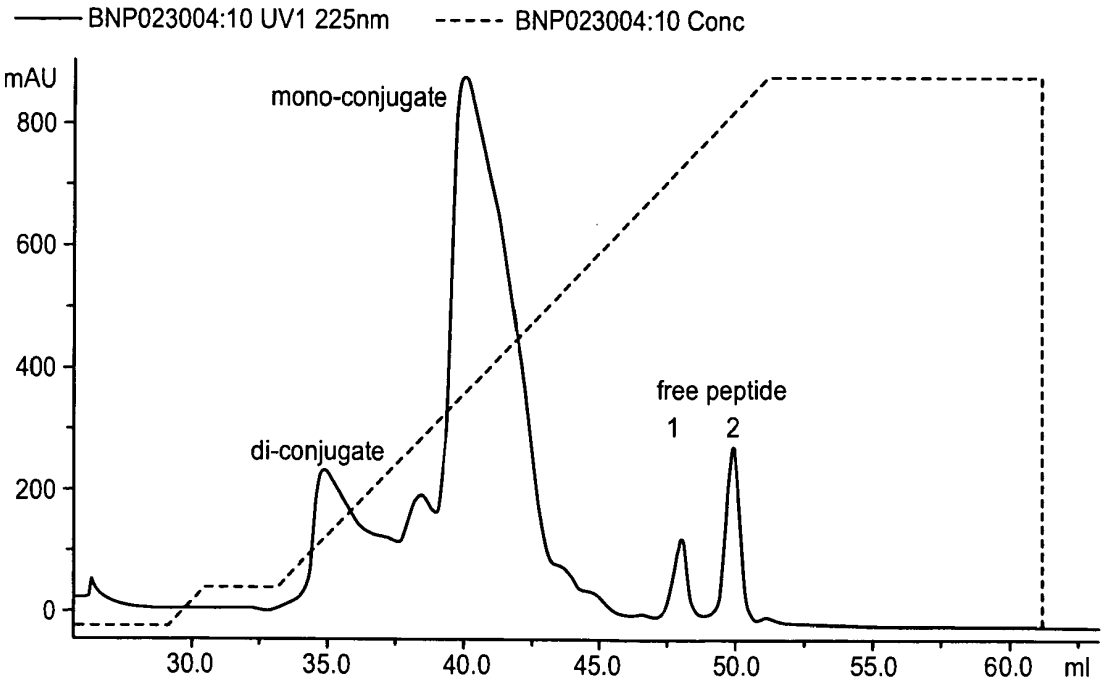



Figure BNP4.4

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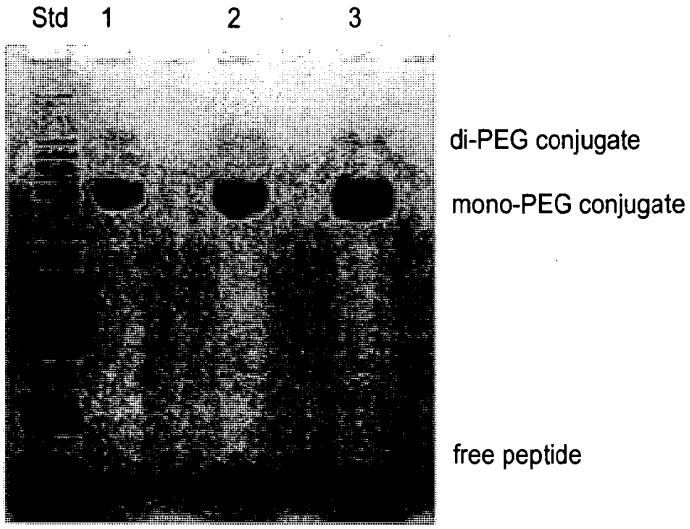



Figure BNP5.2

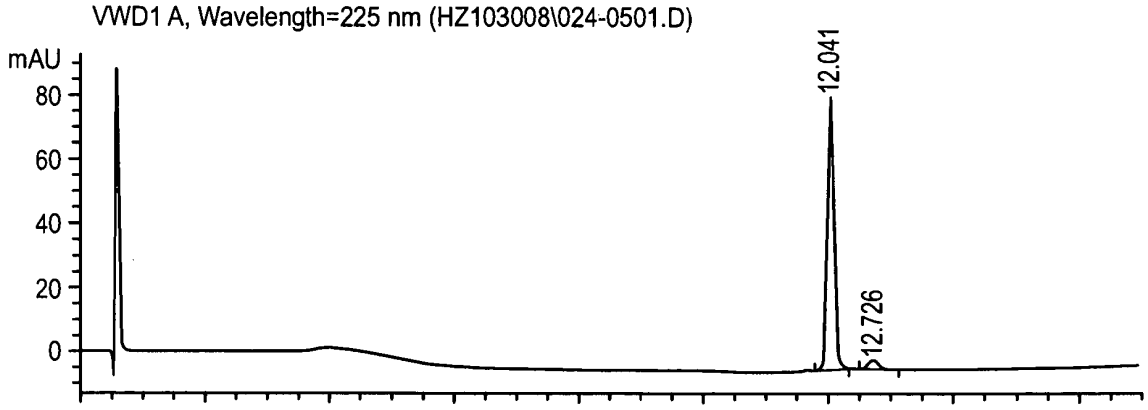



Figure BNP5.3

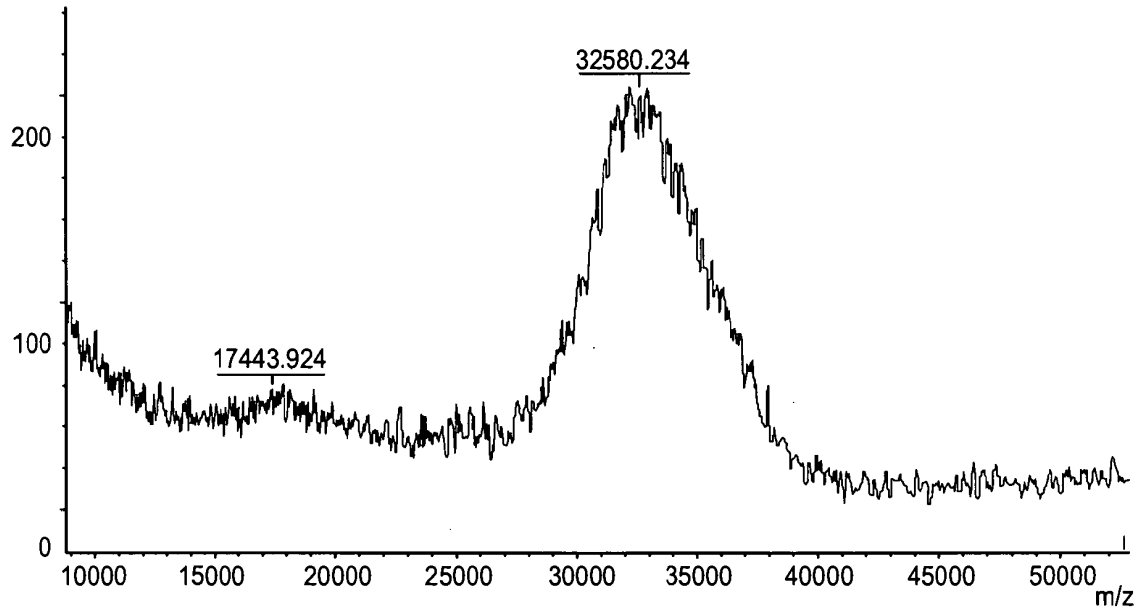
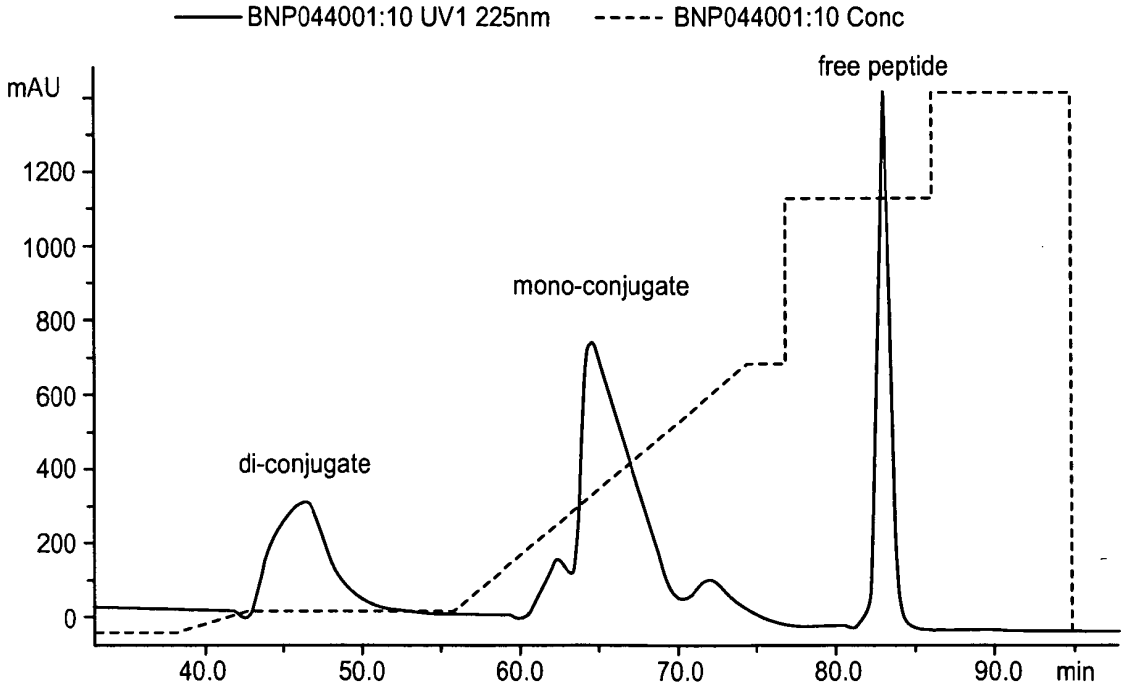



Figure BNP5.4

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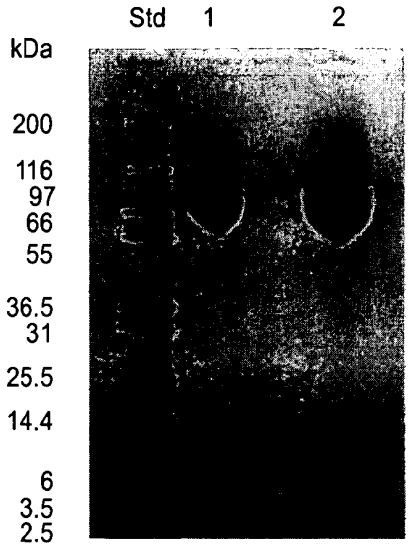


Figure BNP6.2

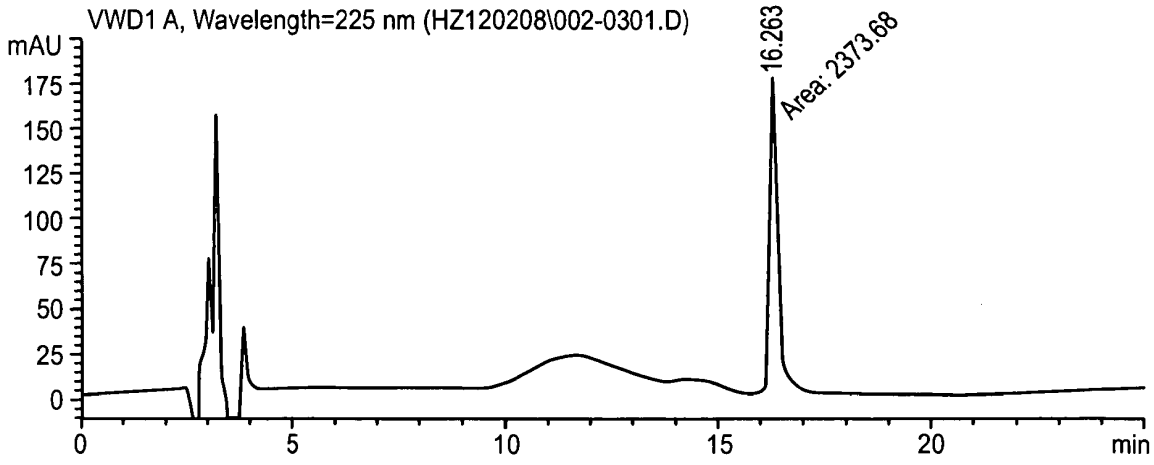


Figure BNP6.3

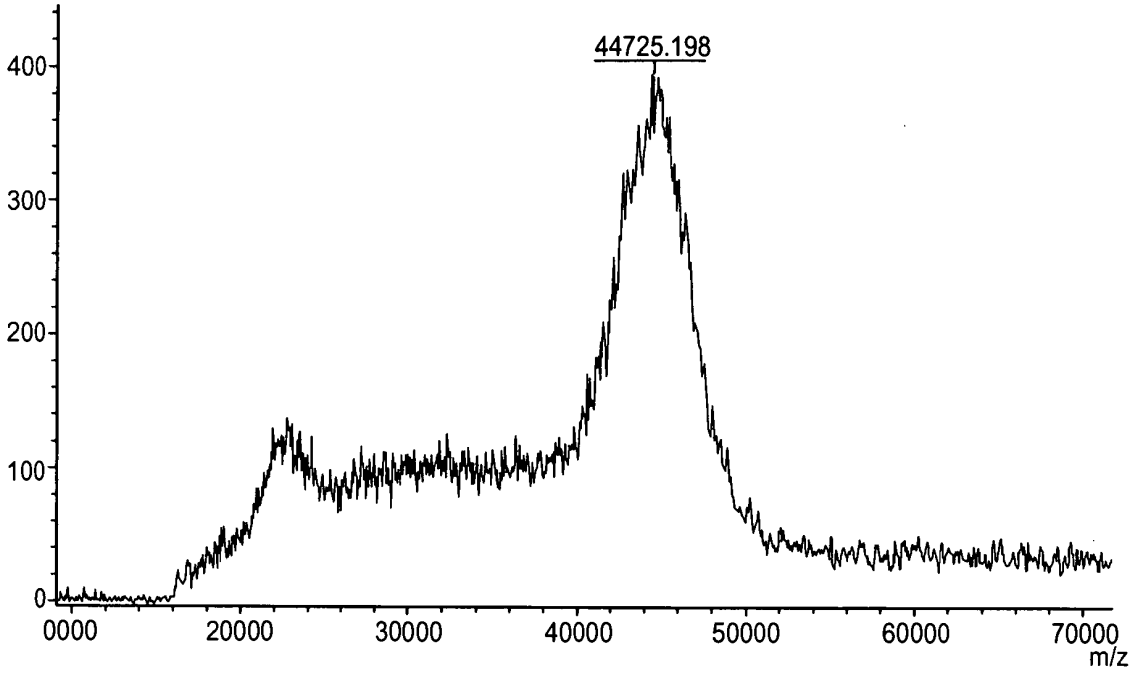


Figure BNP6.4

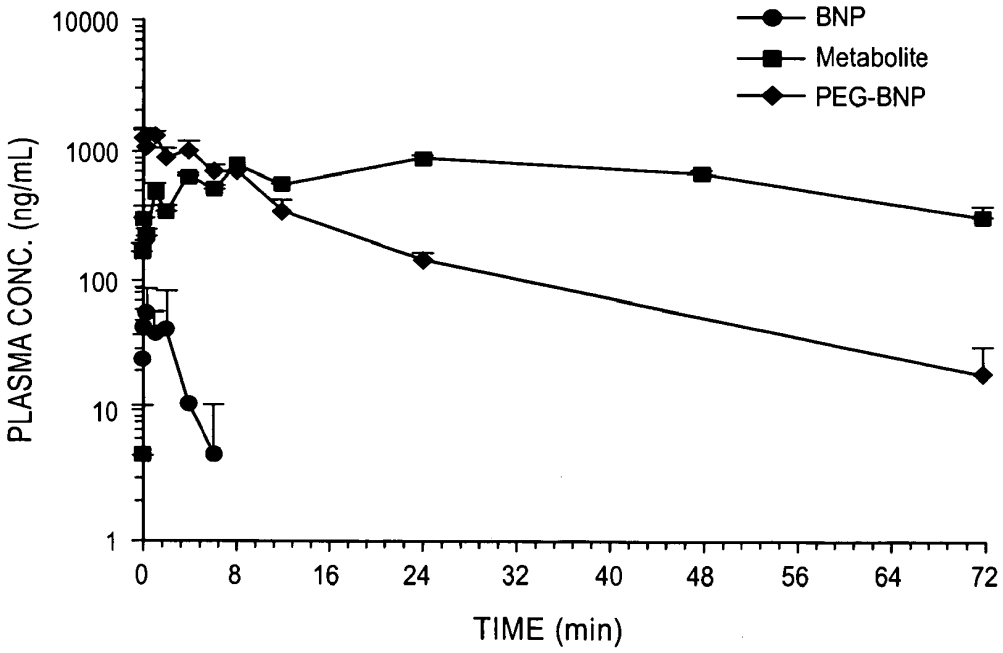


Figure BNP7.1

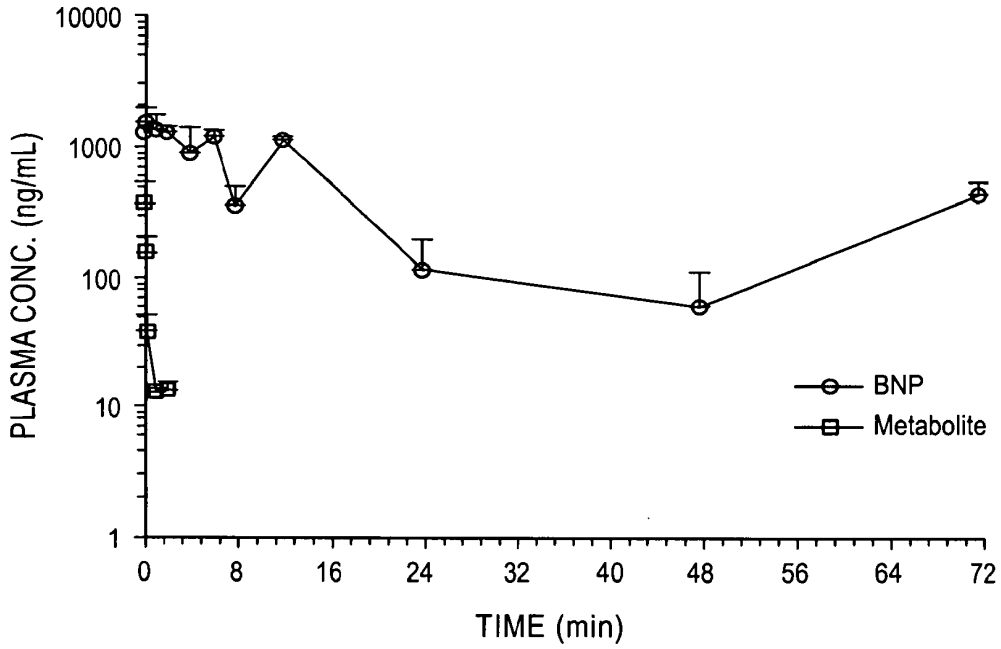


Figure BNP7.2

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/005206

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. A61K47/48 C07K14/575

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | US 2005/059600 A1 (BURNETT JOHN C [US] ET AL BURNETT JR JOHN C [US] ET AL)<br>17 March 2005 (2005-03-17)<br>sequence 2 | 1-28 |
| Y | ROBERTS M J ET AL: "Chemistry for peptide and protein PEGylation"<br>ADVANCED DRUG DELIVERY REVIEWS, ELSEVIER BV, AMSTERDAM, NL,<br>vol. 54, no. 4, 17 June 2002 (2002-06-17),<br>pages 459-476, XP002293146<br>ISSN: 0169-409X<br>abstract | 1-28 |

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 4 December 2009 | 14/12/2009 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Schleifenbaum, A |

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# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/005206

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | WO 2006/110776 A (NEKTAR THERAPEUTICS AL CORP [US]; BOSSARD MARY J [US]; MITCHELL STACY) 19 October 2006 (2006-10-19) tables 1-3 | 17-19 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/005206

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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Form PCT/ISA/210 (patent family annex) (April 2005)

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(22) International Filing Date: 30 January 2009 (30.01.2009)

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(71) Applicant *(for all designated States except US)*: **ASCENDIS PHARMA AS** [DK/DK]; Tuborg Boulevard 12, DK-2900 Hellerup (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CLEEMANN, Felix** [DE/DE]; Turnerstraße 114, 69126 Heidelberg (DE). **HERSEL, Ulrich** [DE/DE]; Fritz-Frey-Straße 2, 69121 Heidelberg (DE). **KADEN, Silvia** [DE/DE]; Langgewann 79-81, 69120 Heidelberg (DE). **RAU, Harald** [DE/DE]; Schwarzwaldstraße 51/2, 69124 Heidelberg (DE). **WEGGE, Thomas** [DE/DE]; Kurfürstenanlage 43, 69115 Heidelberg (DE).

(74) Agent: **HUHN, Michael**; Theodor-Heuss-Anlage 12, 68165 Mannheim (DE).

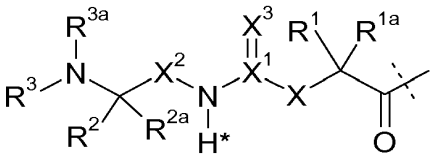
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

WO 2009/095479 A2

(54) Title: PRODRUG COMPRISING A DRUG LINKER CONJUGATE



(I)

(57) Abstract: The present invention relates to a prodrug or a pharmaceutically acceptable salt thereof comprising a drug linker conjugate D-L, wherein -D is an amine containing biologically active moiety; and -L is a non-biologically active linker moiety -$L^1$ represented by formula (I), wherein the dashed line indicates the attachment to the amine of the biologically active moiety and wherein $R^1$, $R^{1a}$, $R^2$, $R^{2a}$, $R^3$, $R^{3a}$, X, $X^1$, $X^2$, $X^3$ have the meaning as indicated in the description and the claims and wherein $L^1$ is substituted with one to four groups $L^2$-Z and optionally further substituted, provided that the hydrogen marked with the asterisk in formula (I) is not replaced by a substituent; wherein $L^2$ is a single chemical bond or a spacer; and Z is a carrier group.The invention also relates to A-L, wherein A is a leaving group, pharmaceutical composition comprising said prodrugs and their use as medicaments.

# Prodrug comprising a drug linker conjugate

The present invention relates to a prodrug or a pharmaceutically acceptable salt thereof comprising a drug linker conjugate D-L. The invention also relates to pharmaceutical compositions comprising said prodrugs and their use as medicaments.

To enhance physicochemical or pharmacokinetic properties of a drug in vivo such drug can be conjugated with a carrier.

Typically, carriers in drug delivery are either used in a non-covalent fashion, with the drug physicochemically formulated into a solvent-carrier mixture, or by covalent attachment of a carrier reagent to one of the drug's functional groups.

However the non-covalent approach requires a highly efficient drug encapsulation to prevent uncontrolled, burst-type release of the drug. Restraining the diffusion of an unbound, water soluble drug molecule requires strong van der Waals contacts, frequently mediated through hydrophobic moieties. Many conformationally sensitive drugs, such as proteins or peptides, are rendered dysfunctional during the encapsulation process and/or during subsequent storage of the encapsulated drug. In addition, such amino-containing drugs readily undergo side reactions with carrier degradation products (see, for example, D.H. Lee et al., J. Contr. Rel., 2003, 92, 291-299). Furthermore, dependence of the release mechanism of the drug upon biodegradation may cause interpatient variability.

Alternatively, the drugs may be conjugated to a carrier through covalent bonds. This approach is applied to various classes of molecules, from so-called small molecules, through natural products up to larger proteins. Covalent drug carrier conjugates can be divided into two groups. Firstly, conjugates, where the covalent bond between carrier and drug is mostly present during the action of the drug ("permanent covalent bond"), i.e. a derivative of the drug exhibits its pharmacological effects as it is known for the drug as such. Secondly, the covalent bond is mostly previously cleaved to release the drug as such, which can exhibit its known pharmacological effects. In the latter case the covalent drug carrier conjugate is called carrier linked prodrug or carrier prodrug.

In order to ensure cleavage of the covalent bond between carrier and drug easy removal of said bond in vivo is required to release the drug (prodrug activation).

Prodrug activation may occur by enzymatic or non-enzymatic cleavage of the bond between the carrier and the drug molecule, or a sequential combination of both, i.e. an enzymatic step followed by a non-enzymatic rearrangement.

Enzymatically induced prodrug activation is characterized in that the cleavage in enzyme-free in-vitro environment such as an aqueous buffer solution, of, e.g., an ester or amide may occur, but the corresponding rate of hydrolysis may be much too slow and not therapeutically useful. In an in-vivo environment, esterases or amidases are typically present and the esterases and amidases may cause significant catalytic acceleration of the kinetics of hydrolysis from twofold up to several orders of magnitude. Therefore, the cleavage is predominantly controlled by the enzymatic reaction.

A major drawback of predominantly enzymatic cleavage is interpatient variability. Enzyme levels may differ significantly between individuals resulting in biological variation of prodrug activation by the enzymatic cleavage. The enzyme levels may also vary depending on the site of administration. For instance it is known that in the case of subcutaneous injection, certain areas of the body yield more predictable therapeutic effects than others. To reduce this unpredictable effect, non-enzymatic cleavage or intramolecular catalysis is of particular interest.

Therefore, enzyme-independent autocatalytic cleavage of carrier and biologically active moiety is preferred. In most cases this is achieved by an appropriately designed linker moiety between the carrier and the biologically active moiety, which is directly attached to the functional group of a biologically active moiety via covalent bond.

Specific linker types are known in the art.

Y. Sohma et al., J. Med. Chem. 46 (2003), 4124-4135 describe ester based prodrugs, where the carrier is water-soluble and the biologically active moiety is derived from HIV-1 protease inhibitor KNI-727. The linker used is attached to the biologically active moiety via ester group. The mechanism of this prodrug system is cyclization-activation by cyclic imide formation for the cleavage of ester bonds.

However this is disadvantageous because of the instability of the ester functional group. Furthermore, ester groups may be less chemoselectively addressable for the conjugation of the carrier or linker and the drug.

A.J. Garman et al. (A.J. Garman, S.B. Kalindjan, FEBS Lett. 1987, 223 (2), 361-365 1987) use PEG5000-maleic anhydride for the reversible modification of amino groups in tissue-type plasminogen activator and urokinase. Regeneration of functional enzyme from PEG-uPA conjugate upon incubation at pH 7.4 buffer by cleavage of the maleamic acid linkeage follows first order kinetics with a half-life of 6.1 h. A disadvantage of the maleamic acid linkage is the lack of stability of the conjugate at lower pH values. This limits the applicability of the maleamic acid linkage to biologically active agents which are stable at basic (high) pH values, as purification of the biologically active agent polymer conjugate has to be performed under basic (high pH) conditions to prevent premature prodrug cleavage.

In WO-A 2004/108070 prodrug system based on N,N-bis-(2-hydroxyethyl)glycine amide (bicine) linker is described. In this system two PEG carrier molecules are linked to a bicine molecule coupled to an amino group of the drug molecule. The first two steps in prodrug activation is the enzymatic cleavage of the first linkages connecting both PEG carrier molecules with the hydroxy groups of the bicine activating group. Different linkages between PEG and bicine are described resulting in different prodrug activation kinetics. The second step in prodrug activation is the cleavage of the second linkage connecting the bicine activating group to the amino group of the drug molecule. The main disadvantage of this system is the connection of the polymer to the bicine linker resulting in a slow hydrolysis rate of this second bicine amide linkage ($t1/2 > 3$ h in phosphate buffer). Consequently the release of a bicine-modified prodrug intermediate may show different pharmacokinetic, immunogenic, toxicological and pharmacodynamic properties as compared to the parent native drug molecule.

Another bicine-based system is described in WO-A 2006/136586.

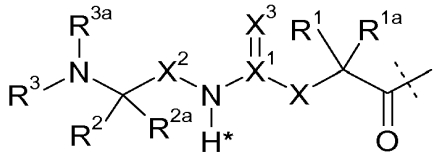
Accordingly, there is a need for alternative carrier-linked prodrugs, where the linker allows an autocatalytic cleavage to release a drug in an unmodified form without remaining residues originating from the linker.

Thus, an object of the present invention is to provide such drug linker conjugates, where the linker is covalently attached via a cleavable bond to a biologically active moiety (representing the drug after release), and where the linker is further covalently attached via a permanent bond to a carrier directly or via a spacer to form the carrier-linked prodrug.

This object is achieved by a prodrug or a pharmaceutically acceptable salt thereof comprising a drug linker conjugate D-L, wherein

-D is a nitrogen containing biologically active moiety; and

-L is a
non-biologically active linker moiety -$L^1$ represented by formula (I),



(I)

,

wherein the dashed line indicates the attachment to the nitrogen of the biologically active moiety by forming an amide bond;

X is $C(R^4R^{4a})$; $N(R^4)$; O; $C(R^4R^{4a})$-$C(R^5R^{5a})$; $C(R^5R^{5a})$-$C(R^4R^{4a})$; $C(R^4R^{4a})$-$N(R^6)$; $N(R^6)$-$C(R^4R^{4a})$; $C(R^4R^{4a})$-O; or O-$C(R^4R^{4a})$;

$X^1$ is C; or S(O);

$X^2$ is $C(R^7, R^{7a})$; or $C(R^7, R^{7a})$-$C(R^8, R^{8a})$;

$X^3$ is O; S; or N-CN;

$R^1$, $R^{1a}$, $R^2$, $R^{2a}$, $R^3$, $R^{3a}$, $R^4$, $R^{4a}$, $R^5$, $R^{5a}$, $R^6$, $R^7$, $R^{7a}$, $R^8$, $R^{8a}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl;

Optionally, one or more of the pairs $R^{1a}/R^{4a}$, $R^{1a}/R^{5a}$, $R^{4a}/R^{5a}$, $R^{7a}/R^{8a}$ form a chemical bond;

Optionally, one or more of the pairs $R^1/R^{1a}$, $R^2/R^{2a}$, $R^4/R^{4a}$, $R^5/R^{5a}$, $R^7/R^{7a}$, $R^8/R^{8a}$ are joined together with the atom to which they are attached to form a $C_{3-7}$ cycloalkyl; or 4 to 7 membered heterocyclyl;

Optionally, $R^4/R^6$ are joined together with the atoms to which they are attached to form a saturated 4 to 7 membered heterocyclyl;

Optionally, one or more of the pairs $R^1/R^4$, $R^1/R^5$, $R^1/R^6$, $R^4/R^5$, $R^4/R^6$, $R^7/R^8$, $R^2/R^3$ are joined together with the atoms to which they are attached to form a ring A;

Optionally, $R^3/R^{3a}$ are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle;

A is selected from the group consisting of phenyl; naphthyl; indenyl; indanyl; tetralinyl; $C_{3-10}$ cycloalkyl; 4 to 7 membered heterocyclyl; and 9 to 11 membered heterobicyclyl; and

wherein $L^1$ is substituted with one to four groups $L^2$-Z and optionally further substituted, provided that the hydrogen marked with the asterisk in formula (I) is not replaced by a substituent; wherein

$L^2$ is a single chemical bond or a spacer; and
Z is a carrier group.

It was surprisingly found, that the scope of cyclization-activation by cyclic imide formation can be extended from ester to even carrier-linked amide prodrugs, despite the much greater stability of the amide bond under aqueous conditions. It was observed that N,N'-biscarboxamides linked to a nucleophile carrying moiety through one amide bond and to the drug molecule through the second amide bond exhibit autohydrolysis in a range that is useful for prodrug applications. In addition, it was discovered that linkers can be designed that include a carrier permanently attached to the N,N' biscarboxamide motif in such a fashion that cyclic imide formation can be employed as a self-activation principle in carrier-linked amide prodrug design.

Examples for such preferred cyclic cleavage products are substituted succinimide or glutarimide ring structures. Prerequisite for such cyclization activation is the presence of an amine-containing nucleophile in the linker structure and another amide bond which is not the amide prodrug bond but an amide bond substituted with a hydrogen atom.

In case of succinimide- or a glutarimide-activated prodrug cleavage, the amine-containing nucleophile serves as a neighbouring group to enhance the nucleophilicity of the nitrogen contained in the permanent amide bond which in turn attacks the prodrug amide carbonyl group and consequently induces intramolecular acylation of the permanent amide bond generating the cyclic imide ring.

Therefore preferred linker structures comprise a permanent linkage to a carrier, an amine-containing nucleophile, and a permanent amide bond with a hydrogen attached to the nitrogen of the amide bond. Corresponding carrier-linked prodrugs comprise a linker containing a permanent linkage to a carrier, an amine-containing nucleophile and said permanent amide bond, and a nitrogen containing biologically active moiety derived from the drug conjugated to the linker by means of a cleavable amide bond.

Fig. 1 shows an example of the cleavage resulting in a cyclic imide. The nitrogen of the biologically active moiety is shown as hydrogen containing amine, which results in a drug having a primary amine functional group. However also, e.g., a secondary amine may be part of the drug. For simplification reasons the one to four mandatory substituents $L^2$-Z including the carrier are not shown.

Preferred properties of the prodrug are given by a half-life of hydrolysis in aqueous buffer at pH 7.4 and 37°C between 1 h and 3 months; similar rates of hydrolysis under physiological conditions in buffer and plasma.

The prodrug according to the present invention may show excellent in vivo/in vitro correlation of linker cleavage, a high degree of enzyme independence and can be stored at lower pH (pH dependent cleavage).

Within the meaning of the present invention the terms are used as follows.

"Biologically active moiety D" means the part of the drug linker conjugate, which results after cleavage in a drug D-H of known biological activity.

"Non-active linker" means a linker which does not show the pharmacological effects of the drug derived from the biologically active agent.

"Alkyl" means a straight-chain or branched carbon chain. Each hydrogen of an alkyl carbon may be replaced by a substituent.

"$C_{1-4}$ alkyl" means an alkyl chain having 1 - 4 carbon atoms, e.g. if present at the end of a molecule: methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl tert-butyl, or e.g. -$CH_2$-, -$CH_2$-$CH_2$-, -$CH(CH_3)$-, -$CH_2$-$CH_2$-$CH_2$-, -$CH(C_2H_5)$-, -$C(CH_3)_2$-, when two moieties of a molecule are linked by the alkyl group. Each hydrogen of a $C_{1-4}$ alkyl carbon may be replaced by a substituent.

"$C_{1-6}$ alkyl" means an alkyl chain having 1 - 6 carbon atoms, e.g. if present at the end of a molecule: $C_{1-4}$ alkyl, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl; tert-butyl, n-pentyl, n-hexyl, or e.g. -$CH_2$-, -$CH_2$-$CH_2$-, -$CH(CH_3)$-, -$CH_2$-$CH_2$-$CH_2$-, -$CH(C_2H_5)$-, -$C(CH_3)_2$-, when two moieties of a molecule are linked by the alkyl group. Each hydrogen of a $C_{1-6}$ alkyl carbon may be replaced by a substituent.

Accordingly, "$C_{1-18}$ alkyl" means an alkyl chain having 1 to 18 carbon atoms and "$C_{8-18}$ alkyl" means an alkyl chain having 8 to 18 carbon atoms. Accordingly, "$C_{1-50}$ alkyl" means an alkyl chain having 1 to 50 carbon atoms.

"$C_{2-50}$ alkenyl" means a branched or unbranched alkenyl chain having 2 to 50 carbon atoms, e.g. if present at the end of a molecule: -CH=$CH_2$, -CH=CH-$CH_3$, -$CH_2$-CH=$CH_2$, -CH=CH-$CH_2$-$CH_3$, -CH=CH-CH=$CH_2$, or e.g. -CH=CH-, when two moieties of a molecule are linked by the alkenyl group. Each hydrogen of a $C_{2-50}$ alkenyl carbon may be replaced by a substituent as further specified. Accordingly, the term "alkenyl" relates to a carbon chain with at least one carbon carbon double bond. Optionally, one or more triple bonds may occur.

"$C_{2-50}$ alkynyl" means a branched or unbranched alkynyl chain having 2 to 50 carbon atoms, e.g. if present at the end of a molecule: -C≡CH, -$CH_2$-C≡CH, $CH_2$-$CH_2$-C≡CH, $CH_2$-C≡C-$CH_3$, or e.g. -C≡C- when two moieties of a molecule are linked by the alkynyl group. Each

hydrogen of a $C_{2-50}$ alkynyl carbon may be replaced by a substituent as further specified. Accordingly, the term “alkynyl” relates to a carbon chaim with at lest one carbon carbon triple bond. Optionally, one or more double bonds may occur.

"$C_{3-7}$ cycloalkyl" or "$C_{3-7}$ cycloalkyl ring" means a cyclic alkyl chain having 3 to 7 carbon atoms, which may have carbon-carbon double bonds being at least partially saturated, e.g. cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclohexenyl, cycloheptyl. Each hydrogen of a cycloalkyl carbon may be replaced by a substituent. The term "$C_{3-7}$ cycloalkyl" or "$C_{3-7}$ cycloalkyl ring" also includes bridged bicycles like norbonane or norbonene. Accordingly, "$C_{3-5}$ cycloalkyl" means a cycloalkyl having 3 to 5 carbon atoms.

Accordingly, “$C_{3-10}$ cycloalkyl” means a cyclic alkyl having 3 to 10 carbon atoms, e.g. $C_{3-7}$ cycloalkyl; cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclohexenyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl. The term “$C_{3-10}$ cycloalkyl” also includes at least partially saturated carbomono- and –bicycles.

"Halogen" means fluoro, chloro, bromo or iodo. It is generally preferred that halogen is fluoro or chloro.

"4 to 7 membered heterocyclyl" or "4 to 7 membered heterocycle" means a ring with 4, 5, 6 or 7 ring atoms that may contain up to the maximum number of double bonds (aromatic or non-aromatic ring which is fully, partially or un-saturated) wherein at least one ring atom up to 4 ring atoms are replaced by a heteroatom selected from the group consisting of sulfur (including -S(O)-, -$S(O)_2$-), oxygen and nitrogen (including =N(O)-) and wherein the ring is linked to the rest of the molecule via a carbon or nitrogen atom. Examples for a 4 to 7 membered heterocycles are azetidine, oxetane, thietane, furan, thiophene, pyrrole, pyrroline, imidazole, imidazoline, pyrazole, pyrazoline, oxazole, oxazoline, isoxazole, isoxazoline, thiazole, thiazoline, isothiazole, isothiazoline, thiadiazole, thiadiazoline, tetrahydrofuran, tetrahydrothiophene, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, thiadiazolidine, sulfolane, pyran, dihydropyran, tetrahydropyran, imidazolidine, pyridine, pyridazine, pyrazine, pyrimidine, piperazine, piperidine, morpholine, tetrazole, triazole, triazolidine, tetrazolidine, diazepane, azepine or homopiperazine.

"9 to 11 membered heterobicyclyl" or "9 to 11 membered heterobicycle" means a heterocyclic system of two rings with 9 to 11 ring atoms, where at least one ring atom is shared by both rings and that may contain up to the maximum number of double bonds (aromatic or non-aromatic ring which is fully, partially or un-saturated) wherein at least one ring atom up to 6 ring atoms are replaced by a heteroatom selected from the group consisting of sulfur (including -S(O)-, -$S(O)_2$-), oxygen and nitrogen (including =N(O)-) and wherein the ring is linked to the rest of the molecule via a carbon or nitrogen atom. Examples for a 9 to 11 membered heterobicycle are indole, indoline, benzofuran, benzothiophene, benzoxazole, benzisoxazole, benzothiazole, benzisothiazole, benzimidazole, benzimidazoline, quinoline, quinazoline, dihydroquinazoline, quinoline, dihydroquinoline, tetrahydroquinoline, decahydroquinoline, isoquinoline, decahydroisoquinoline, tetrahydroisoquinoline, dihydroisoquinoline, benzazepine, purine or pteridine. The term 9 to 11 membered heterobicycle also includes spiro structures of two rings like 1,4-dioxa-8-azaspiro[4.5]decane or bridged heterocycles like 8-aza-bicyclo[3.2.1]octane.

In case the compounds according to formula (I) contain one or more acidic or basic groups, the invention also comprises their corresponding pharmaceutically or toxicologically acceptable salts, in particular their pharmaceutically utilizable salts. Thus, the compounds of the formula (I) which contain acidic groups can be used according to the invention, for example, as alkali metal salts, alkaline earth metal salts or as ammonium salts. More precise examples of such salts include sodium salts, potassium salts, calcium salts, magnesium salts or salts with ammonia or organic amines such as, for example, ethylamine, ethanolamine, triethanolamine or amino acids. Compounds of the formula (I) which contain one or more basic groups, i.e. groups which can be protonated, can be present and can be used according to the invention in the form of their addition salts with inorganic or organic acids. Examples for suitable acids include hydrogen chloride, hydrogen bromide, phosphoric acid, sulfuric acid, nitric acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acids, oxalic acid, acetic acid, tartaric acid, lactic acid, salicylic acid, benzoic acid, formic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, malic acid, sulfaminic acid, phenylpropionic acid, gluconic acid, ascorbic acid, isonicotinic acid, citric acid, adipic acid, and other acids known to the person skilled in the art. If the compounds of the formula (I) simultaneously contain acidic and basic groups in the molecule, the invention also includes, in addition to the salt forms mentioned, inner salts or betaines (zwitterions). The respective salts according to the formula (I) can be

obtained by customary methods which are known to the person skilled in the art like, for example by contacting these with an organic or inorganic acid or base in a solvent or dispersant, or by anion exchange or cation exchange with other salts. The present invention also includes all salts of the compounds of the formula (I) which, owing to low physiological compatibility, are not directly suitable for use in pharmaceuticals but which can be used, for example, as intermediates for chemical reactions or for the preparation of pharmaceutically acceptable salts.

The term "pharmaceutically acceptable" means approved by a regulatory agency such as the EMEA (Europe) and/or the FDA (US) and/or any other national regulatory agency for use in animals, preferably in humans.

"Pharmaceutical composition" means one or more active ingredients, and one or more inert ingredients, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a compound of the present invention and a pharmaceutically acceptable excipient (pharmaceutically acceptable carrier).

The term "excipient" refers to a diluent, adjuvant, or vehicle with which the therapeutic is administered. Such pharmaceutical excipient can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, including but not limited to peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred excipient when the pharmaceutical composition is administered orally. Saline and aqueous dextrose are preferred excipients when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions are preferably employed as liquid excipients for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with

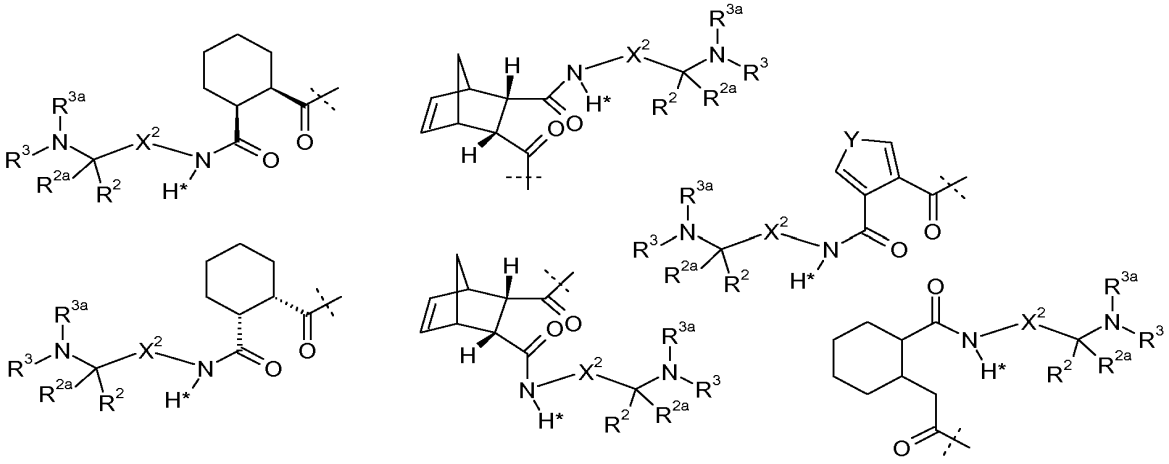
traditional binders and excipients such as triglycerides. Oral formulation can include standard excipients such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical excipients are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic, preferably in purified form, together with a suitable amount of excipient so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

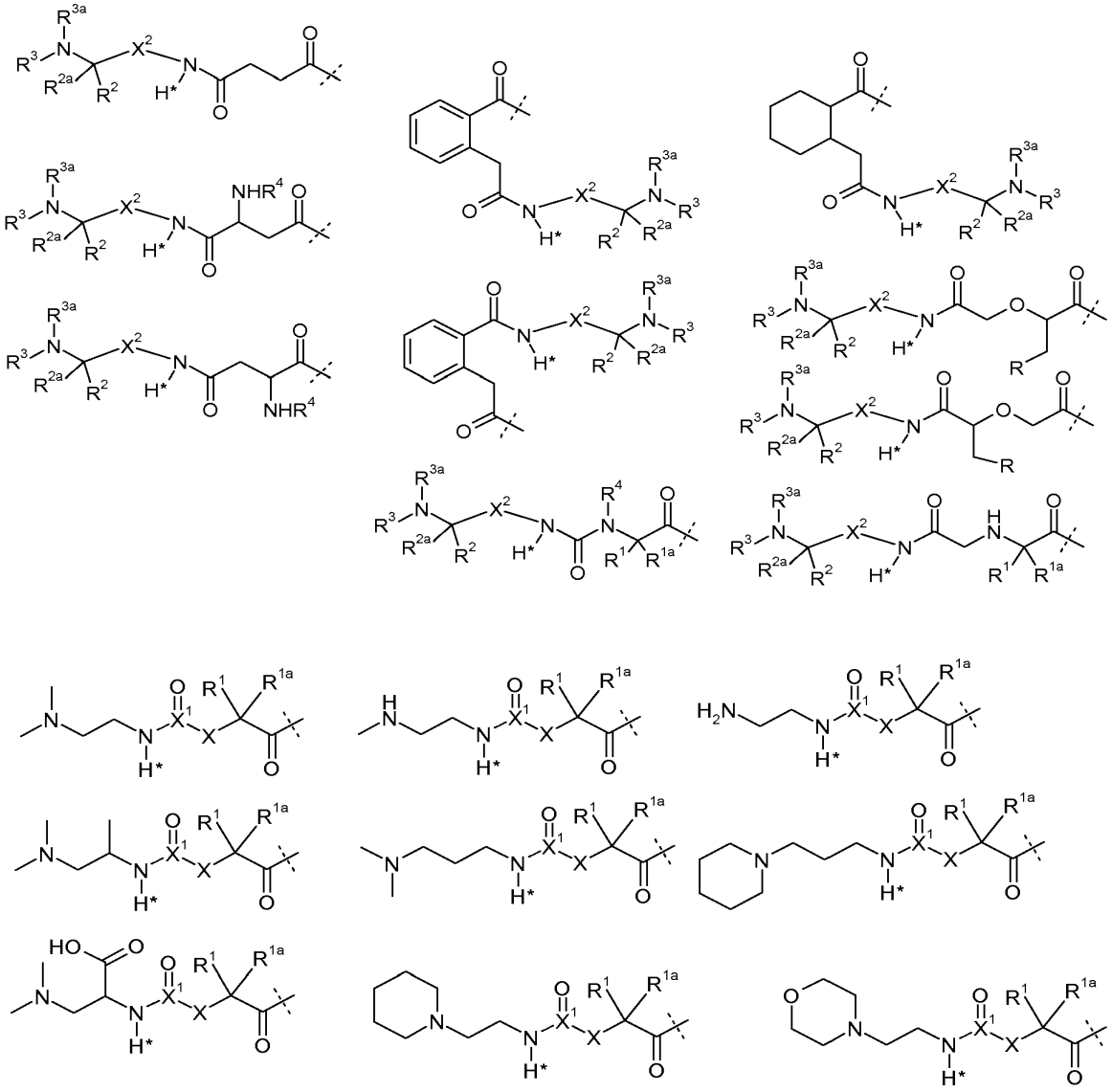
Preferably, $X^3$ is O.

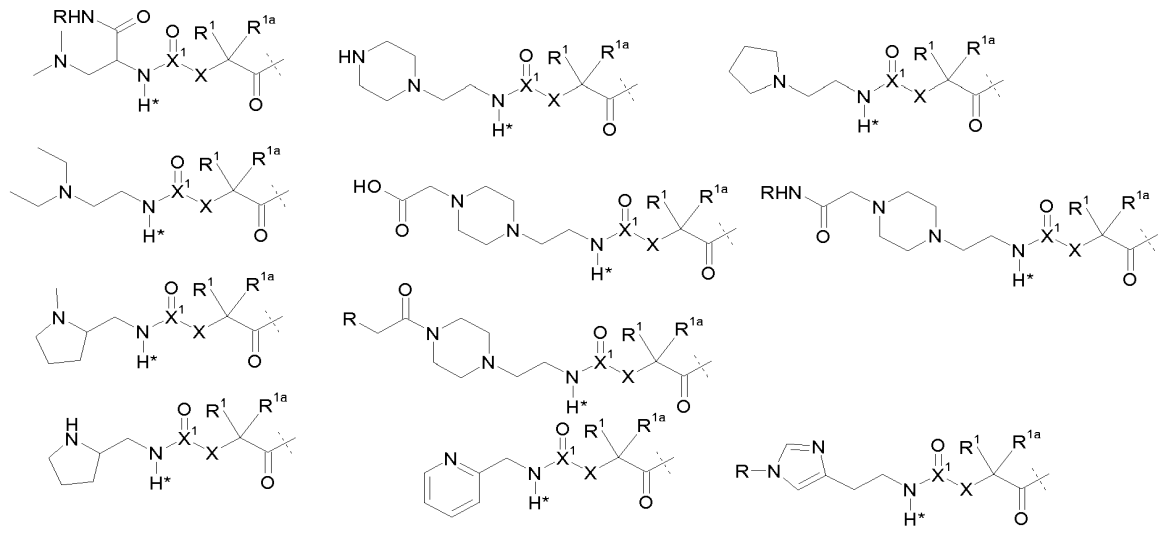
Preferably, X is $N(R^4)$, $X^1$ is C and $X^3$ is O.

Preferably, $X^2$ is $C(R^7R^{7a})$.

Preferably, $L^1$ is selected from the group consisting of

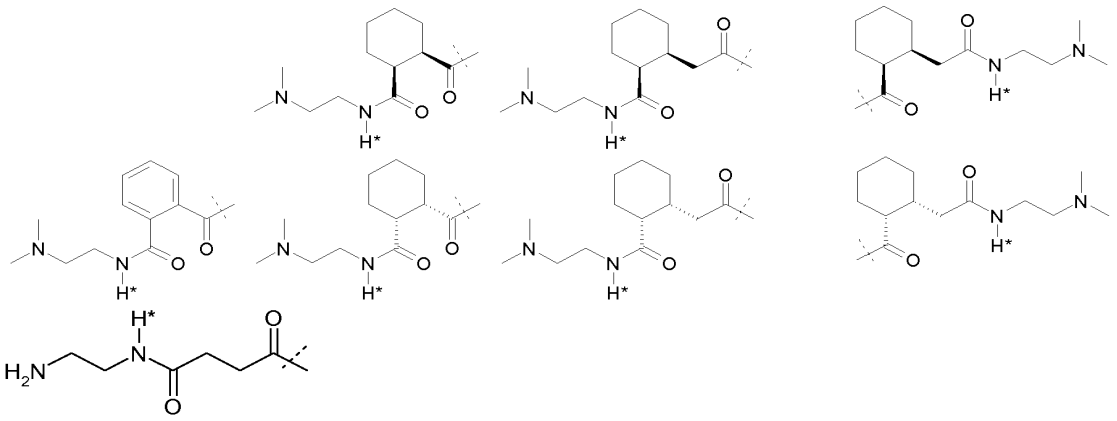


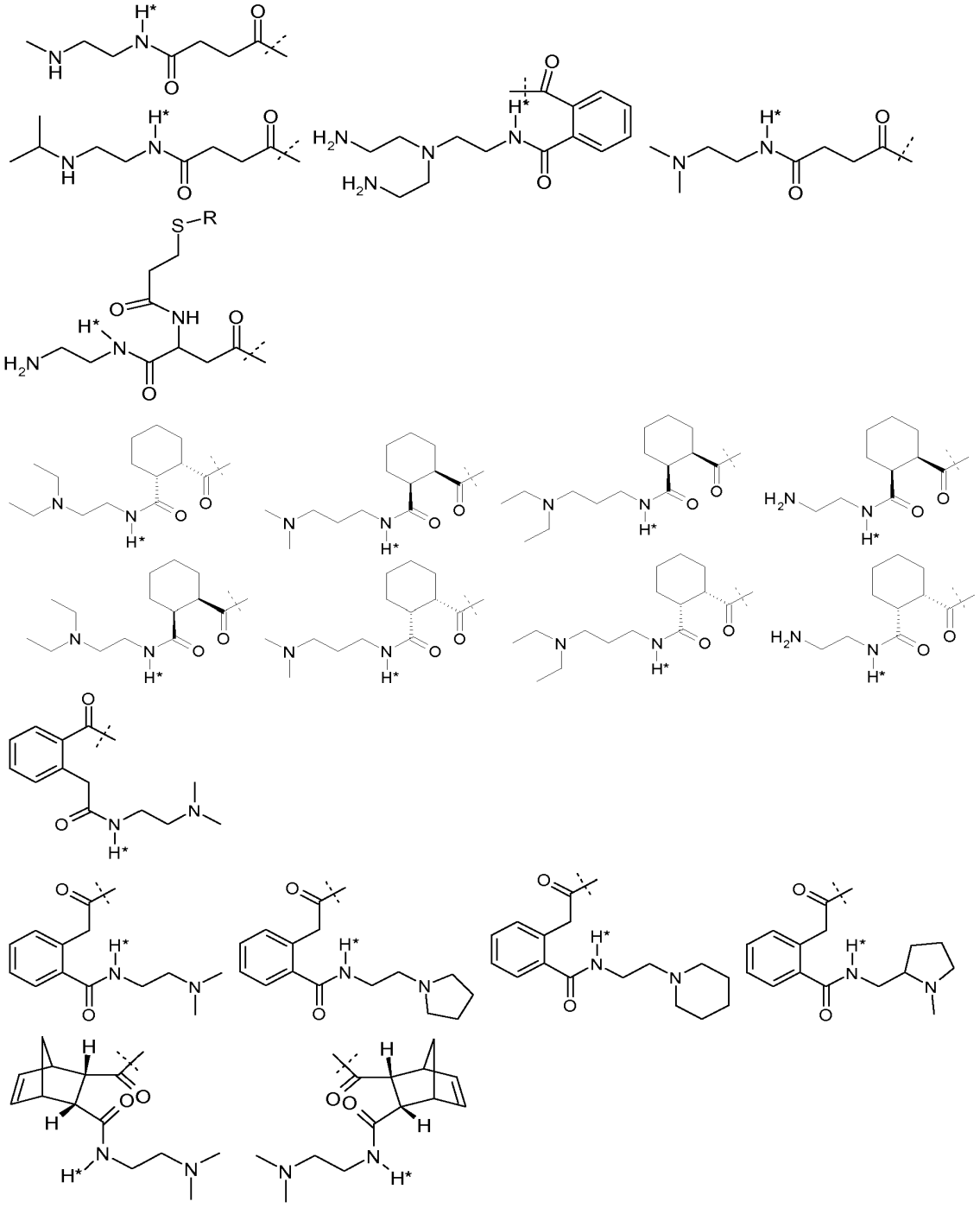


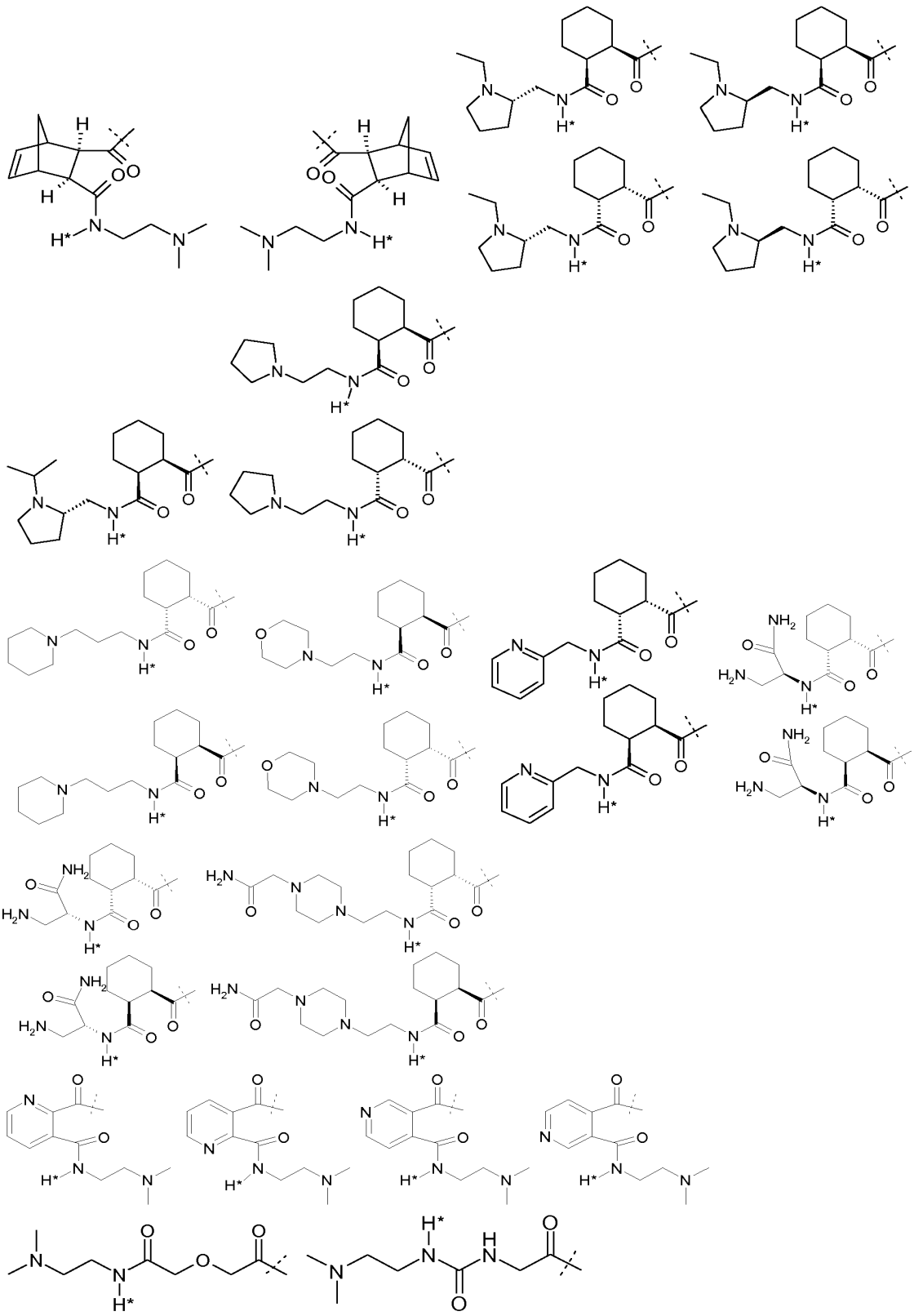


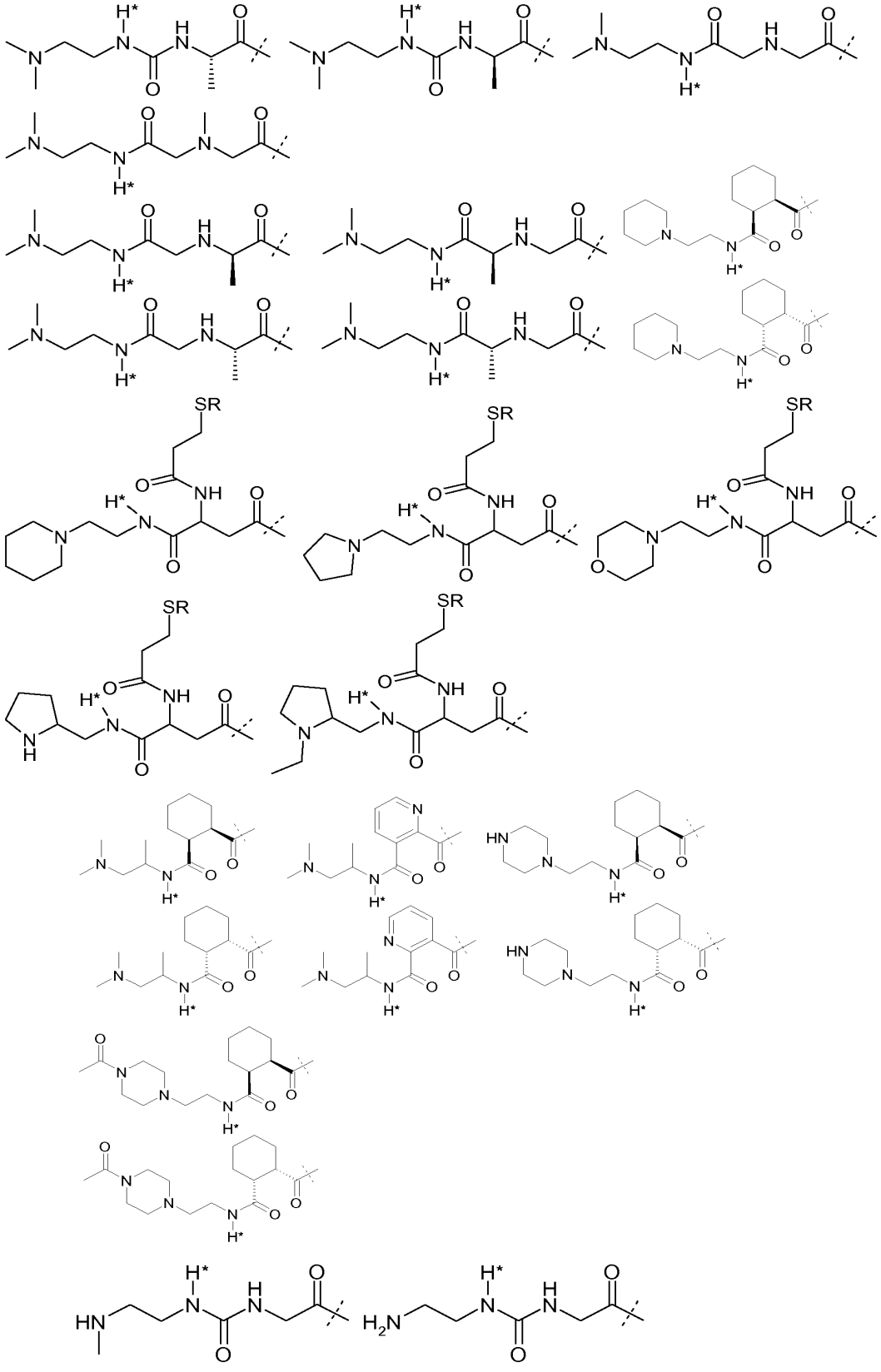
wherein R is H; or $C_{1-4}$ alkyl; Y is NH; O; or S; and $R^1$, $R^{1a}$, $R^2$, $R^{2a}$, $R^3$, $R^{3a}$, $R^4$, X, $X^1$, $X^2$ have the meaning as indicated above.

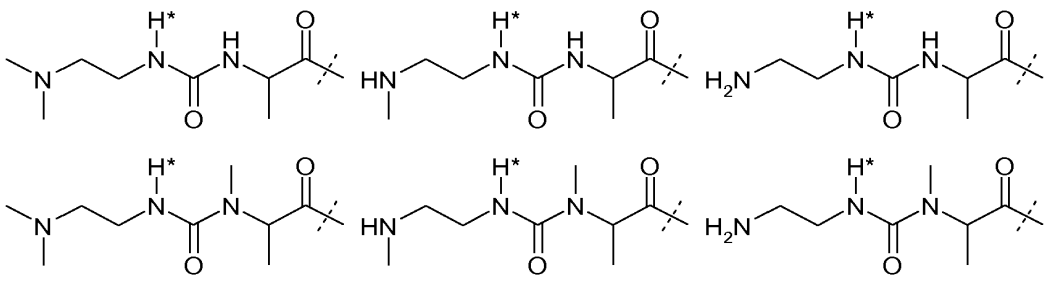
Even more preferred, $L^1$ is selected from the group consisting of











wherein R has the meaning as indicated above.

At least one (up to four) hydrogen is replaced by a group $L^2$-Z. In case more than one group $L^2$-Z is present each $L^2$ and each Z can be selected independently. Preferably, only one group $L^2$-Z is present resulting in the formula D-$L^1$-$L^2$-Z.

In general, $L^2$ can be attached to $L^1$ at any position apart from the replacement of the hydrogen marked with an asterisk in formula (I). Preferably, one to four of the hydrogen given by R, $R^1$ to $R^8$ directly or as hydrogen of the $C_{1-4}$ alkyl or further groups and rings given by the definition of R and $R^1$ to $R^8$ are replaced by $L^2$-Z.

Furthermore, $L^1$ may be optionally further substituted. In general, any substituent may be used as far as the cleavage principle is not affected.

Preferably, one or more further optional substituents are independently selected from the group consisting of halogen; CN; $COOR^9$; $OR^9$; $C(O)R^9$; $C(O)N(R^9R^{9a})$; $S(O)_2N(R^9R^{9a})$; $S(O)N(R^9R^{9a})$; $S(O)_2R^9$; $S(O)R^9$; $N(R^9)S(O)_2N(R^{9a}R^{9b})$; $SR^9$; $N(R^9R^{9a})$; $NO_2$; $OC(O)R^9$; $N(R^9)C(O)R^{9a}$; $N(R^9)S(O)_2R^{9a}$; $N(R^9)S(O)R^{9a}$; $N(R^9)C(O)OR^{9a}$; $N(R^9)C(O)N(R^{9a}R^{9b})$; $OC(O)N(R^9R^{9a})$; T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally interrupted by one or more groups selected from the group consisting of T, -C(O)O-; -O-; -C(O)-; -$C(O)N(R^{11})$-; -$S(O)_2N(R^{11})$-; -$S(O)N(R^{11})$-; -$S(O)_2$-; -S(O)-; -$N(R^{11})S(O)_2N(R^{11a})$-; -S-; -$N(R^{11})$-; -$OC(O)R^{11}$; -$N(R^{11})C(O)$-; -$N(R^{11})S(O)_2$-; -$N(R^{11})S(O)$-; -$N(R^{11})C(O)O$-; -$N(R^{11})C(O)N(R^{11a})$-; and -$OC(O)N(R^{11}R^{11a})$;

$R^9$, $R^{9a}$, $R^{9b}$ are independently selected from the group consisting of H; T; and $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally interrupted by one or more groups selected from the group consisting of T, -C(O)O-; -O-; -C(O)-; -C(O)N($R^{11}$)-; -S(O)$_2$N($R^{11}$)-; -S(O)N($R^{11}$)-; -S(O)$_2$-; -S(O)-; -N($R^{11}$)S(O)$_2$N($R^{11a}$)-; -S-; -N($R^{11}$)-; -OC(O)$R^{11}$; -N($R^{11}$)C(O)-; -N($R^{11}$)S(O)$_2$-; -N($R^{11}$)S(O)-; -N($R^{11}$)C(O)O-; -N($R^{11}$)C(O)N($R^{11a}$)-; and -OC(O)N($R^{11}R^{11a}$);

T is selected from the group consisting of phenyl; naphthyl; indenyl; indanyl; tetralinyl; $C_{3-10}$ cycloalkyl; 4 to 7 membered heterocyclyl; or 9 to 11 membered heterobicyclyl, wherein T is optionally substituted with one or more $R^{10}$, which are the same or different;

$R^{10}$ is halogen; CN; oxo (=O); COO$R^{12}$; O$R^{12}$; C(O)$R^{12}$; C(O)N($R^{12}R^{12a}$); S(O)$_2$N($R^{12}R^{12a}$); S(O)N($R^{12}R^{12a}$); S(O)$_2R^{12}$; S(O)$R^{12}$; N($R^{12}$)S(O)$_2$N($R^{12a}R^{12b}$); S$R^{12}$; N($R^{12}R^{12a}$); $NO_2$; OC(O)$R^{12}$; N($R^{12}$)C(O)$R^{12a}$; N($R^{12}$)S(O)$_2R^{12a}$; N($R^{12}$)S(O)$R^{12a}$; N($R^{12}$)C(O)O$R^{12a}$; N($R^{12}$)C(O)N($R^{12a}R^{12b}$); OC(O)N($R^{12}R^{12a}$); or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different;

$R^{11}$, $R^{11a}$, $R^{12}$, $R^{12a}$, $R^{12b}$ are independently selected from the group consisting of H; or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different.

The term "interrupted" means that between two carbons a group is inserted or at the end of the carbon chain between the carbon and hydrogen.

$L^2$ is a single chemical bond or a spacer. In case $L^2$ is a spacer, it is preferably defined as the one or more optional substituents defined above, provided that $L^2$ is substituted with Z.

Accordingly, when $L^2$ is other than a single chemical bond, $L^2$-Z is COO$R^9$; O$R^9$; C(O)$R^9$; C(O)N($R^9R^{9a}$); S(O)$_2$N($R^9R^{9a}$); S(O)N($R^9R^{9a}$); S(O)$_2R^9$; S(O)$R^9$; N($R^9$)S(O)$_2$N($R^{9a}R^{9b}$); S$R^9$; N($R^9R^{9a}$); OC(O)$R^9$; N($R^9$)C(O)$R^{9a}$; N($R^9$)S(O)$_2R^{9a}$; N($R^9$)S(O)$R^{9a}$; N($R^9$)C(O)O$R^{9a}$; N($R^9$)C(O)N($R^{9a}R^{9b}$); OC(O)N($R^9R^{9a}$); T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are

optionally interrupted by one or more groups selected from the group consisting of -T-, -C(O)O-; -O-; -C(O)-; -C(O)N($R^{11}$)-; -$S(O)_2N(R^{11})$-; -S(O)N($R^{11}$)-; -$S(O)_2$-; -S(O)-; -$N(R^{11})S(O)_2N(R^{11a})$-; -S-; -N($R^{11}$)-; -OC(O)$R^{11}$; -N($R^{11}$)C(O)-; -N($R^{11}$)$S(O)_2$-; -N($R^{11}$)S(O)-; -N($R^{11}$)C(O)O-; -N($R^{11}$)C(O)N($R^{11a}$)-; and -OC(O)N($R^{11}R^{11a}$);

$R^9$, $R^{9a}$, $R^{9b}$ are independently selected from the group consisting of H; Z; T; and $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally interrupted by one or more groups selected from the group consisting of T, -C(O)O-; -O-; -C(O)-; -C(O)N($R^{11}$)-; -$S(O)_2N(R^{11})$-; -S(O)N($R^{11}$)-; -$S(O)_2$-; -S(O)-; -$N(R^{11})S(O)_2N(R^{11a})$-; -S-; -N($R^{11}$)-; -OC(O)$R^{11}$; -N($R^{11}$)C(O)-; -N($R^{11}$)$S(O)_2$-; -N($R^{11}$)S(O)-; -N($R^{11}$)C(O)O-; -N($R^{11}$)C(O)N($R^{11a}$)-; and -OC(O)N($R^{11}R^{11a}$);

T is selected from the group consisting of phenyl; naphthyl; indenyl; indanyl; tetralinyl; $C_{3-10}$ cycloalkyl; 4 to 7 membered heterocyclyl; or 9 to 11 membered heterobicyclyl, wherein t is optionally substituted with one or more $R^{10}$, which are the same or different;

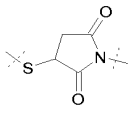
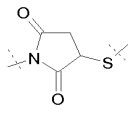
$R^{10}$ is Z; halogen; CN; oxo (=O); $COOR^{12}$; $OR^{12}$; $C(O)R^{12}$; C(O)N($R^{12}R^{12a}$); $S(O)_2N(R^{12}R^{12a})$; S(O)N($R^{12}R^{12a}$); $S(O)_2R^{12}$; $S(O)R^{12}$; $N(R^{12})S(O)_2N(R^{12a}R^{12b})$; $SR^{12}$; N($R^{12}R^{12a}$); $NO_2$; $OC(O)R^{12}$; $N(R^{12})C(O)R^{12a}$; $N(R^{12})S(O)_2R^{12a}$; $N(R^{12})S(O)R^{12a}$; $N(R^{12})C(O)OR^{12a}$; $N(R^{12})C(O)N(R^{12a}R^{12b})$; $OC(O)N(R^{12}R^{12a})$; or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different;

$R^{11}$, $R^{11a}$, $R^{12}$, $R^{12a}$, $R^{12b}$ are independently selected from the group consisting of H; Z; or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different;

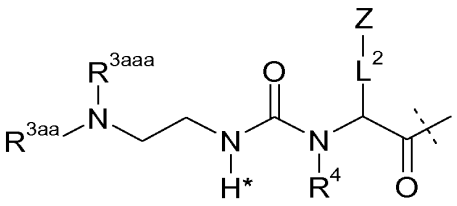
provided that one of $R^9$, $R^{9a}$, $R^{9b}$, $R^{10}$, $R^{11}$, $R^{11a}$, $R^{12}$, $R^{12a}$, $R^{12b}$ is Z.

More preferably, $L^2$ is a $C_{1-20}$ alkyl chain, which is optionally interrupted by one or more groups independently selected from -O-; and C(O)N($R^{3aa}$); optionally substituted with one or more groups independently selected from OH; and C(O)N($R^{3aa}R^{3aaa}$); and wherein $R^{3aa}$, $R^{3aaa}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl.

Preferably, $L^2$ has a molecular weight in the range of from 14 g/mol to 750 g/mol.

Preferably, $L^2$ is attached to Z via a terminal group selected from  ; and  .
In case $L^2$ has such terminal group it is furthermore preferred that $L^2$ has a molecular weight in the range of from 14 g/mol to 500 g/mol calculated without such terminal group.

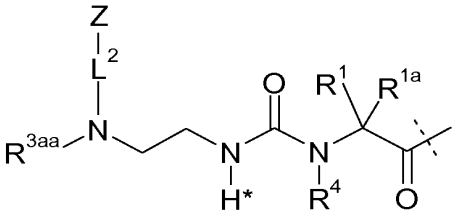
Preferably, L is represented by formula (Ia)


(Ia)

,

wherein $R^4$, $L^2$, and Z have the meaning as indicated above, and wherein $R^{3aa}$, $R^{3aaa}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl; or are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle. Preferably, $R^4$ is H; or methyl.

Preferably, L is represented by formula (Ib)


(Ib)

,

wherein $R^1$, $R^{1a}$, $R^4$, $L^2$ and Z have the meaning as indicated above, and wherein $R^{3aa}$ is H; or $C_{1-4}$ alkyl. Preferably, $R^4$ is H; or methyl.

Preferably, $R^1$ in formula (I) is $L^2$-Z.

Preferably, $R^3$ in formula (I) is $L^2$-Z.

Preferably, $R^3$, $R^{3a}$ in formula (I) are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle, wherein the heterocycle is substituted with $L^2$-Z.

Preferably, D-H is a small molecule bioactive agent or a biopolymer.

Preferably, D-H is a biopolymer selected from the group of biopolymers consisting of proteins, polypeptides, oligonucleotides, and peptide nucleic acids.

"Oligonucleotides" means either DNA, RNA, single-stranded or double-stranded, siRNA, miRNA, aptamers, and any chemical modifications thereof with preferably 2 to 1000 nucleotides. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base- pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping and change of stereochemistry.

Preferably, D-H is a polypeptide selected from the group of polypeptides consisting of ACTH, adenosine deaminase, agalsidase, alfa-1 antitrypsin (AAT), alfa-1 proteinase inhibitor (API), alteplase, amylins (amylin, symlin), anistreplase, ancrod serine protease, antibodies (monoclonal or polyclonal, and fragments or fusions), antithrombin III, antitrypsins, aprotinin, asparaginases, atosiban, biphalin, bivalirudin, bone-morphogenic proteins, bovine pancreatic trypsin inhibitor (BPTI), cadherin fragments, calcitonin (salmon), collagenase, complement C1 esterase inhibitor, conotoxins, cytokine receptor fragments, DNase, dynorphine A, endorphins, enfuvirtide, enkephalins, erythropoietins, exendins, factor VII, factor VIIa, factor VIII, factor VIIIa, factor IX, fibrinolysin, fibroblast growth factor (FGF), growth hormone releasing peptide 2 (GHRP2), fusion proteins, follicle-stimulating hormones, gramicidin, ghrelin, desacyl-ghrelin, granulocyte colony stimulating factor (G-CSF), galactosidase, glucagon, glucagon-like peptides, glucocerebrosidase, granulocyte macrophage colony stimulating factor (GM-CSF), human heat shock proteins (HSP),

phospholipase-activating protein (PLAP), gonadotropin chorionic (hCG), hemoglobins, hepatitis B vaccines, hirudin, human serine protease inhibitor, hyaluronidases, idurnonidase, immune globulins, influenza vaccines, interleukins (1 alfa, 1 beta, 2, 3, 4, 6, 10, 11, 12, 13, 21), IL-1 receptor antagonist (rhIL-1ra), insulins, insulin like growth factors, insulin-like growth factor binding protein (rhIGFBP), interferons (alfa 2a, alfa 2b, alfa 2c, beta 1a, beta 1b, gamma 1a, gamma 1b), intracellular adhesion molecule, keratinocyte growth factor (KGF), P-selectin glycoprotein ligand (PSGL), transforming growth factors, lactase, leptin, leuprolide, levothyroxine, luteinizing hormone, lyme vaccine, natriuretic peptides (ANP, BNP, CNP and fragments), neuropeptide Y, pancrelipase, pancreatic polypeptide, papain, parathyroid hormone, PDGF, pepsin, peptide YY, platelet activating factor acetylhydrolase (PAF-AH), prolactin, protein C, thymalfasin, octreotide, secretin, sermorelin, soluble tumor necorsis factor receptor (TNFR), superoxide dismutase (SOD), somatropins (growth hormone), somatoprim, somatostatin, streptokinase, sucrase, terlipressin, tetanus toxin fragment, tilactase, thrombins, thymosin, thyroid stimulating hormone, thyrotropin, tumor necrosis factor (TNF), TNF receptor-IgG Fc, tissue plasminogen activator (tPA), TSH, urodilatin, urate oxidase, urokinase, vaccines, vascular endothelial growth factor (VEGF), vasoactive intestinal peptide, vasopressin, ziconotide, lectin and ricin.

Preferably, D-H is a protein prepared by recombinant DNA technologies.

Preferably, D-H is a protein selected from the group of proteins consisting of antibody fragments, single chain antigen binding proteins, catalytic antibodies and fusion proteins.

Preferably, D-H is a small molecule bioactive agent selected from the group of agents consisting of central nervous system-active agents, anti-infective, anti-allergic, immunomodulating, anti-obesity, anticoagulants, antidiabetic, anti-neoplastic, antibacterial, anti-fungal, analgesic, contraceptive, anti-inflammatory, steroidal, vasodilating, vasoconstricting, and cardiovascular agents with at least one primary or secondary amino group.

Preferably, D-H is a small molecule bioactive agent selected from the group of agents consisting of acarbose, alaproclate, alendronate, amantadine, amikacin, amineptine, aminoglutethimide, amisulpride, amlodipine, amotosalen, amoxapine, amoxicillin,

amphetamine, amphotericin B, ampicillin, amprenavir, amrinone, anileridine, apraclonidine, apramycin, articaine, atenolol, atomoxetine, avizafone, baclofen, benazepril, benserazide, benzocaine, betaxolol, bleomycin, bromfenac, brofaromine, carvedilol, cathine, cathinone, carbutamid, cefalexine, clinafloxacin, ciprofloxacin, deferoxamine, delavirdine, desipramine, daunorubicin, dexmethylphenidate, dexmethylphenidate, diaphenylsulfon, dizocilpine, dopamin, dobutamin, dorzolamide, doxorubicin, duloxetine, eflornithine, enalapril, epinephrine, epirubicin, ergoline, ertapenem, esmolol, enoxacin, ethambutol, fenfluramine, fenoldopam, fenoterol, fingolimod, flecainide, fluvoxamine, fosamprenavir, frovatriptan, furosemide, fluoexetine, gabapentin, gatifloxacin, gemiflocacin, gentamicin, grepafloxacin, hexylcaine, hydralazine, hydrochlorothiazide, icofungipen, idarubicin, imiquimod, inversine, isoproterenol, isradipine, kanamycin A, ketamin, labetalol, lamivudine, levobunolol, levodopa, levothyroxine, lisinopril, lomefloxacin, loracarbef, maprotiline, mefloquine, melphalan, memantine, meropenem, mesalazine, mescaline, methyldopa, methylenedioxymethamphetamine, metoprolol, milnacipran, mitoxantron, moxifloxacin, norepinephrine, norfloxacin, nortriptyline, neomycin B, nystatin, oseltamivir, pamidronic acid, paroxetine, pazufloxacin, pemetrexed, perindopril, phenmetrazine, phenelzine, pregabalin, procaine, pseudoephedrine, protriptyline, reboxetine, ritodrine, sabarubicin, salbutamol, serotonin, sertraline, sitagliptin, sotalol, spectinomycin, sulfadiazin, sulfamerazin, sertraline, sprectinomycin, sulfalen, sulfamethoxazol, tacrine, tamsulosin, terbutaline, timolol, tirofiban, tobramycin, tocainide, tosufloxacin, trandolapril, tranexamic acid, tranylcypromine, trimerexate, trovafloxacin, valaciclovir, valganciclovir, vancomycin, viomycin, viloxazine, and zalcitabine.

Preferably, Z is a polymer of at least 500 Da or a $C_{8-18}$ alkyl group.

Preferably, Z is selected from the group of optionally crosslinked polymers consisting of poly(propylene glycol), poly(ethylene glycol), dextran, chitosan, hyaluronic acid, alginate, xylan, mannan, carrageenan, agarose, cellulose, starch, hydroxyalkyl starch (HAS), poly(vinyl alcohols), poly(oxazolines), poly(anhydrides), poly(ortho esters), poly(carbonates), poly(urethanes), poly(acrylic acids), poly(acrylamides), poly(acrylates), poly(methacrylates), poly(organophosphazenes), polyoxazoline, poly(siloxanes), poly(amides), poly(vinylpyrrolidone), poly(cyanoacrylates), poly(esters), poly(iminocarbonates), poly(amino acids), collagen, gelatin, hydrogel or a blood plasma protein, and copolymers thereof.

Preferably, Z is a protein.

Preferably, Z is a protein selected from the group consisting of albumin, transferrin, immunoglobulin.

Preferably, Z is a linear or branched poly(ethylene glycol) with a molecular weight from 2,000 Da to 150,000 Da.

Even more preferred is a prodrug of the present invention, wherein D-H is a GLP-1 receptor agonist; L is $L^1$ represented by formula (I) as indicated above; and Z is a hydrogel. Even more preferably, in formula (I) X is $N(R^4)$, $X^1$ is C and $X^3$ is O. Even more preferably, L is represented by formula (Ia) as indicated above.

GLP-1 is one of the intestinal peptide hormones that are released into the circulatory system after food intake. It augments the postprandial release of insulin, when nutritions (especially carbohydrates) are absorbed and their level postprandially elevated. GLP-1 associates with GLP-1 receptor sites located on pancreatic β-cells and elevates endogenous cAMP levels in a dose dependent manner. In isolated rat islets in the presence of above normoglycemic glucose levels, GLP-1 stimulates the release of insulin. A therapeutic potential for GLP-1 in type 2 diabetes patients was suggested before, owing to the profound efficacy of this insulinotropic peptide to stimulate secretion of insulin when glucose levels are elevated and to cease doing so upon return to normoglycemia. The antidiabetogenic effect of glucagon-like peptide-1 (7-36) amide in normal subjects and patients with diabetes mellitus is described e. g. in N. Engl. J. Med. 326(20):1316-1322. In vitro studies and animal experiments suggest that GLP-1 improves insulin sensitivity and has an anabolic effect on pancreatic β-cells. In humans, GLP-1 was also reported to suppress glucagon secretion, decelerate gastric empyting, and induce satiety, leading to weight loss if administered for weeks and months.

Exendin-4 is reported to associate with GLP-1 receptors located on pancreatic beta-cells with 2.5 times higher affinity than GLP-1. In isolated rat islets and beta-cells in presence of glucose, exendin enhances secretion of insulin in a dose-dependent fashion. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells (see J. Biol. Chem. 268(26):19650-19655). Studies in type 2 diabetic rodents revealed that exendin-4 is 5530-fold more potent than GLP-1 in lowering blood glucose levels. Also, the duration of glucose-lowering action after a single administration of exendin-4 is significantly longer compared to GLP-1 (see e.g. Diabetes 48(5):1026-1034). Plasma half-life of exendin-4 in humans was

described to be only 26 minutes. Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers (see e.g. Am. J. Physiol. Endocrinol. Metab. 281(1):E155-61).

Accordingly in an even more preferred embodiment the GLP-1 receptor agonist is Exendin-4.

Hydrogels to be used are known in the art. Suitable hydrogels may be used which are described in WO-A 2006/003014. Accordingly, a hydrogel may be defined as a three-dimensional, hydrophilic or amphiphilic polymeric network capable of taking up large quantities of water. The networks are composed of homopolymers or copolymers, are insoluble due to the presence of covalent chemical or physical (ionic, hydrophobic interactions, entanglements) crosslinks. The crosslinks provide the network structure and physical integrity. Hydrogels exhibit a thermodynamic compatibility with water which allow them to swell in aqueous media. The chains of the network are connected in such a fashion that pores exist and that a substantial fraction of these pores are of dimensions between 1 nm and 1000 nm.

Another object of the present invention is a pharmaceutical composition comprising a prodrug of the present invention or a pharmaceutical salt thereof together with a pharmaceutically acceptable excipient.

Yet another object of the present invention is a prodrug of the present invention or a pharmaceutical composition of the present invention for use as a medicament.

Yet another object of the present invention is a method of treating, controlling, delaying or preventing in a mammalian patient in need of the treatment of one or more conditions comprising administering to said patient a therapeutically effective amount of a prodrug of the present invention or a pharmaceutical composition of the present invention or a pharmaceutically acceptable salt thereof.

Another object of the present invention is a prodrug precursor of formula Act-L, wherein L has the meaning as indicated above and Act is a leaving group.

Preferably, Act is chloride, bromide, fluoride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy, 2-thiooxo-thiazolidinyl, or N-hydroxysulfosuccinimidyl.

Examples

**Materials and Methods**

Materials: Side chain protected Exendin-4 (J. Eng et al., J. Biol.Chem. 1992, 267, 11, 7402-7405) on Rink amide resin, side chain protected BNP-32a (human, Cys10 and Cys26 exchanged for Ala) on chlorotrityl resin, side chain protected BNP-32b with ivDde side-chain protecting group on Lys14 (human, Cys10 and Cys26 exchanged for Ala) on chlorotrityl resin, and side chain protected human growth hormone releasing factor fragment 1-29 amide (GRF(1-29)) on Rink amide (each synthesized by Fmoc-strategy) were obtained from Peptide Specialty Laboratories GmbH, Heidelberg, Germany. Standard side chain protecting groups were used except for Lys27 of Exendin-4 and Lys21 of GRF(1-29) where Mmt side-chain protecting groups were used.
40kDa methoxy poly(ethylene glycol) maleimido-propionamide (PEG40kDa-maleimide) was obtained from Chirotech Technology Ltd, Cambridge, UK.

2-Chlorotrityl chloride resin, Sieber amide resin and amino acids were from Merck Biosciences GmbH, Schwalbach/Ts, Germany, if not stated otherwise. Fmoc-D-Homocysteine(Trt)-OH and S-Trityl-3-mercaptopropionic acid (Trt-MPA) were obtained from Bachem AG, Bubendorf, Switzerland. O-(N-Fmoc-2-aminoethyl)-O´-(2-carboxyethyl)-undecaethyleneglycol (Fmoc-Pop-OH) was obtained from Polypure AS, Oslo, Norway.
Fmoc-4-(2-aminoethyl)-1-carboxymethyl-piperazine (Fmoc-Acp-OH) was purchased from NeoMPS SA, Strasbourg, France. cis-Cyclohexane-1,2-dicarboxylic anhydride was obtained from Alfa Aesar GmbH & Co KG, Karlsruhe, Germany.

All other chemicals were from Sigma-ALDRICH Chemie GmbH, Taufkirchen, Germany.

Solid phase synthesis was performed on 2-Chlorotrityl chloride resin with a loading of 1.3 mmol/g or Sieber amide resin with a loading of 0.55 mmol/g. Syringes equipped with polypropylene frits were used as reaction vessels.

Loading of the first amino acid to resins was performed according to manufacturer's instructions.

Fmoc deprotection:

For Fmoc protecting-group removal, the resin was agitated with 2/2/96 (v/v/v) piperidine/DBU/DMF (two times, 10 min each) and washed with DMF (ten times).

ivDde deprotection:

For ivDde protecting-group removal, the resin was agitated with 98/2 (v/v) DMF/hydrazine hydrate (3 times, 10 min each) and washed with DMF (ten times).

Boc protection:

The N-terminus of a peptide was boc-protected by agitating the resin with 30 eq $(boc)_2O$ and 60 eq pyridine in DCM. After 1 h the resin was washed with DCM (10 times).

Standard coupling condition for acids:

Coupling of acids (aliphatic acids, Fmoc-amino acids) to free amino groups on resin was achieved by agitating resin with 3 eq of acid, 3 eq PyBOP and 6 eq DIEA in relation to free amino groups on resin (calculated based on theoretical loading of the resin) in DMF at room temperature. After 1 hour resin was washed with DMF (10 times).

3-maleimido propionic acid coupling:

Coupling of 3-maleimido propionic acid to free amino groups on resin was achieved by agitating resin with 2 eq of acid, 2 eq DIC and 2 eq HOBt in relation to free amino groups in DMF at room temperature. After 30 min, resin was washed with DMF (10 times).

Standard protocol for synthesis of ureas on resin:

Synthesis of ureas on resin was achieved by agitating resin with 2.5 eq of bis(pentafluorophenyl) carbonate, 5 eq DIEA, and 0.25 eq DMAP in relation to free amino groups in DCM/ACN 1/1 at room temperature. After 15 min resin was washed with DMF (10 times). 5 eq of amine was dissolved in DMF. Mixture was added to resin and agitated for 60 min at room temperature. Resin was washed with DMF (10 times).

Cleavage protocol for Sieber amide resin:

Upon completed synthesis, the resin was washed with DCM (10 times), dried in vacuo and treated repeatedly (five times a 15 minutes) with 97/2/1 (v/v) DCM/TES/TFA. Eluates were combined, volatiles were removed under a nitrogen stream and product was purified by RP-HPLC. HPLC fractions containing product were combined and lyophilized.

Cleavage protocol for 2-chlorotrityl chloride resin:
Upon completed synthesis, the resin was washed with DCM, dried in vacuo and treated two times for 30 minutes with 6/4 (v/v) DCM/HFIP. Eluates were combined, volatiles were removed under a nitrogen stream and product was purified by RP-HPLC. HPLC fractions containing product were combined and lyophilized.

Cleavage protocol for Rink amide resin:
Upon completed synthesis, the resin was washed with DCM, dried in vacuo and treated with 2 ml of TFA cleavage cocktail (TFA/TES/Water/DTT 95/2/2/1) per 100 mg resin for 60 min at room temperature. Volatiles were removed under a nitrogen stream. Unpolar side products and protecting groups were removed by precipitating peptide from diethyl ether. Precipitate was dried in vacuo and dissolved in ACN/water 1/1 and purified by RP-HPLC.

Amine containing products obtained as TFA salts were converted to the corresponding HCl salts using ion exchange resin (Discovery DSC-SAX, Supelco, USA). This step was performed in case the residual TFA was expected to interfere with e.g. a subsequent coupling reactions.
RP-HPLC purification:
RP-HPLC was done on a 100x20 or a 100x40 mm C18 ReproSil-Pur 300 ODS-3 5µ column (Dr. Maisch, Ammerbuch, Germany) connected to a Waters 600 HPLC System and Waters 2487 Absorbance detector. Linear gradients of solution A (0.1% TFA in $H_2O$) and solution B (0.1% TFA in acetonitrile) were used. HPLC fractions containing product were lyophilized.

Analytics: Electrospray ionization mass spectrometry (ESI-MS) was performed on a Waters ZQ 4000 ESI instrument and spectra were, if necessary, interpreted by Waters software MaxEnt.

Size exclusion chromatography (SEC) was performed using an Amersham Bioscience AEKTAbasic system equipped with a Superdex200 10/300 column (Amersham

Bioscience/GE Healthcare), if not stated otherwise. 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, 3 mM EDTA was used as mobile phase

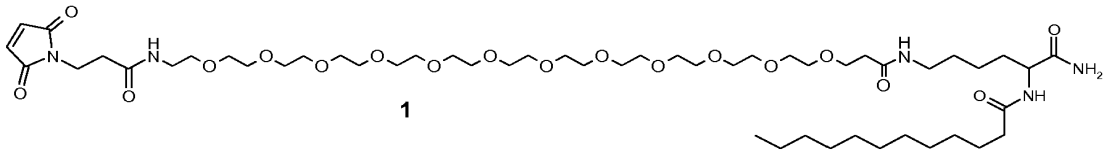
For Cation Exchange Chromatography, an Amersham Bioscience AEKTAbasic system was equipped with a Source 15S filled HR16/10 column (Amersham Bioscience/GE Healthcare).

Desalting was performed using an Amersham Bioscience AEKTAbasic system equipped with a HiPrep 26/10 Desalting column and 0.1% acetic acid in water as mobile phase.

In vitro linker hydrolysis and release of drug: Compounds were dissolved in buffer A (10 mM sodium phosphate, 140 mM NaCl, pH 7.4, 3 mM EDTA) or buffer B (0.1 M Acetat 3 mM EDTA, pH 4.0), and solution was filtered through a 0.2 µm filter and incubated at 37 °C. Samples were taken at time intervals and analyzed by RP-HPLC at 215 nm and ESI-MS. UV-signals correlating to liberated drug molecule were integrated and plotted against incubation time. In case of identical retention times of prodrug and drug, ratio of mass signals was used to determine release kinetics.
For hydrogel conjugates, compounds were suspended in buffer A and incubated at 37°C. Samples were taken after centrifugation of the suspension and analyzed by RP-HPLC at 215 nm. UV-signals correlating to liberated drug molecule were integrated and plotted against incubation time.
Curve-fitting software was applied to estimate the corresponding halftime of release.

**Example 1**

**Synthesis of fatty acid carrier (1)**



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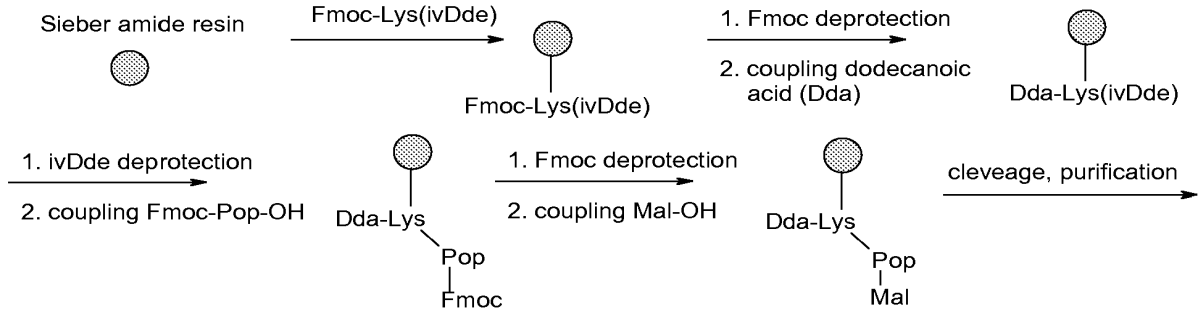


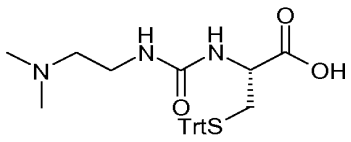

**1** was synthesized on sieber amide resin (477 mg, 0.262 mmol) by coupling of Fmoc-Lys(ivDde)-OH, fmoc deprotection, coupling of dodecanoic acid, ivDde deprotection, coupling of Fmoc-Pop-OH, fmoc deprotection, coupling of 3-maleimido propionic acid, cleavage from resin and purification as depicted above and described in "Materials and Methods".

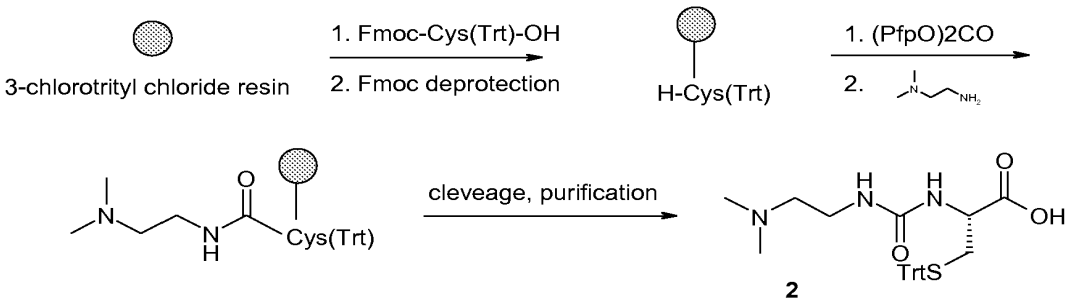
Yield: 128 mg (0.119 mmol).

MS: m/z 1101.0 = $[M+Na]^+$ (MW calculated = 1078.4 g/mol).

**Example 2**

**Synthesis of Linker reagent (2)**






**2**

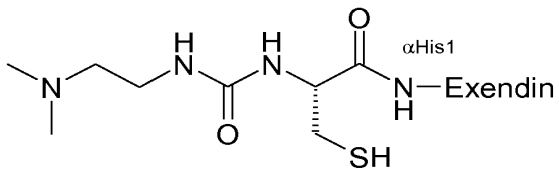
Linker reagent **2** was synthesized on 3-chlorotrityl chloride resin (300 mg, 0.39 mmol) by loading of resin with Fmoc-Cys(Trt)-OH, fmoc deprotection, and on-resin urea formation using N,N-dimethyl-ethylenediamine as amine, cleavage from resin as depicted above and described in "Materials and Methods". For RP-HPLC separation, 0.01% HCl in water was used as solution A and 0.01% HCl in acetonitrile was used as solution B.

Yield: 82 mg of HCl salt (0.16 mmol).

MS: m/z 478.2 = $[M+H]^+$ (MW calculated = 477.6 g/mol).

**Example 3**

**Synthesis of Exendin-4 linker intermediate (3)**




**3**

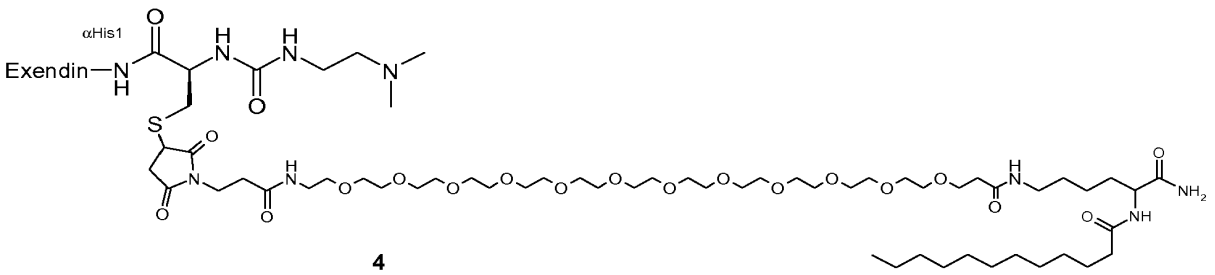
**2** (14 mg, 0.027 mmol), PyBOP (14 mg, 0.027 mmol), and DIEA (17 µl, 0.10 mmol) were dissolved in 0.2 ml of dry DMF. Mixture was added to 22 mg side-chain protected Exendin-4 on-resin (0.1 mmol/g, 2.2 µmol) and agitated for 30 min at room temperature. Resin was washed with DMF (10 times) and DCM (10 times). **3** was cleaved from resin and purified by RP-HPLC as described in "Materials and Methods".

Yield: 1.7 mg **3** as TFA salt (0.38 µmol).

MS: m/z 1468.7 = $[M+3H]^{3+}$ (MW calculated = 4403 g/mol).

**Example 4**

**Synthesis of fatty acid-PEG-linker-Exendin-4 conjugate (4)**

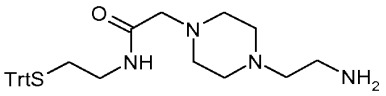



4

**3** (1.7 mg, 0.38 µmol) and **1** (0.6 mg, 0.58 µmol) were dissolved in 500 µl of acetonitrile/water 7/3 (v/v). 40 µl of 0.5 M phosphate buffer (pH 7.4) were added and the mixture was incubated at RT for 10 min. Conjugate **4** was purified by RP-HPLC.
MS: m/z 1828.7 = $[M+3H]^{3+}$ (MW calculated = 5480 g/mol).

**Example 5**

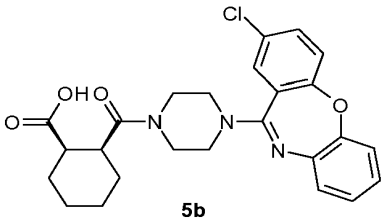
**Synthesis of linker intermediate (5a)**




**5a**

Fmoc-Acp-OH · 2 HCl (100 mg, 0.21 mmol) was suspendend in 400 µl DMF/DMSO 1/1 (v/v). S-tritylcysteamine · HCl (75 mg, 0.21 mmol), PyBOP (109 mg, 0.21 mmol) and DIEA (146 µl, 0.86 mmol) were added and mixture was agitated for 60 min at RT. Fmoc group was removed by adding 75 µl piperidine and 25 µl DBU. After 15 min mixture was hydrolyzed and acidified (AcOH) and compound was purified by RP-HPLC. After lyophilization 98 mg (0.14 mmol, double TFA salt) were obtained.
MS: m/z 511.6 = $[M+Na]^{+}$ (MW calculated = 488.7 g/mol).

**Synthesis of cis-cyclohexane diacarboxylic acid amoxapine monoamide (5b)**

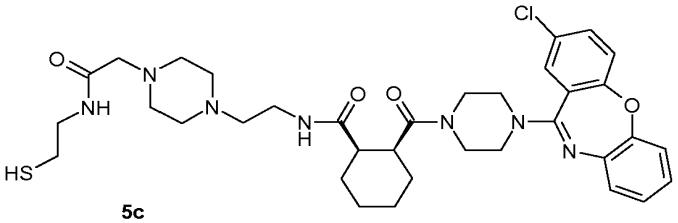


5b + enantiomer

Amoxapine (200 mg, 0.64 mmol) and cis-cyclohexane-1,2-dicarboxylic anhydride (108 mg, 0.70 mmol) were dissolved in 700 µl of dry DMF. Pyridine (130 µl, 1.6 mmol) was added and mixture was stirred for 60 min at RT. Mixture was quenched with 2 ml of acetonitrile/acetic acid/water (1/1/1) and purified by RP-HPLC. After lyophilization 344 mg (0.49 mmol, double TFA salt) of **5b** were obtained.

MS: m/z 468.5 = $[M+H]^+$ (MW calculated = 468.0 g/mol).

**Synthesis of linker-amoxapine conjugate (5c)**



5c + enantiomer

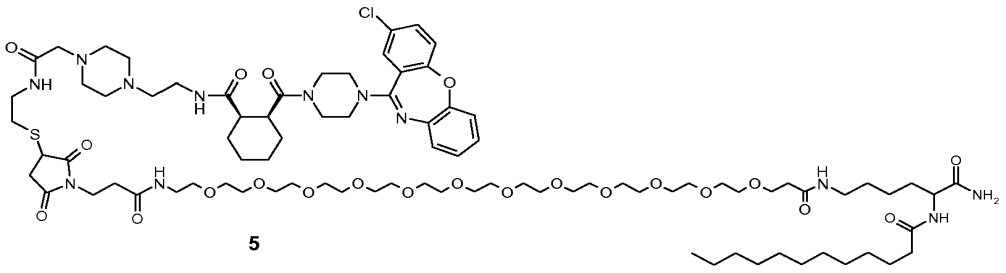
**5b** (7 mg, 0.010 mmol) was preactivated by incubating with PyBOP (12.5 mg, 0.024 mmol) and DIEA (5 µl, 0.03 mmol) in 200 µl of dry DMF for 45 min at RT. **5a** (20 mg, 0.028 mmol) and DIEA (15 µl, 0.09 mmol) were added and mixture was incubated for further 60 min. Mixture was quenched with 0.5 ml of acetonitrile/acetic acid/water (1/1/1) and purified by RP-HPLC. After lyophilization 3 mg (0.0026 mmol, double TFA salt) of **5c** were obtained.

MS: m/z 939.3 = $[M+H]^+$ (MW calculated = 938.6 g/mol).

For trityl deprotection, lyophilisate was incubated in 1 ml HFIP and 3 µl TES for 30 min. Mixture was evaporated and thiol was purified by RP-HPLC. After lyophilization 2 mg (2.2 µmol, double TFA salt) of amoxapine-linker conjugate **5c** were obtained.

MS: m/z 697.1 = $[M+H]^+$ (MW calculated = 696.3 g/mol).

**Synthesis of fatty acid-PEG-amoxapine conjugate (5)**



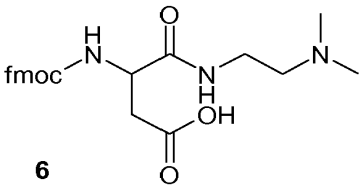
5

Amoxapine-linker conjugate **5c** (2 mg, 2.2 µmol) and **1** (3.5 mg, 3.2 µmol) were dissolved in 900 µl of acetonitrile/water 7/3 (v/v). 60 µl of 0.5 M phosphate buffer (pH 7.4) were added and the mixture was incubated at RT for 10 min. **5** was purified by RP-HPLC.

MS: m/z 1774.9 = $[M+H]^+$ (MW calculated = 1774.7 g/mol).

**Example 6**

**Synthesis of linker reagent (6)**



6

Fmoc-Asp(tBu)-OH (411 mg, 1 mmol), HOBt (153 mg, 1 mmol), and DIC (160 µl, 1 mmol) were dissolved in 2 ml of DMF and incubated for 10 min at RT. N,N-dimethyl ethylenediamine (160 µl, 1.5 mmol) was added and stirred at RT for 30 min. Acetic acid (300 µl) was added and Fmoc-Asp(tBu)-NH-$(CH_2)_2$-$N(CH_3)_2$ was purified by RP-HPLC.

Yield: 220 mg (0.46 mmol)

MS Fmoc-Asp(tBu)-NH-$(CH_2)_2$-$N(CH_3)_2$: m/z 504.6 = $[M+Na]^+$ (MW calculated = 481.6 g/mol).

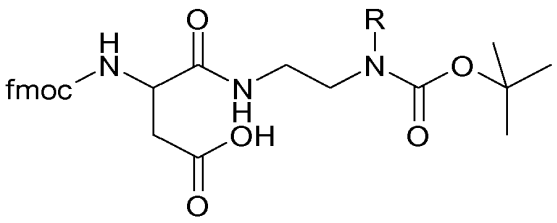
Fmoc-Asp(tBu)-NH-$(CH_2)_2$-$N(CH_3)_2$ (220 mg, 0.46 mmol) was dissolved in 3 ml of 98/2 (v/v) TFA/TES. After 30 min the solvent was removed under a nitrogen stream and **6** was purified by RP-HPLC using 0.01% HCl in water as solvent A and 0.01% HCl in acetonitril as solvent B.

Yield: 146 mg (0.32 mmol, HCl salt)

MS: m/z 426.5 = $[M+H]^+$ (MW calculated = 425.5 g/mol).

**Example 7**

**Synthesis of linker reagents 7a and 7b**




**7a**: R = H
**7b**: R = $CH_3$

Synthesis of **7a:**

Fmoc-Asp(tBu)-OH (300 mg, 0.73 mmol), HOBt (1112 mg, 0.73 mmol), and DIC (117 µl, 0.73 mmol) were dissolved in 2 ml of DMF and incubated for 10 min at RT. Boc-ethylenediamine (230 mg, 1.44 mmol) was added and stirred at RT for 30 min. Acetic acid (300 µl) was added and Fmoc-Asp(tBu)-NH-$(CH_2)_2$-NH-boc was purified by RP-HPLC.

Yield: 205 mg (0.37 mmol)

MS intermediate: m/z 576.6 = $[M+Na]^+$ (MW calculated = 553.7 g/mol).

Fmoc-Asp(tBu)-NH-$(CH_2)_2$-NH-boc (205 mg, 0.37 mmol) was dissolved in 3 ml of 98/2 (v/v) TFA/TES. After 30 min the solvent was removed under a nitrogen stream and Fmoc-Asp(H)-NH-$(CH_2)_2$-$NH_2$ was purified by RP-HPLC.

Yield: 140 mg (0.27 mmol, TFA salt)

MS intermediate: m/z 398.8 = $[M+H]^+$ (MW calculated = 397.4 g/mol).

Fmoc-Asp(H)-NH-$(CH_2)_2$-$NH_2$ (140 mg, 0.27 mmol, TFA salt) was dissolved in 1 ml of DMF and DIEA (140 µl, 0.81 mmol) and $boc_2O$ (100 mg, 0.46 mmol) added. The solution was stirred at RT for 15 min and then acidified with acetic acid (300 µl). **7a** was purified by RP-HPLC.

Yield **7a**: 120 mg (0.24 mmol)

MS **7a**: m/z 520.5 = $[M+Na]^+$ (MW calculated = 497.6 g/mol).

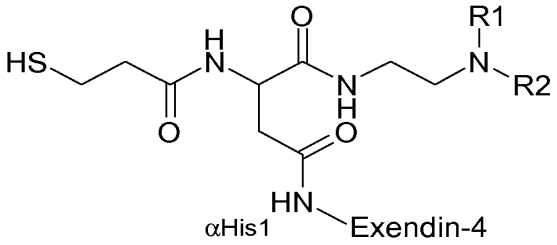
**7b** was synthesized as described above except for the use of $H_2N$-$(CH_2)_2$-$N(CH_3)$-boc instead of boc-ethylenediamine as amine in the first step.

Yield **7b**: 115 mg

MS **7b**: m/z 534.5 = $[M+Na]^+$ (MW calculated = 511.6 g/mol).

**Example 8**

**Synthesis of Exendin-linker conjugates 8a, 8b and 8c**




**8a**: R1 = H, R2 = H
**8b**: R1 = H, R2 = $CH_3$
**8c**: R1 = $CH_3$, R2 = $CH_3$

Synthesis of **8a**:

**7a** (30 mg, 60 µmol), HOBt (9 mg, 60 µmol), DIEA (12 µl, 70 µmol), and DIC (10 µl, 63 µmol) were dissolved in 200 µl of DMF and immediately added to side-chain protected Exendin-4 on resin (40 mg, 4 µmol) and incubated for 1 h at room temperature. Resin was washed ten times with DMF and then incubated for 5 min with 500 µl of 1/1/2 acetic anhydride/pyridine/DMF. Resin was washed 10 times with DMF and fmoc group was removed. Trt-mercaptopropionic acid was coupled and **8a** was cleaved from resin and purified by RP-HPLC.

Yield: 3.6 mg

MS **8a**: m/z 1108.5 = $[M+4H]^{4+}$; 1477.8 = $[M+3H]^{3+}$ (MW calculated = 4432 g/mol).

**8b** was synthesized as described above for **8a** except for the use of **7b** instead of **7a**.

Yield: 3.5 mg

MS **8b**: m/z 1112.5 = $[M+4H]^{4+}$; 1482.5 = $[M+3H]^{3+}$ (MW calculated = 4446 g/mol).

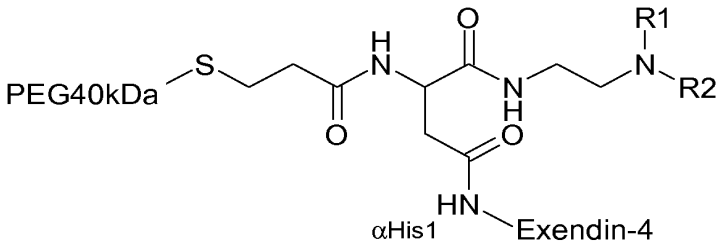
**8c** was synthesized as described above for **8a** except for the use of **6** instead of **7a**.

Yield: 3.2 mg

MS **8c**: m/z 1116.2 = $[M+4H]^{4+}$; 1487.8 = $[M+3H]^{3+}$ (MW calculated = 4460 g/mol).

**Example 9**

**Synthesis of PEG40kDa-linker-Exendin conjugates 9a, 9b, and 9c**




**9a**: R1 = H, R2 = H
**9b**: R1 = H, R2 = $CH_3$
**9c**: R1 = $CH_3$, R2 = $CH_3$

Synthesis of **9a**:

**8a** (3.6 mg) was dissolved in 300 µl 2/1 water/acetonitrile and 50 mg PEG40kDa-maleimide was added. 100 µl 0.25 M sodium phosphate buffer pH 7 were added and after 5 min the solution was acidfied with 50 µl acetic acid.

**9a** was purified by ion exchange chromatography using 10 mM sodium citrate pH 3 as solvent A and 10 mM sodium citrate pH 3 and 1 M NaCl as solvent B and a step-gradient (0 to 40% B). Fractions containing **9a** were desalted and lyophilized:

Yield: 14 mg

**9b** was synthesized as described above except for the use of **8b**.

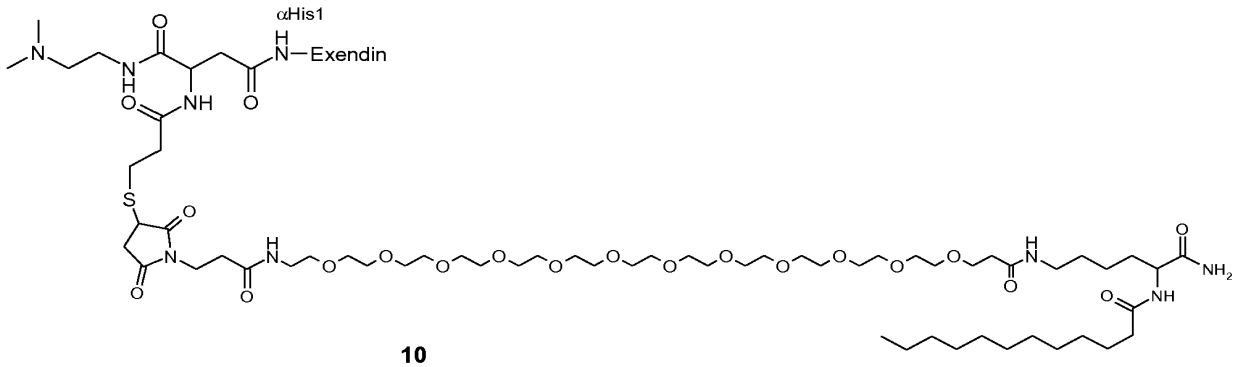
Yield: 15 mg

**9c** was synthesized as described above except for the use of **8c**.

Yield: 13 mg

**Example 10**

**Synthesis of fatty acid-linker-Exendin conjugate 10**




**10**

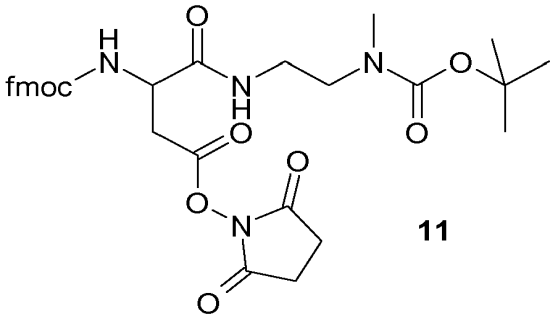
**8c** (1 mg) was dissolved in 100 µl 1/1 acetonitrile/water and **1** (1 mg) in 100 µl of 3/1 acetonitrile/water was added. 100 µl of 0.25 M sodium phosphate buffer was added, the reaction was stirred for 5 min, after which **10** was purified by RP-HPLC.

Yield: 1.3 mg

MS **10**: m/z 1385.9 = $[M+4H]^{4+}$; 1846.3 = $[M+3H]^{3+}$ (MW calculated = 5528.3 g/mol).

**Example 11**

**Synthesis of NHS-activated linker reagent 11**




**11**

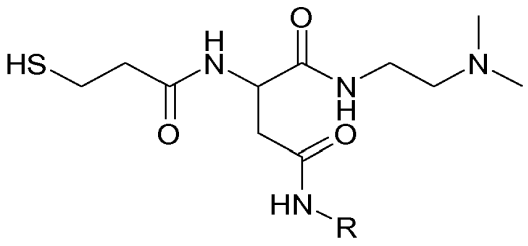
**7b** (20 mg, 40 µmol), N,N´-dicyclohexylcarbodiimide (10 mg, 48 µmol), and NHS (8 mg, 70 µmol) were dissolved in 300 µl of dry DCM and stirred at RT for 1 h. Solvent was removed under a nitrogen stream and **11** was purified by RP-HPLC and lyophilized.

Yield: 22 mg (36 µmol)

MS: m/z 631.5 = $[M+Na]^{+}$ (MW calculated = 608.7 g/mol).

**Example 12**

**Synthesis of linker-Exendin(Fluorescein) conjugate (12a) and linker-GRF(1-29)(Fluorescein) conjugate (12b)**



**12a**: R = Exendin(εK27-fluorescein)
**12b**: R = GRF(1-29)(εK21-fluorescein)

**6** (60 mg, 130 µmol HCl salt), HOBt (20 mg, 130 µmol), DIEA (40 µl, 230 µmol), and DIC (20 µl, 126 µmol) were dissolved in 700 µl of DMF and immediately added to side-chain protected Exendin-4 on resin (120 mg, 12 µmol) and incubated for 1 h at room temperature. Resin was washed ten times with DMF and then incubated for 5 min with 1 ml of 1/1/2 (v/v/v) acetic anhydride/pyridine/DMF. Resin was washed ten times with DMF and fmoc group was removed. Trt-mercaptopropionic acid was coupled according to standard coupling method and resin was washed five times with DMF and ten times with DCM. Mmt protecting group of Lys27 was removed by incubation of resin five times in 2 ml of 9/1 (v/v) DCM/HFIP for 5 min. Resin was washed five times with DCM and five times with DMF and 5,6-carboxy-flourescein-NHS ester (20 mg, 42 µmol) and DIEA (20 µl, 115 µl) in 300 µl DMF were added to resin and incubated for 30 min. **12a** was cleaved from resin and purified by RP-HPLC

Yield: 12 mg

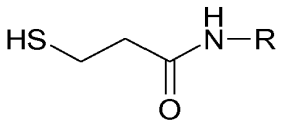
MS **12a**: m/z 1205.9 = $[M+4H]^{4+}$; 1607.0 = $[M+3H]^{3+}$ (MW calculated = 4818.3 g/mol).

**12b** was synthesized as described for **12a** except for the use of GRF(1-29) on resin (120mg, 12 µmol).

Yield: 11 mg

MS **12b**: m/z 998.6 = $[M+4H]^{4+}$; 1330.5 = $[M+3H]^{3+}$ (MW calculated = 3989.6 g/mol).

**Synthesis of mercaptopropionyl-Exendin(Fluorescein) (12c) and mercaptopropionyl-GRF(1-29)(Fluorescein) (12d)**



**12c**: R = Exendin(εK27-fluorescein)
**12d**: R = GRF(1-29)(εK21-fluorescein)

Trt-mercaptopropionic acid was coupled according to standard coupling method to side-chain protected Exendin-4 on resin (120 mg, 12 μmol). Mmt protecting group removal of Lys27 and 5,6-carboxy-flourescein-NHS ester coupling was performed as described for **12a**. **12c** was cleaved from resin and purified by RP-HPLC

Yield: 13 mg

MS **12c**: m/z 1545.6 = $[M+3H]^{3+}$ (MW calculated = 4633 g/mol).

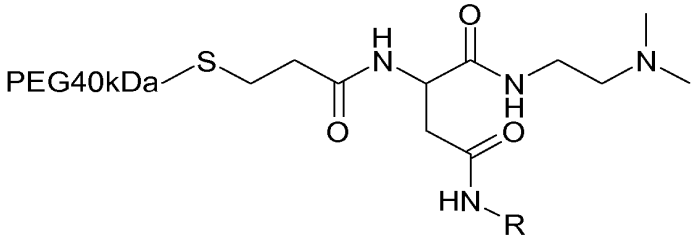
**12d** was synthesized as described for **12c** except for the use of GRF(1-29) on resin (120mg, 12 μmol).

Yield: 11 mg

MS **12d**: m/z 1269.1 = $[M+3H]^{3+}$ (MW calculated = 3804.3 g/mol).

**Example 13**

**Synthesis of reversible PEG40kDa-linker-Exendin(Fluorescein) conjugate (13a) And reversible PEG40kDa-linker-GRF(1-29)(Fluorescein) conjugate (13b)**



**13a**: R = Exendin(εK27-fluorescein)
**13b**: R = GRF(1-29)(εK21-fluorescein)

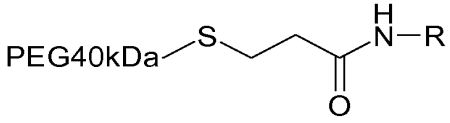
**12a** (12 mg) was dissolved in 500 µl of 1/1 acetonitrile/water and 120 mg PEG40kDa-maleimide in 1 ml of 1/1 acetonitrile/water was added. 300 µl of 0.25 M sodium phosphate buffer pH 7.0 were added and solution was acidified after 10 min with 300 µl acetic acid. **13a** was purified by cation exchange chromatography, desalted, and then lyophilized.

Yield: 51 mg

**13b** was synthesized as described for **13a** except for the use of **12b** instead of **12a.**

Yield: 46 mg

**Synthesis of permanent PEG40kDa- Exendin(Fluorescein) conjugate (13c) and permanent PEG40kDa-GRF(1-29)(Fluorescein) conjugate (13d)**




**13c**: R = Exendin(εK27-fluorescein)
**13d**: R = GRF(1-29)(εK21-fluorescein)

**13c** was synthesized as described for **13a** except for the use of **12c** instead of **12a**.

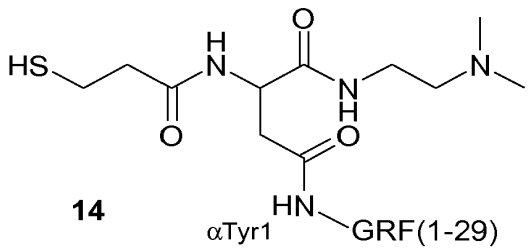
Yield: 55 mg

**13d** was synthesized as described for **13a** except for the use of **12d** instead of **12a.**

Yield: 45 mg

**Example 14**

**Synthesis of linker-GRF(1-29) conjugate 14**




**14**

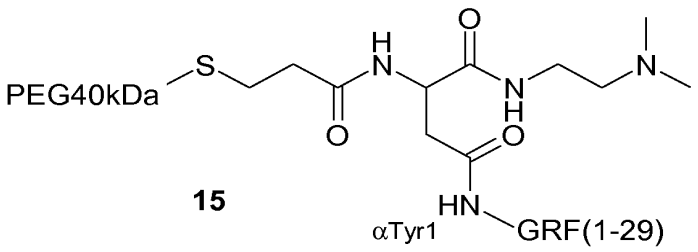
**14** was synthesized as described for **8c** except for the use of side-chain protected GRF(1-29) resin.

Yield: 10 mg

MS **14**: m/z 908.2 = $[M+4H]^{4+}$; 1211.2 = $[M+3H]^{3+}$ (MW calculated = 3631.3 g/mol).

**Example 15**

**Synthesis of PEG40kDa-linker-GRF(1-29) conjugate (15)**



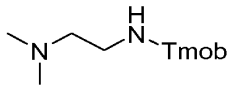

**15**

**15** was synthesized as described for **9c** except for the use of **14** and 10 mM sodium citrate pH 4 as solvent A and 10 mM sodium citrate pH 4 and 1 M sodium chloride as solvent B for cation exchange chromatography.

Yield: 11 mg

**Example 16**

**Synthesis of linker intermediate 16**




**16**

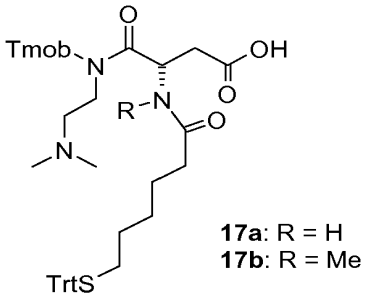
N,N-dimethylethylenediamine (198 µL, 1.8 mmol) and $NaCNBH_3$ (58 mg, 0.9 mmol) were dissolved in methanol (5 mL) and brought to pH 5.5 by addition of AcOH (250 µL). A suspension of 2,4,6,-trimethoxybenzaldehyde (294 mg, 1.5 mmol) in EtOH (5 mL) was added and the reaction was stirred at RT for 1 h. 5 N HCl (0.5 mL) was added and the mixture was stirred for further 12 h. The solvent was removed under reduced pressure; the residue was dissolved in sat. $NaHCO_3$ and extracted 3x with DCM. The combined organic phases were dried over $NaSO_4$ and the solvent was evaporated under reduced pressure.

Yield: 303 mg (1.13 mmol)

MS: m/z 269.3 = $[M+H]^+$ (MW calculated = 268.4 g/mol)

**Example 17**

**Synthesis of linker 17a and 17b**




**17a**: R = H
**17b**: R = Me

Synthesis of **17a**:

Fmoc-Asp(OtBu)-OH (322 mg, 0.78 mmol), Tmob-protected diamine **16** (150 mg, 0.56 mmol), HATU (255 mg, 0.67 mmol) and DIEA (290 µL, 1.68 mmol) were dissolved in DMF (1.5 mL). The mixture was stirred for 30 min, acidified with AcOH and purified by RP-HPLC.

Yield: 463 mg (5.97 mmol,TFA salt, ca. 90% pure)

MS Fmoc-Asp(OtBu)-N(TMOB)$CH_2CH_2N(CH_3)_2$ : m/z 662.5 = $[M+H]^+$ (MW calculated = 661.8 g/mol)

Fmoc-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$ (225 mg, 0.29 mmol) was dissolved in a solution of piperidine (50 µL) and DBU (15 µL) in DMF (1.5 mL). The mixture was stirred at RT for 1.5 h. AcOH was added and H-Asp(OtBu)-N(TMOB)$CH_2CH_2N(CH_3)_2$ was purified by RP-HPLC.

Yield: 114 mg (0.21 mmol, TFA salt)

MS H-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$: m/z 462.4 = $[M+Na]^+$ (MW calculated = 439.6 g/mol)

The TFA salt of H-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$ (114 mg, 0.21 mmol) was dissolved in sat. $NaHCO_3$ (10 mL) and extracted 3x with DCM (3x 10 mL). The combined organic layers were dried over $NaSO_4$ and the solvent was removed under reduced pressure. The residue was dissolved in DMF (1.0 mL), 6-tritylmercaptohexanoic acid (121 mg, 0.31 mmol), HATU (118 mg, 0.31 mmol) and DIEA (108 µL, 0.62 mmol) were added. The mixture was

stirred for 30 min. AcOH was added (200 µL) and TrtS($CH_2$)$_5$CONH-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$ was purified by RP-HPLC.

Yield: 95 mg (0.10 mmol, TFA salt)

MS TrtS($CH_2$)$_5$CONH-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$: m/z 812.64 = $[M+H]^+$ (MW calculated = 812.1 g/mol)

TrtS($CH_2$)$_5$CONH-Asp(OtBu)-N(Tmob)$CH_2CH_2N(CH_3)_2$ (95 mg, 0.10 mmol) was dissolved in a 3:1 mixture of MeOH/$H_2O$ (1.0 mL), LiOH (7.4 mg, 0.31 mmol) was added and the mixture was stirred for 5 h at 60°C. AcOH was added (100 µL) and **17a** was purified by RP-HPLC.

Yield: 64 mg (0.07 mmol, TFA salt)

MS **17a**: m/z 756.5 = $[M+H]^+$ (MW calculated = 756.0 g/mol)

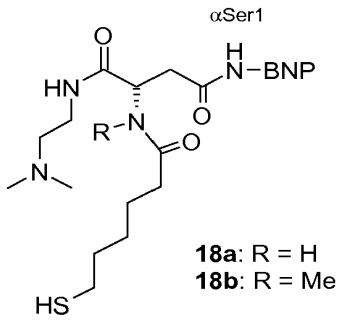
**17b** was synthesized as described above except for the use of Fmoc-NMe-Asp(OtBu)-OH instead of Fmoc-Asp(OtBu)-OH in the first step.

Yield **17b**: 16 mg (18 µmol, TFA salt)

MS **17b**: m/z 770.5 = $[M+H]^+$ (MW calculated = 770.0 g/mol)

**Example 18**

**Synthesis of linker-BNP conjugates 18a and 18b**




**18a**: R = H
**18b**: R = Me

Synthesis of **18a**:

**17a** (8.0 mg, 0.01 mmol), PyBOP (5.2 mg, 10 µmol) and DIEA (7 µL, 40 µmol) were dissolved in DMF (400 µL) and immediately added to resin bound, side chain protected BNP-32a (50 mg, 5 µmol). After incubation for 2 h at RT, the resin was washed with 10x DMF, 10x DCM and dried in vacuo. The product was cleaved from the resin and purified by RP-HPLC.

Yield: 10.6 mg

MS **18a**: m/z 930.4 = $[M+4H]^{4+}$; 1240.1 = $[M+3H]^{3+}$ (MW calculated = 3717.2 g/mol)

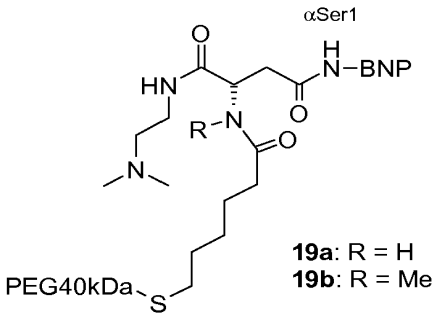
**18b** was synthesized as described above except for the use of **17b** instead of **17a**.

Yield: 4.7 mg

MS **18b**: m/z 933.9 = $[M+4H]^{4+}$; 1244.7 = $[M+3H]^{3+}$ (MW calculated = 3731.0 g/mol)

**Example 19**

**Synthesis of PEG40kDa-linker-BNP conjugates 19a and 19b**



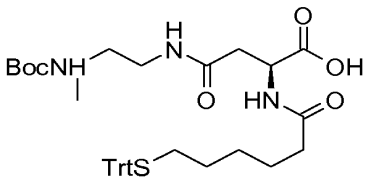

**18a** (5.2 mg) was dissolved in 1:1 $H_2O$/acetonitrile containing 0.1 % TFA (200 µL). A solution of PEG40kDa-maleimide (70 mg) in 1:1 $H_2O$/acetonitrile (1.5 mL) and phosphate buffer (30 µL, pH 7.4, 0.5 M) was added. The solution was incubated at RT, after 5 min AcOH (30 µL) was added. **19a** was purified by cation exchange chromatography, desalted, and lyophilized.

Yield: 19.2 mg

**19b** was synthesized as described for **19a** except for the use of **18b** instead of **18a**.

**Example 20**

**Synthesis of linker 20**




**20**

Fmoc-Asp(OH)OtBu (100 mg, 0.24 mmol), $H_2N$-$(CH_2)_2$-N($CH_3$)-boc (36 µL, 0.20 mmol), HATU (92 mg, 0.24 mmol) and DIEA (105 µL, 0.60 mmol) were dissolved in 1 mL DMF. The mixture was stirred for 1 h at RT, acidified with AcOH (100 µL) and purified by HPLC.
Yield: 91 mg (0.13 mmol)
MS Fmoc-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu: 590.3 = $[M+Na]^+$ (MW calculated = 567.7 g/mol)

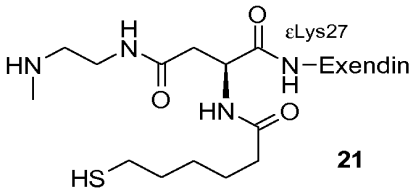
Fmoc-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu (91 mg, 0.13 mmol) was dissolved in DMF (1.0 mL), piperidine (50 µL) and DBU (15 µL) were added and the mixture was stirred for 45 min at RT. AcOH (100 µL ) was added and $NH_2$-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu was purified by RP-HPLC.
Yield: 39 mg (0.09 mmol, TFA salt)
MS $NH_2$-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu: m/z 368.1 = $[M+Na]^+$ (MW calculated = 345.4 g/mol)

$NH_2$-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu (36 mg, 0.09 mmol) was dissolved in DMF (0.5 mL), 6-tritylmercaptohexanoic acid (55 mg, 0.14 mmol), HATU (53 mg, 0.14 mmol) and DIEA (49 µL, 0.28 mmol) were added. The mixture was stirred for 45 min. AcOH was added (100 µL) and Trt$S(CH_2)_5$CONH-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu was purified by RP-HPLC.
Yield: 41 mg (0.06 mmol)
MS Trt$S(CH_2)_5$CONH-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu: m/z 740.6 = $[M+Na]^+$ (MW calculated = 718.0 g/mol)

Trt$S(CH_2)_5$CONH-Asp(NH$(CH_2)_2$N($CH_3$)-boc)OtBu (41 mg, 0.06 mmol) was dissolved in 1:1 dioxane/$H_2O$ (1.0 mL), LiOH (4.1 mg, 0.17 mmol) was added and the mixture was stirred at 60°C for 1h. AcOH (50 µL) was added and **20** was purified by RP-HPLC.
Yield: 31 mg (0.05 mmol)
MS **20**: m/z 684.5 = $[M+Na]^+$ (MW calculated = 661.9 g/mol)

**Example 21**

**Synthesis of linker-exendin conjugate 21**




21

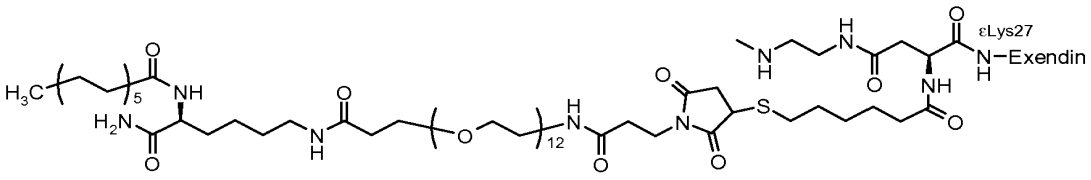
Resin bound, side chain protected exendin (50 mg, 5 μmol) with a Mmt protecting-group on Lys27 was first boc-protected at the N-terminus (see Materials and Methods) and then incubated five times (5 min) with 2 mL of 9/1 (v/v) DCM/HFIP to remove the Mmt protecting group from Lys27. **20** (6.6 mg, 10 μmol), PyBOP (5.2 mg, 10 μmol) and DIEA (7 μL, 40 μmol) were dissolved in DMF (400 μL) and immediately added to the resin. Incubation for 3 h at RT, the resin was washed with 10x DMF, 10x DCM and dried in vacuo. The product was cleaved from the resin and purified by RP-HPLC.

Yield: 2.4 mg

MS **21**: m/z 1497.2 = $[M+3H]^{3+}$ (MW calculated = 4488.0 g/mol)

**Example 22**

**Synthesis of fatty acid-linker-Exendin conjugate 22**




22

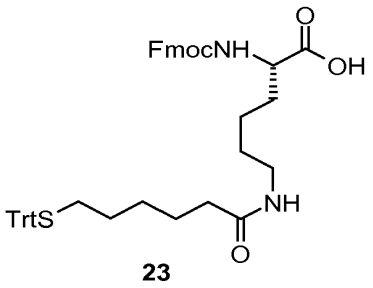
**21** (2.6 mg) was dissolved in 200 μl 1/1 acetonitrile/water and **1** (0.8 mg) in 400 μl of 7/3 acetonitrile/water was added. 100 μl of 0.25 M sodium phosphate buffer was added, the reaction was stirred for 5 min after which **22** was purified by RP-HPLC.

Yield: 2.4 mg

MS **22**: m/z 1388.3 = $[M+4H]^{4+}$; 1857.1 = $[M+3H]^{3+}$ (MW calculated = 5566.4 g/mol).

**Example 23**

**Synthesis of precursor 23**




**23**

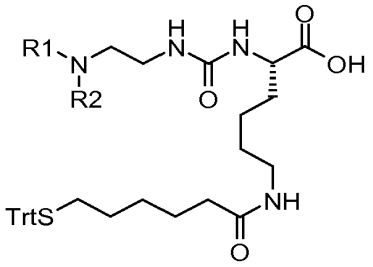
6-Tritylmercaptohexanoic acid (200 mg, 0.51 mmol), $(PfpO)_2CO$ (202 mg, 0.51 mmol) and collidine (340µL, 2.65 mmol) were dissolved in DMSO (1 mL) and stirred for 30 min at RT. The mixture was added to a solution of Fmoc-Lys-OH (170 mg, 0.46 mmol) in $H_2O$/pyridine/*t*BuOH (3:3:1, 6 mL). The reaction was heated at 60°C for 2h, diluted with EtOAc, extracted 2x with 0.1M $H_2SO_4$, 2x with brine and dried over $Na_2SO_4$. The solvent was evaporated under reduced pressure and the residue was purified by RP-HPLC.

Yield: 109 mg

MS **23**: m/z 741.3 $[M+H]^+$ (MW calculated = 741.0 g/mol)

**Example 24**

**Synthesis of linker 24a-24c**




**24a**: R1 = H, R2 = boc
**24b**: R1 = Me, R2 = boc
**24c**: R1 = R2 = Me

**23** (186 mg, 0.25 mmol) and DIEA (160 µL, 0.92 mmol) were dissolved in DCM (2 mL), added to 2-chlorotrityl chloride resin (312 mg, 1.3 mmol/g) and agitated for 45 min at RT. MeOH (0.6 mL) was added and the resin was incubated for another 15 min. The resin was washed with DCM (10x) and DMF (10x). Fmoc-deprotection and urea formation was achieved according to general procedures (see Materials and Methods) by reaction with N-boc-ethylenediamine (57 µL, 0.34 mmol), the product was cleaved from the resin and purified by RP-HPLC.

Yield: 14 mg

MS **24a**: m/z 705.4 $[M+H]^+$, 727.3 $[M+Na]^+$ (MW calculated = 704.9 g/mol)

**24b** was synthesized as described for **24a** except for the use of N-boc-N-methylethylenediamine instead of instead of N-boc-ethylenediamine.

Yield: 21 mg

MS **24b**: m/z 719.3 $[M+H]^+$, 741.4 $[M+Na]^+$ (MW calculated = 719.0 g/mol)

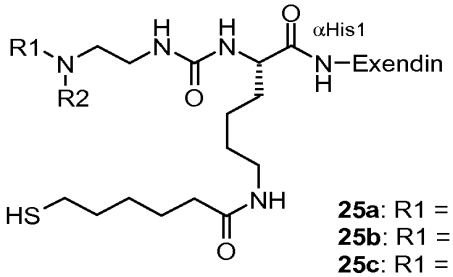
**24c** was synthesized as described for **24a** except for the use of N,N-dimethylethylenediamine instead of N-boc-ethylenediamine.

Yield: 10 mg

MS **24c**: m/z 633.2 $[M+H]^+$ (MW calculated = 632.9 g/mol)

**Example 25**

**Synthesis of exendin-linker conjugates 25a-25c**




**25a**: R1 = R2 = H
**25b**: R1 = Me, R2 = H
**25c**: R1 = R2 = Me

**24a** (7.0 mg, 0.01 mmol), PyBOP (5.2 mg, 10 μmol) and DIEA (7 μL, 40 μmol) were dissolved in DMF (250 μL), immediately added to resin bound, side chain protected exendin (50 mg, 5 μmol) and incubated for 2 h at RT. The resin was washed with DMF (10x), DCM (10x) and dried in vacuo. The product was cleaved from the resin and purified by RP-HPLC.

Yield: 1.6 mg

MS **25a**: m/z 1511.8 = $[M+3H]^{3+}$ (MW calculated = 4530.8 g/mol)

**25b** was synthesized as described for **25a** except for the use of **24b** instead of **24a**.

Yield: 4.3 mg

MS **25b**: m/z 1516.3 = $[M+3H]^{3+}$ (MW calculated = 4544.8 g/mol)

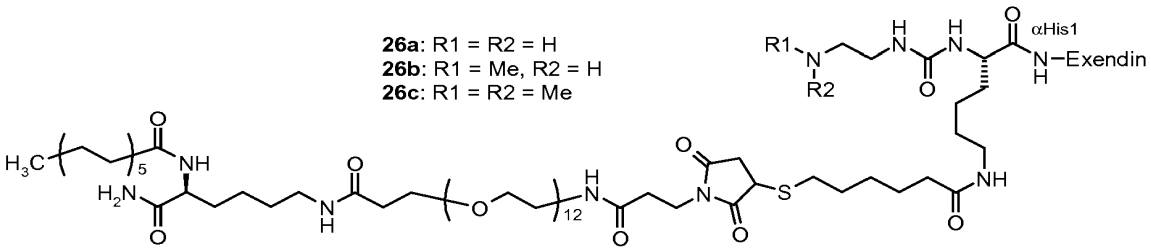
**25c** was synthesized as described for **25a** except for the use of **24c** instead of **24a**.

Yield: 1.3 mg

MS **25c**: m/z 1520.4 = $[M+3H]^{3+}$ (MW calculated = 4558.8 g/mol)

**Example 26**

**Synthesis of fatty acid-linker conjugates 26a-26c**




**25a** (1.6 mg) was dissolved in 200 µl 1/1 acetonitrile/water and **1** (0.11 mg) in 200 µl of 7/3 acetonitrile/water was added. 30 µl of 0.25 M sodium phosphate buffer was added, the reaction was stirred for 5 min, after which **26a** was purified by RP-HPLC.

MS **26a**: m/z 1870.0 = $[M+3H]^{3+}$ (MW calculated = 5609.2 g/mol).

**26b** was synthesized as described for **26a** except for the use of **25b** instead of **25a**.

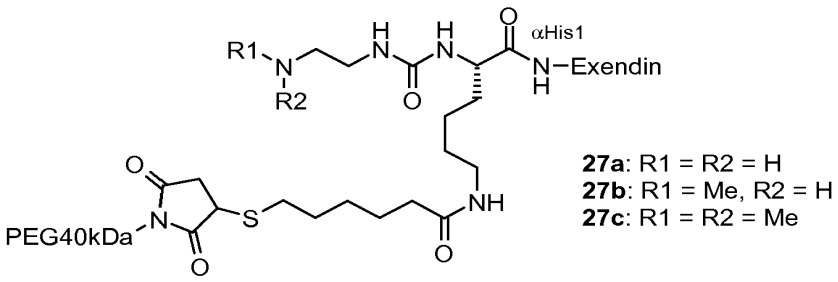
MS **26b**: m/z 1875.9 = $[M+3H]^{3+}$, 1406.7 = $[M+4H]^{4+}$ (MW calculated = 5623.2 g/mol)

**26c** was synthesized as described for **26a** except for the use of **25c** instead of **25a**.

MS **26c**: m/z 1879.4 = $[M+3H]^{3+}$, 1410.5 = $[M+4H]^{4+}$ (MW calculated = 5637.2 g/mol)

**Example 27**

**Synthesis of 20KDa-PEG-linker-exendin conjugates 27a-27c**



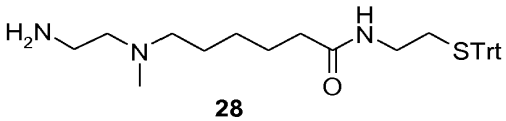
**25a** (2.0 mg) was dissolved in 1:1 $H_2O$/MeCN containing 0.1 % TFA (200 µl). A solution of PEG40KDa-maleimide (18 mg) in 1:1 $H_2O$/MeCN (1 ml) and phosphate buffer (15 µl, pH 7.4, 0.5 M) was added. The solution was incubated at RT, after 5 min AcOH (20 µl) was added and **27a** was purified by cation exchange chromatography, desalted and lyophilized.

**27b** was synthesized as described for **27a** except for the use of **25b** instead of **25a**.

**27c** was synthesized as described for **27a** except for the use of **25c** instead of **25a**.

**Example 28**

**Synthesis of linker 28:**




**28**

6-Bromohexanoyl chloride (46 µl, 0.31 mmol) was dissolved in 0.2 ml $CH_2Cl_2$ and added to a solution of $H_2N$-$CH_2$-$CH_2$-STrt (100 mg, 0.28 mmol), DIEA (97 µl, 0.56 mmol) in $CH_2Cl_2$ (0.8 ml). The mixture was stirred for 2 h at RT. The reaction mixture was acidified with AcOH (50 µl) and the solvent was removed in vacuo. The residue was purified on silica gel (heptane/EtOAc = 1 :1) to obtain Br-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt.

Yield: 137 mg (0.276 mmol, 98 %)

MS Br-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt: 518.9 = $[M+Na]^+$, (MW calculated = 496.5 g/mol)

*N*-Boc-ethylenediamine (81 µl, 0.51 mmol) was added to a solution of Br-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt (230 mg, 0.46 mmol) and $Na_2CO_3$ (196 mg, 1.85 mmol) in DMF (0.8 ml). The reaction mixture was stirred for 10 h at 70 °C. After cooling to RT the mixture was diluted with 4 ml (MeCN/$H_2O$ = 25:75, with 0.1 % TFA) and purified by RP-HPLC to get Boc-NH-$(CH_2)_2$-NH-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt.

Yield: 189 mg (0.27 mmol, 59 %, TFA-salt)

MS Boc-NH-$(CH_2)_2$-NH-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt: 576.5 = $[M+H]^+$, (MW calculated = 575.5 g/mol)

Boc-NH-$(CH_2)_2$-NH-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt (189 mg, 0.27 mmol) and HCHO (35 % aqueous, 113 µl) were dissolved in MeCN (1.5 ml) and $NaCNBH_3$ (34 mg, 0.54 mmol) was added. The reaction mixture was stirred for 5 h at RT. After completion of the reaction (MS) the solution was diluted with $H_2O$ (5 ml) and extracted with $CH_2Cl_2$ (3 x 5 ml). The combined organic layers were dried over $MgSO_4$, filtered and the solvent was removed in vacuo. The residue was purified by RP-HPLC to get Boc-NH-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt.

Yield: 62.8 mg (0.11 mmol, 39 %)

MS Boc-NH-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt: 590.6 = $[M+H]^+$, (MW calculated = 589.0 g/mol)

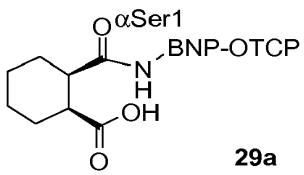
Boc-NH-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt (62.8 mg, 0.11 mmol) was dissolved in THF (6 ml) and HCl in dioxane (130 µl, 4 M solution) was added. The reaction mixture was stirred for 12 h at RT. 200 µl HCl in dioxane was added and the solvent was removed in vacuo. The residue was purified by RP-HPLC to give $H_2N$-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt and not consumed starting material Boc-NH-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt.

Yield: 32.8 mg (0.062 mmol, 44 %, HCl-salt) **28** and 14.7 mg (0.025 mmol, 23 %, TFA-salt) starting material

MS **28**: 490.5 = $[M+H]^+$, (MW calculated = 489.0 g/mol)

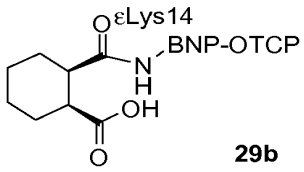
**Example 29**

**General procedure for the synthesis carboxylic acid substituted BNP precursors 29a and 29b**




**29a**

*Cis*-cyclohexane-1,2-dicarboxylic anhydride (231 mg, 1.5 mmol) and pyridine (271 µl, 2 mmol) were dissolved in DCM (2 ml) and added to resin bound, side chain protected BNP-32a (300 mg). Incubation for 1 h at RT, washed with 10 x DCM and dried in vacuo.

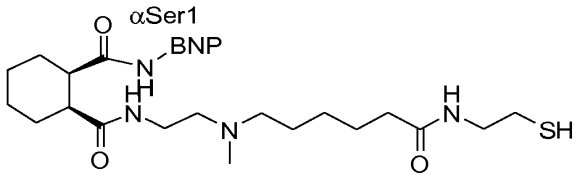



**29b**

Resin bound, side chain protected BNP-32b, carrying an ivDde protecting group at Lys14, was first boc-protected at the N-terminus, deprotected at Lys14 position (see Materials and Methods) and then reacted with *cis*-cyclohexane-1,2-dicarboxylic anhydride as described above for **29a**.

**Example 30**

**Synthesis of BNP-linker-thiols 30a and 30b.**

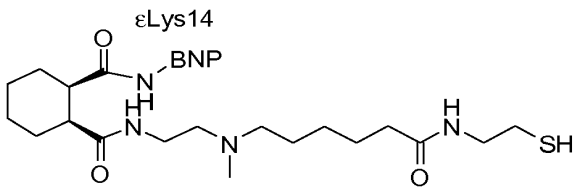



**30a**

$H_2N$-$(CH_2)_2$-N$(CH_3)$-$(CH_2)_5$-CONH-$(CH_2)_2$-STrt **28** (5.2 mg, 0.01 mmol), PyBOP (5.2 mg, 0.01 mmol) and DIEA (7.0 µl, 0.04 mmol) were dissolved in DMF (300 µl) and added to resin bound, side chain protected BNP **29a** (50 mg, 0.005 mmol). Incubation for 2 h at RT, the resin was washed with DMF (10x), DCM (10x) and dried in vacuo. The product was cleaved from the resin and purified by RP-HPLC.

Yield: 9.8 mg

MS **30a**: m/z 947.6 = $[M+4H]^{4+}$, 1263.1 = $[M+3H]^{3+}$ (MW calculated = 3786.3 g/mol)




**30b**

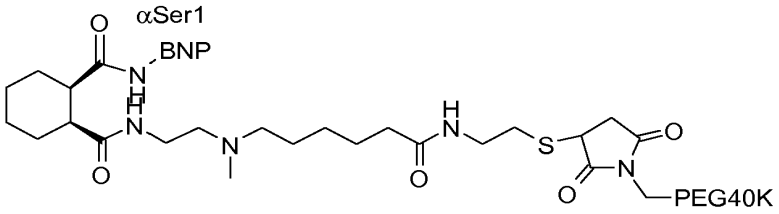
**30b** was synthesized as described above except for the use of resin bound BNP derivative **29b** instead of **29a**.

Yield: 7.4 mg

MS **30b**: m/z 947.5 = $[M+4H]^{4+}$, 1263.0 = $[M+3H]^{3+}$ (MW calculated = 3786.3 g/mol)

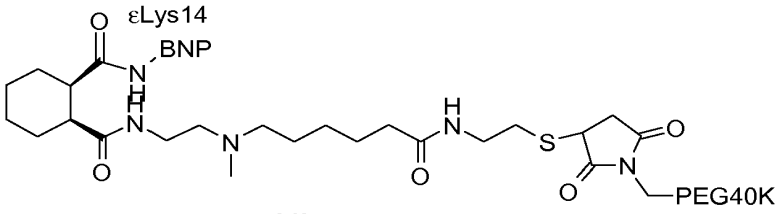
**Example 31**

**Synthesis of 40KDa-PEG-linker-BNP conjugates 31a and 31b**




**31a**

**30a** (4 mg) was dissolved in 1:1 $H_2O$/MeCN containing 0.1 % TFA (200 µl). A solution of PEG40KDa-maleimide (42.2 mg) in 1:1 $H_2O$/MeCN (1 ml) and phosphate buffer (15 µl, pH 7.4, 0.5 M) was added. The solution was incubated at RT, after 5 min AcOH (20 µl) was added and **31a** was purified by cation exchange chromatography, desalted and lyophilized.
Yield: 2.0 mg

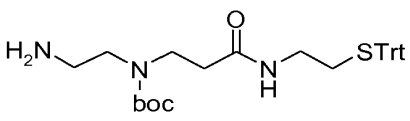



**31b**

**31b** was synthesized as described for **31a** except for the use of **30b** instead of **30a**.
Yield: 16.8 mg

**Example 32**

**Synthesis of linker 32**




**32**

3-Bromopropionylchloride (62.5 µl, 0.62 mmol) was dissolved in 0.5 ml $CH_2Cl_2$ and added to a solution of $H_2N$-$CH_2$-$CH_2$-STrt (200 mg, 0.56 mmol), DIEA (196 µl, 1.1 mmol) in $CH_2Cl_2$ (1 ml). The mixture was stirred for 1 h at RT. The reaction mixture was acidified with AcOH (100 µl) and the solvent was removed in vacuo. The residue was purified over silica gel (heptane/EtOAc = 1:1) to obtain Br-$(CH_2)_2$CONH-$(CH_2)_2$-STrt.
Yield: 223 mg (0.49 mmol, 87 %)
MS Br-$(CH_2)_2$CONH-$(CH_2)_2$-STrt: 478.7 = $[M+Na]^+$, (MW calculated = 454.7 g/mol)

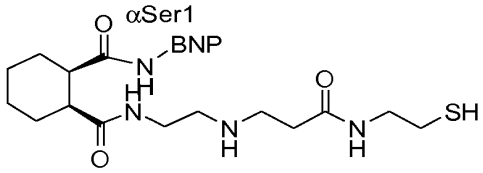
*N*-Alloc-ethylenediamine HCl-salt (43.5 mg, 0.24 mmol) and DIEA (38 µl, 0.22 mmol) were added to a solution of Br-$(CH_2)_2$CONH-$(CH_2)$-STrt (100 mg, 0.22 mmol) and $Na_2CO_3$ (93 mg, 0.87 mmol) in DMF (1 ml). The reaction mixture was stirred for 10 h at 70 °C. After cooling down to RT the reaction mixture was diluted with 4 ml (MeCN/$H_2O$ = 25:75, with 0.1 % TFA) and purified by HPLC to get Alloc-NH-$(CH_2)_2$-NH-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt.
Yield: 61 mg (0.096 mmol, 44 %, TFA salt)
MS Alloc-NH-$(CH_2)_2$-NH-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt: 540.8 = $[M+Na]^+$, (MW calculated = 517.8. g/mol)

Alloc-NH-$(CH_2)_2$-NH-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt (60.9 mg, 0.096 mmol) was dissolved in $CH_2Cl_2$ and $Boc_2O$ (42 mg, 0.19 mmol) was added. The solution was stirred for 20 h at RT. After completion the reaction was quenched by addition of 70 µl AcOH and the solvent was removed in vacuo. The residue was diluted with 4 ml MeCN/$H_2O$ (25:75, with 0.1 % TFA) and purified by RP-HPLC to give Alloc-NH-$(CH_2)_2$-N(Boc)-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt.
Yield: 53.3 mg (0.086 mmol, 89 %)
MS Alloc-NH-$(CH_2)_2$-N(Boc)-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt: 640.6 = $[M+Na]^+$, (MW calculated = 617.9. g/mol)

Alloc-NH-$(CH_2)_2$-N(Boc)-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt (48.3 mg, 0.078 mmol) was dissolved in THF, triethylammoniumformat (62 µl) and $Pd(PPh_3)_4$ (16 mg) were added. The solution was stirred for 12 h at RT and monitored by MS. After completion the solvent was removed in vacuo. The residue was dissolved in MeCN/$H_2O$ (50:50, with 0.1 % TFA) and purified by RP-HPLC to give $H_2N$-$(CH_2)_2$-N(Boc)-$(CH_2)_2$-CONH-$(CH_2)_2$-STrt (**31**).
Yield: 20.1 mg (0.031 mmol, 40 %, TFA-salt)
MS **31**: 534.6 = $[M+H]^+$, 556.6 = $[M+Na]^+$, (MW calculated = 533.5 g/mol)

**Example 33**

**Synthesis of BNP-linker-thiols 33a and 33b**

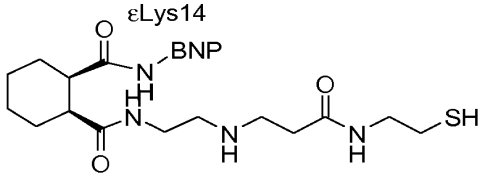



**33a**

**33a** was synthesized as described for **30a** except for the use of **32** instead of **28**.

Yield: 8.0 mg

MS **33a**: m/z 933.5 = $[M+4H]^{4+}$, 1244.3 = $[M+3H]^{3+}$ (MW calculated = 3729.9 g/mol)




**33b**

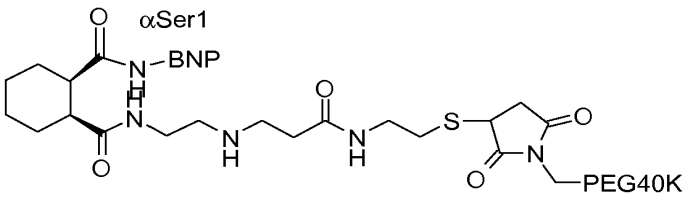
**33b** was synthesized as described for **30b** except for the use of **32** instead of **28**.

Yield: 5.0 mg

MS **33b**: m/z 933.5 = $[M+4H]^{4+}$, 1244.3 = $[M+3H]^{3+}$ (MW calculated = 3715.9 g/mol)

**Example 34**

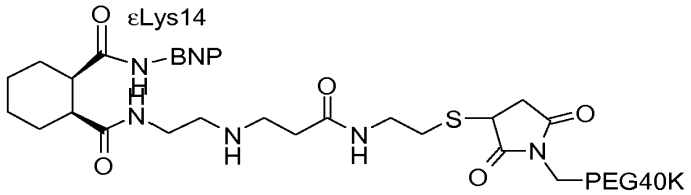
**Synthesis of 40KDa-PEG-linker-BNP conjugates 34a and 34b**




**34a**

**33a** (4.3 mg) was dissolved in 1:1 $H_2O$/MeCN containing 0.1 % TFA (200 µl). A solution of PEG40KDa-maleimide (46.8 mg) in 1:1 $H_2O$/MeCN (1 ml) and phosphate buffer (20 µl, pH

7.4, 0.5 M) was added. The solution was incubated at RT, after 5 min AcOH (20 µl) was added and **34a** was purified by cation exchange chromatography, desalted and lyophilized.
Yield: 9.7 mg

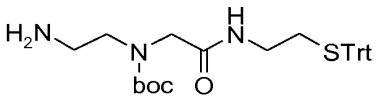



**34b**

**34b** was synthesized as described for **34a** except for the use of **33b** instead of **33a**.
Yield: 11.5 mg

## Example 35

### Synthesis of Linker 35




**35**

Bromoacetylbromide (54 µl, 0.62 mmol) was dissolved in 0.5 ml $CH_2Cl_2$ and added to a solution of $H_2N$-$CH_2$-$CH_2$-STrt (200 mg, 0.56 mmol) and DIEA (196 µl, 1.1 mmol) in $CH_2Cl_2$ (1 ml). The mixture was stirred for 1 h at RT. The reaction mixture was acidified with AcOH (100 µl) and the solvent was removed in vacuo. The residue was purified over silica gel (heptane/EtOAc = 1 :1) to obtain product Br-$CH_2$-CONH-$(CH_2)_2$-STrt.
Yield: 245 mg (0.55 mmol, 99 %)
MS Br-$CH_2$-CONH-$(CH_2)_2$-STrt: 462.4 = $[M+Na]^+$, (MW calculated = 440.4 g/mol)

*N*-Alloc-ethylenediamine HCl-salt (45 mg, 0.25 mmol) and DIEA (79 µl, 0.45 mmol) was added to a solution of Br-$CH_2$-CONH-$(CH_2)_2$-STrt (100 mg, 0.23 mmol) in DMF (1 ml). The reaction mixture was stirred for 10 h at 70 °C. After cooling down to RT the reaction mixture was diluted with $H_2O$/$Et_2O$ (1:1, 40 ml) and the layers were separated. The aqueous layer was extracted several times with $Et_2O$. The combined organic layers were dried with $MgSO_4$,

filtered and the solvent was removed in vacuo. The residue was purified over silica gel (DCM/MeOH = 95:5) to give Alloc-NH-$(CH_2)_2$-NH-$CH_2$-CONH-$(CH_2)_2$-STrt.

Yield: 94 mg (0.186 mmol, 82 %, contains residual DMF)

MS Alloc-NH-$(CH_2)_2$-NH-$CH_2$-CONH-$(CH_2)_2$-STrt: 526.8 = $[M+Na]^+$, (MW calculated = 503.8. g/mol)

Alloc-NH-$(CH_2)_2$-NH-$CH_2$-CONH-$(CH_2)_2$-STrt (94 mg, 0.186 mmol, with DMF)) was dissolved in $CH_2Cl_2$ and $Boc_2O$ (81 mg, 0.37 mmol) was added. The solution was stirred for 20 h at RT. After completion the reaction was quenched by addition of 100 µl AcOH and the solvent was removed in vacuo. The residue was diluted with 4 ml MeCN/$H_2O$ (25:75, with 0.1 % TFA) and purified by RP-HPLC to give Alloc-NH-$(CH_2)_2$-N(Boc)-$CH_2$-CONH-$(CH_2)_2$-STrt.

Yield: 34.7 mg (0.057 mmol, 26 %)

MS Alloc-NH-$(CH_2)_2$-N(Boc)-$CH_2$-CONH-$(CH_2)_2$-STrt: 603.9 = $[M+Na]^+$, (MW calculated = 603.9. g/mol)

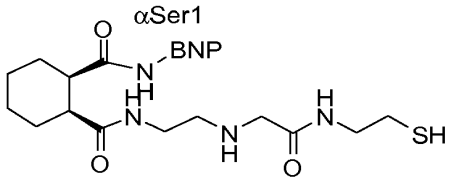
Alloc-NH-$(CH_2)_2$-N(Boc)-$CH_2$-CONH-$(CH_2)_2$-STrt (34.7 mg, 0.048 mmol) was dissolved in THF, triethylammoniumformate (38 µl) and $Pd(PPh_3)_4$ (5 mg) were added. The solution was stirred for 12 h at RT and monitored by MS. After completion the solvent was removed in vacuo. The residue was dissolved in MeCN/$H_2O$ (50:50, with 0.1 % TFA) and purified by RP-HPLC to give $H_2N$-$(CH_2)_2$-N(Boc)-$CH_2$-CONH-$(CH_2)_2$-STrt **35**.

Yield: 12.6 mg (0.019 mmol, 42 %, TFA-salt)

MS **35**: 520.1 = $[M + H]^+$, 542.2 = $[M + Na]^+$, (MW calculated = 519.2 g/mol)

**Example 36**

**Synthesis of BNP-linker-thiols 36a and 36b**

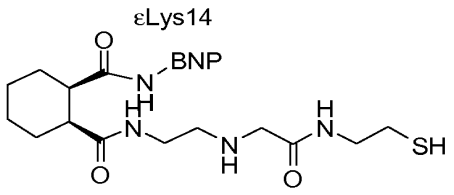



**36a**

**36a** was synthesized as described for **30a** except for the use of **35** instead of **28**.

Yield: 9.1 mg

MS **36a**: m/z 930.0 = $[M+4H]^{4+}$, 1239.6 = $[M+3H]^{3+}$ (MW calculated = 3715.9 g/mol)




**36b**

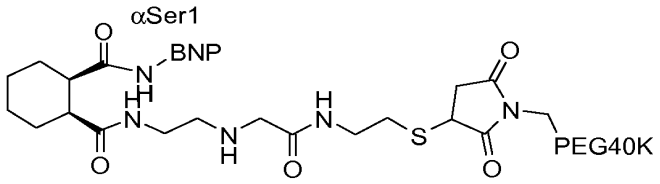
**36b** was synthesized as described for **30b** except for the use of **35** instead of **28**.

Yield: 8.0 mg

MS **36b**: m/z 929.9 = $[M+4H]^{4+}$, 1239.5 = $[M+3H]^{3+}$ (MW calculated = 3715.9 g/mol)

**Example 37**

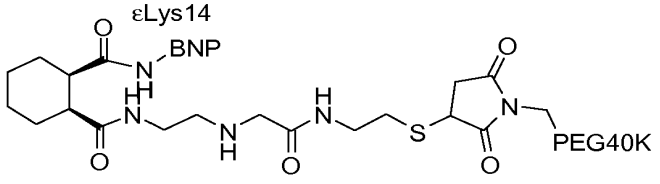
**Synthesis of 40KDa-PEG-linker-BNP conjugates 37a and 37b**




**37a**

**36a** (4.2 mg) was dissolved in 1:1 $H_2O$/MeCN containing 0.1 % TFA (200 µl). A solution of PEG40KDa-maleimide (68 mg) in 1:1 $H_2O$/MeCN (1 ml) and phosphate buffer (20 µl, pH 7.4, 0.5 M) was added. The solution was incubated at RT, after 5 min AcOH (20 µl) was added and **37a** was purified by ion exchange chromatography, desalted and lyophilized.

Yield: 16 mg




**37b**

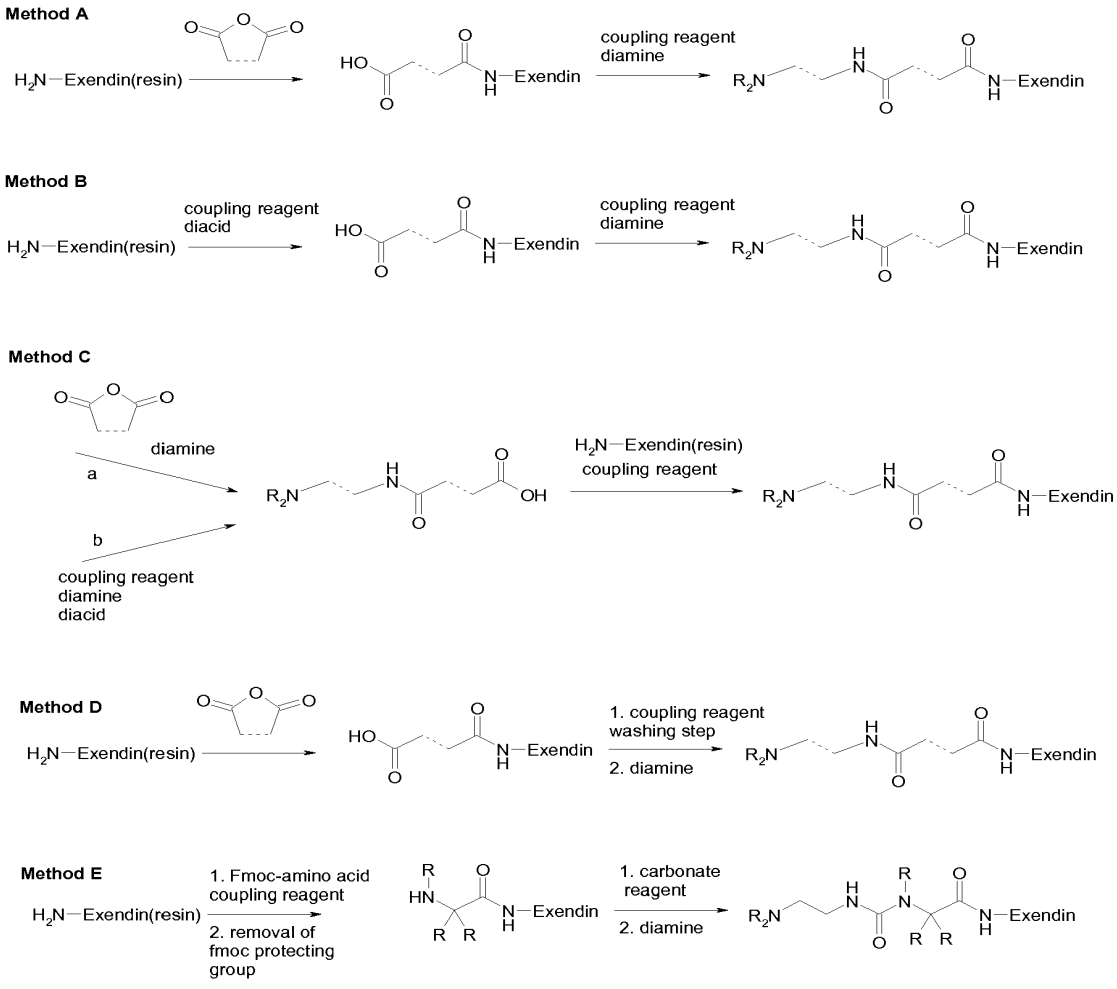
**37b** was synthesized as described for **37a** except for the use of **36b** instead of **36a**.

Yield: 18.5 mg

**Example 38**

**Synthesis of linker-exendin conjugates**

Linker-exendin conjugates were synthesized according to general synthesis method A, B, C, D, E or F.




**Method A**

Synthesis: Diacid anhydride (0.2 mmol) and pyridine (0.2 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to side chain protected exendin-4 on resin (2 µmol) and agitated for 30 min at room temperature. Resin was washed with DMF (10 times). PyBOP (0.1 mmol)

and diamine (0.1 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to resin and agitated for 30 min at room temperature. Resin was washed with DMF (10 times).
Exendin-linker conjugates were cleaved and purified by RP-HPLC as described in "Materials and Methods".

**Method B**

Synthesis: As described for Method A except that diacid anhydride and pyridine are replaced by diacid (0.2 mmol), HOBt (0.2 mmol), DIC (0.2 mmol), and collidine (0.4 mmol).

**Method C**

Synthesis. Diamine (0.6 mmol) was dissolved in 1 ml of dry DCM and diacid anhydride (0.4 mmol) was added. Mixture was stirred for 60 min at room temperature. DCM was removed, the residue was dissolved in ACN/water/AcOH, and amino acid was purified by RP-HPLC and lyophilized.

Amino acid (0.1 mmol), HOBt (0.1 mmol), DIC (0.1 mmol), and collidine (0.2 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to exendin-4 on resin (2 μmol) and agitated for 30 min at room temperature. Resin was washed with DMF (10 times).
Exendin-linker conjugates were cleaved and purified by RP-HPLC as described in "Materials and Methods".

**Method D**

Synthesis: Diacid anhydride (0.2 mmol) and pyridine (0.2 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to exendin-4 on resin (2 μmol) and agitated for 30 min at room temperature. Resin was washed with DMF (10 times). PyBOP (0.1 mmol), HOBt (0.1 mmol), and collidine (0.4 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to resin and agitated for 30 min at room temperature. Resin was washed with DMF (10 times). Diamine (0.1 mmol) and DIEA (0.3 mmol) were dissolved in a mixture of 0.4 ml of DMF and 0.4 ml of EtOH. Mixture was added to resin and agitated for 30 min at room temperature. Resin was washed with DMF (10 times).
Exendin-linker conjugates were cleaved and purified by RP-HPLC as described in "Materials and Methods".

**Method E**

Synthesis: Fmoc amino acid (0.1 mmol), PyBOP (0.1 mmol) and DIEA (0.2 mmol) were dissolved in 0.3 ml of dry DMF. Mixture was added to exendin-4 on resin (2 μmol) and agitated for 30 min at room temperature. Fmoc protecting group was removed by incubating resin in DMF/piperidine 4/1 (v/v) for 2 x 10 min. Resin was washed with DMF (10 times) and DCM (10 times). p-Nitrophenyl chloroformate (0.1 mmol) was dissolved in 0.3 ml of dry THF and DIEA (0.2 mmol). Mixture was added to resin and agitated for 30 min at room temperature. Resin was washed with DCM (10 times). Diamine (0.1 mmol) was dissolved in 0.3 ml of DMF. Mixture was added to resin and agitated for 30 min at room temperature. Resin was washed with DMF (10 times).

Exendin-linker conjugates were cleaved and purified by RP-HPLC as described in "Materials and Methods".

**Method F**

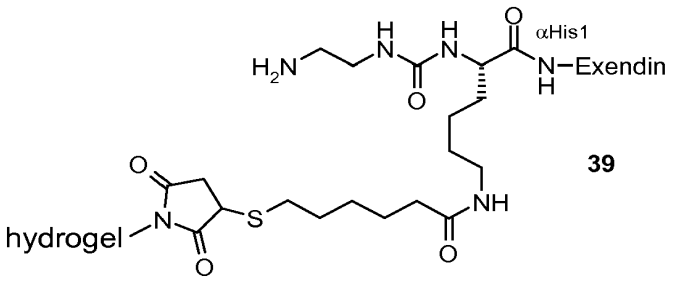
Synthesis: as described for Method A, followed by fmoc-deprotection and acetylation: Fmoc protecting group was removed as by incubating resin in DMF/piperidine 4/1 (v/v) for 2 x 10 min. Resin was washed with DMF (10 times). Acetylation was performed by incubating resin with acetic anhydride/pyridine/DMF 1/1/2 (v/v/v) for 30 min. Resin was washed with DMF (10 times).

Exendin-linker conjugates were cleaved and purified by RP-HPLC as described in "Materials and Methods".

Further details concerning compound numerals, starting materials, synthesis method, molecular weight (MW) and MS data are given in Fig. 2.

**Example 39**

**Synthesis of hydrogel-linker-exendin conjugates 39**

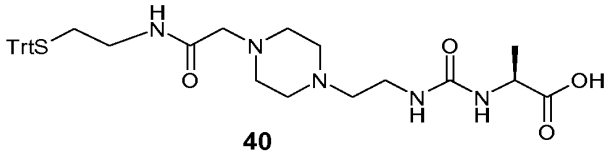



Maleimide-functionalized hydrogel microparticles were synthesized as described in EP 1 625 856 A1.

30 mg of maleimide-derivatized hydrogel microparticles (loading 40 µmol/g, 1.2 µmol) were reacted with 6 mg of compound **25a** (1.32 µmol, 1.1 eq) in 600µl 20/80 (v/v) acetonitrile/50 mM phosphate buffer (pH 7.4) for 10 min to give exendin-linker loaded hydrogel microparticles **39**. The loaded hydrogel **39** was washed 5 times with 50/50 (v/v) acetonitrile/water and three times with water.

**Example 40**

**Synthesis of Linker 40**

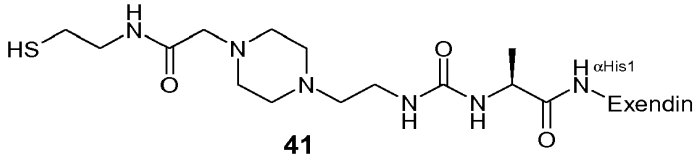



**40**

Fmoc-Ala-OH (250 mg, 0.8 mmol) and DIEA (170 µL, 1,0 mmol) were dissolved in DCM (2 mL), added to 2-chlorotrityl chloride resin (312 mg, 1.3 mmol/g) and agitated for 45 min at RT. Methanol (0.6 mL) was added and the resin was incubated for another 15 min. The resin was washed with DCM (10x) and DMF (10x). Fmoc-deprotection and urea formation was achieved according to general procedures (see Materials and Methods) by reaction with linker intermediate **5a**, the product was cleaved from the resin and purified by RP-HPLC.
Yield: 53 mg
MS **40**: m/z 604,4 $[M+H]^+$ (MW calculated = 603,8 g/mol)

**Example 41**

**Synthesis of exendin-linker conjugate 41**




**41**

**40** (HCl salt, 14.0 mg, 0.02 mmol), PyBOP (10.2 mg, 0.02 mmol) and DIEA (17 µL, 0.1 mmol) were dissolved in DMF (300 µL), immediately added to resin bound, side chain protected exendin (100 mg, 10 µmol) and incubated for 4 h at RT. The resin was washed with

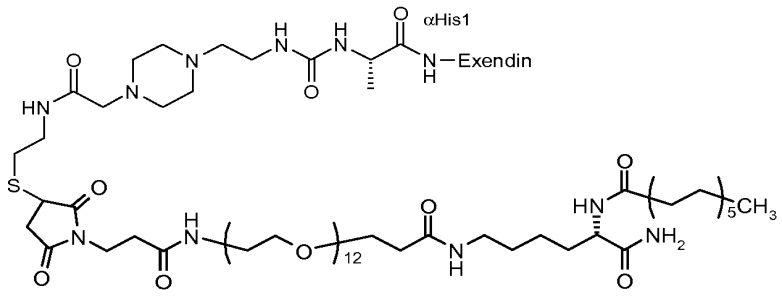
DMF (10x), DCM (10x) and dried in vacuo. The product was cleaved from the resin and purified by RP-HPLC.

Yield: 5.4 mg

MS **40**: m/z 1510.9 = $[M+3H]^{3+}$ (MW calculated = 4530.1 g/mol)

**Example 42**

**Synthesis of fatty acid-linker conjugate 42**



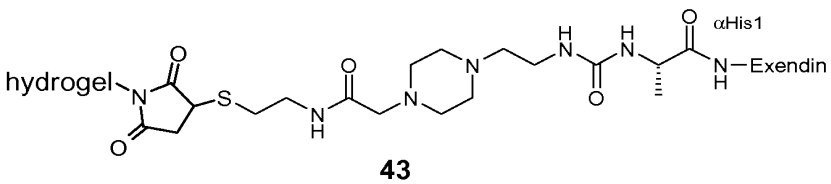

**42**

**41** (1.6 mg) was dissolved in 200 µl 3/1 acetonitrile/water and **1** (0.11 mg) in 200 µl of 3/1 acetonitrile/water was added. 30 µl of 0.25 M sodium phosphate buffer was added, the reaction was stirred for 5 min, after which **42** was purified by RP-HPLC.

MS **42**: m/z 1870.2 = $[M+3H]^{3+}$ (MW calculated = 5608.4 g/mol).

**Example 43**

**Synthesis of hydrogel-linker-exendin conjugate 43**

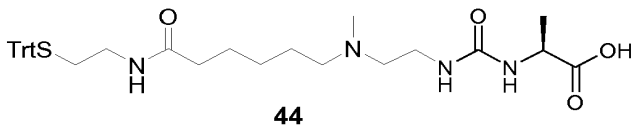



**43**

**43** was synthesized as described for **39** except for the use of **41** instead of **25a**.

**Example 44**

**Synthesis of Linker 44**



**44**

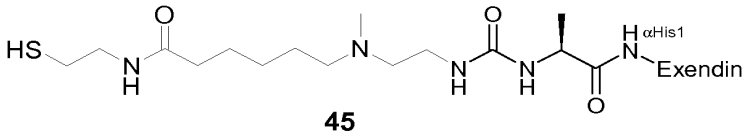
**44** was synthesized as described for **40** except for the use of **28** instead of **5a**.

Yield: 74 mg

MS **44**: m/z 605,4 $[M+H]^+$ (MW calculated = 604,8 g/mol)

**Example 45**

**Synthesis of exendin-linker conjugate 45**



**45**

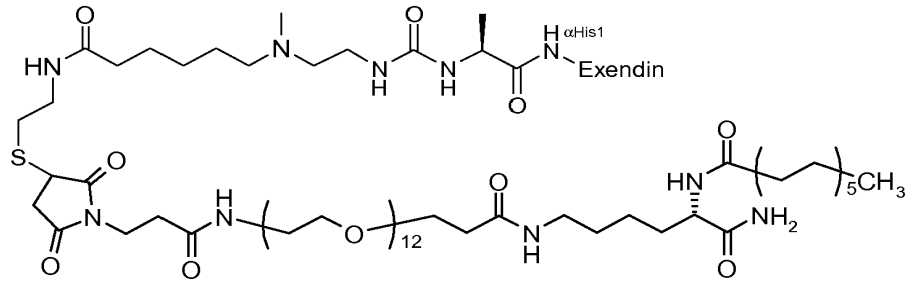
**45** was synthesized as described for **41** except for the use of **44** instead of **40**.

Yield: 6.0 mg

MS **45**: m/z 1511.3 = $[M+3H]^{3+}$ (MW calculated = 4531.1 g/mol)

**Example 46**

**Synthesis of fatty acid-linker conjugate 46**



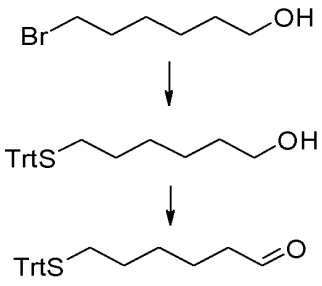
**46**

**46** was synthesized as described for **42** except for the use of **45** instead of **41**.

MS **46**: m/z 1870.5 = $[M+3H]^{3+}$ (MW calculated = 5609,5 g/mol).

**Example 47**

**Synthesis of linker intermediate 47**




**47**

Tritylsulfide (247 mg, 0.89 mmol) was suspended in 1 ml DMSO. DBU (152 µl, 1.02 mmol) and 6-bromohexan-1-ol (173 mg, 0.96) were added and mixture was stirred for 5 min at RT. Reaction mixture was dissolved in 20 ml ethylacetate and washed with 1 N $H_2SO_4$ (2x) and brine (3x). Organic layer was dried ($Na_2SO_4$) and volatiles were removed in vacuo. Product was purified by flash chromatography on silica (heptane/AcOEt 1/1).

Yield 283 mg (S-trityl)-6-mercaptohexan-1-ol

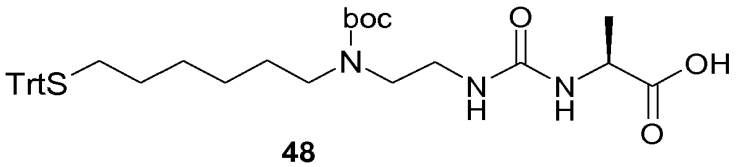
(S-Trityl)-6-mercaptohexan-1-ol (466 mg, 1.24 mmol) was dissolved in 3.5 ml DCM, 0.5 ml DMSO and 0.6 ml $NEt_3$, and cooled in an ice bath. $SO_3$-pyridine (408 mg, 2.57 mmol) was suspended in 0.5 ml DMSO and added to reaction mixture. Ice bath was removed and reaction was stirred for 60 min at RT. Reaction mixture was dissolved in 20 ml $Et_2O$ and extracted with 1 N $H_2SO_4$ (2x) and brine (3x). Organic layer was dried ($Na_2SO_4$) and volatiles were removed in vacuo. Product was purified by flash chromatography on silica (heptane/AcOEt 1/1).

Yield: 390 mg (S-trityl)-6-mercaptohexan-1-al **47**

MS **47**: m/z 243,1 = $[Trt]^+$ , 413.1 = $[M+K]^+$ (MW calculated = 374.4 g/mol)

**Example 48**

**Synthesis of linker 48**




**48**

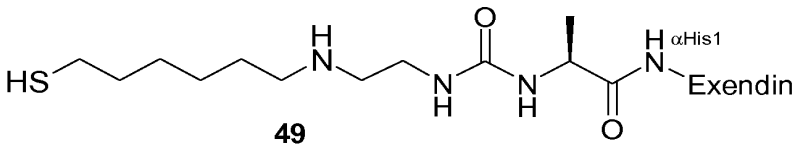
Fmoc-Ala-OH (250 mg, 0.8 mmol) and DIEA (170 µL, 1,0 mmol) were dissolved in DCM (2 mL), added to 2-chlorotrityl chloride resin (312 mg, 1.3 mmol/g) and agitated for 45 min at RT. Methanol (0.6 mL) was added and the resin was incubated for another 15 min. The resin was washed with DCM (10x) and DMF (10x). Fmoc-deprotection and urea formation was achieved according to general procedures (see Materials and Methods) by reaction with ethylene diamine. For reductive alkylation **47** (299 mg, 0.8 mmol) and $Na(OAc)_3BH$ (340 mg, 1.6 mmol) were dissolved in 0,5 mL DMF, 0.5 ml MeOH and 10 µL AcOH, added to resin and agitated for 2 h at RT. Resin was washed with DMF (10x) and DCM (10x). Boc protection was performed by agitating resin in a solution of boc anhydride (218 mg, 1.0 mmol) and DIEA (170 µL, 1.0 mmol) in DCM. Resin was washed with DCM (10x) and product was cleaved from the resin and purified by RP-HPLC.

Yield: 34 mg

MS **48**: m/z 634.2 $[M+H]^+$ (MW calculated = 633.9 g/mol)

**Example 49**

**Synthesis of exendin-linker conjugate 49**




**49**

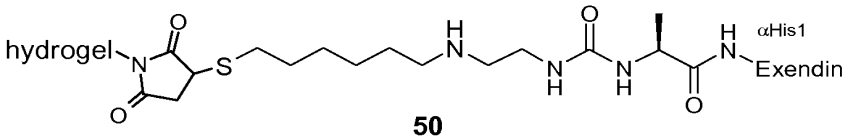
**49** was synthesized as described for **41** except for the use of **48** instead of **40**.

Yield: 4.8 mg

MS **49**: m/z 1487.3 = $[M+3H]^{3+}$ (MW calculated = 4460.0 g/mol)

**Example 50**

**Synthesis of hydrogel-linker-exendin conjugate 50**




**50**

**50** was synthesized as described for **39** except for the use of **49** instead of **25a**.

**Example 51**

**Release kinetics in vitro**

Release of drug molecule from **38a** to **38z**, **38aa** to **38ab**, **4**, **5**, **9a**, **9b**, **9c**, **10**, **13a**,**15**, **19a**, **19b**, **22**, **26a** to **26c**, **31a**, **31b**, **34a**, **34b**, **37a**, **37b, 42, 43, 46, and 50** was effected by hydrolysis in buffer at pH 7.4 and 37°C or pH 4 and 37°C as described in "Materials and Methods".

| Compound | $t_{1/2}$ buffer A (pH 7.4) | $t_{1/2}$ buffer B (pH 4.0) |
|---|---|---|
| **38a** | < 1h | 13 h |
| **38b** | 20 h | 72 d |
| **38c** | >3 m | > 3m |
| **38d** | 58 d | n.d. |
| **38e** | 41 d | n. d. |
| **38f** | 23 h | 114 d |
| **38g** | 19 d | none |
| **38h** | 47 d | none |
| **38i** | 69 h | 108 d |
| **38j** | 16 d | n.d. |
| **38k** | 40 min | 6 d |
| **38l** | 16 h | n.d. |
| **38m** | 17 h | 66 d |
| **38n** | 18 d | n.d. |
| **38o** | 11-12 h | 22 d |
| **38p** | 26 d | 178 d |
| **38q** | 26 d | 210 d |
| **38r** | 26 h | 47 d |
| **38s** | 80 min | 80h |
| **38t** | 96 min | 67 h |
| **38u** | 51 d | none |
| **38v** | 47 d | none |
| **38w** | 8 d | 3,2 a |
| **38x** | 72 d | n.d. |
| **38y** | 11-14 h | 105 d |
| **38z** | 11 d | 1,6 a |
| **38aa** | 40 h | 65 d |
| **38ab** | 20 h | 20 d |
| **38ac** | 14h | n.d. |
| **38ad** | 18h | n.d. |
| **4** | 15 d | n.d. |
| **5** | 22 h | n.d. |
| **9a** | 340 h | n.d |
| **9b** | 360 h | n.d |
| **9c** | 120 h | n.d |
| **10** | 130 h | n.d |

| | | |
|---|---|---|
| **13a** | 120 h | n.d |
| **13b** | 160 h | n.d. |
| **15** | 160 h | n.d |
| **19a** | 31h | n.d. |
| **19b** | 18h | n.d. |
| **22** | 40d | n.d. |
| **26a** | 34d | n.d. |
| **26b** | 40d | n.d. |
| **26c** | 18d | n.d. |
| **31a** | 22h | n.d. |
| **31b** | 95h | n.d. |
| **34a** | 42h | n.d. |
| **34b** | 205h | n.d. |
| **37a** | 138h | n.d. |
| **37b** | 639h | n.d. |
| **42** | 10d | n.d. |
| **43** | 17d | n.d. |
| **46** | 13d | n.d. |
| **50** | 35d | n.d. |

**Example 52**

**Release kinetics in vivo – in vitro/in vivo correlation**

Release kinetics in vivo were determined by comparing the pharmacokinetics of **13a** with the pharmacokinetics of **13c** and **13b** with **13d**, respectively, after intravenous injection into rat. Animal studies were performed at Heidelberg Pharma AG, Heidelberg, Germany.

**13a** (27 mg) was dissolved in 3.5 ml PBS and 500 µl of the resulting solution were injected intravenously into six rats each. Male SD rats with approximately 270 g weight were used. Blood samples were drawn at t = 0, 2 h, 24 h, 32 h, 48 h, 72 h, 96 h, 120 h, and 168 h, plasma was prepared, and plasma was analyzed for fluorescein fluorescence using a Perkin-Elmer LS 50B spektrometer.

Pharmacokinetics of **13c** were determined as described for **13a**. Pharmacokinetics of **13b** and **13d** were determined as described for **13a**, except for the use of 20 mg **13 b** and **13d** each in 2.5 ml PBS and four rats.

Linker hydrolysis half-life was calculated from the ratio of fluorescence of **13a** compared to fluorescence of **13c** and **13b** compared to **13d**, respectively, at the respective time points.

Half-life of in vivo linker hydrolysis was determined to be 115 h and 160 h for **13a** and **13b**, respectively, which is in excellent correlation to the half-life of in vitro linker hydrolysis of 120 h and 160 h for **13a** and **13b**, respectively.

Fig. 3 shows in vivo and in vitro linker cleavage data of **13b**, wherein in vivo (triangles) and in vitro (diamonds) cleavage kinetics are shown by semilogarithmic representation.

Abbreviations:

| | |
|---|---|
| Acp | 4-(2-aminoethyl)-1-carboxymethyl-piperazine |
| AcOH | acetic acid |
| Boc | t-butyloxycarbonyl |
| Dab | 2,4-diaminobutyric acid |
| DBU | 1,3-diazabicyclo[5.4.0]undecene |
| DCM | dichloromethane |
| Dda | dodecanoic acid |
| DIC | diisopropylcarbodiimide |
| DIEA | diisopropylethylamine |
| DMAP | dimethylamino-pyridine |
| DMF | N,N-dimethylformamide |
| DMSO | dimethylsulfoxide |
| EDTA | ethylenediaminetetraacetic acid |
| eq | stoichiometric equivalent |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| HATU | O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate |
| HFIP | hexafluoroisopropanol |
| HEPES | N-(2-hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid) |
| HOBt | N-hydroxybenzotriazole |
| ivDde | 1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methylbutyl |
| LCMS | mass spectrometry-coupled liquid chromatography |
| Mal | 3-maleimido propionyl |
| Mmt | 4-methoxytrityl |
| MS | mass spectrum |
| MW | molecular mass |
| n.d. | not determined |
| PfpOH | pentafluorophenol |
| PyBOP | benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate |
| RP-HPLC | reversed-phase high performance liquid chromatography |

| | |
|---|---|
| RT | room temperature |
| SEC | size exclusion chromatography |
| Suc | succinimidopropionyl |
| TCP | 2-chlorotrityl chloride resin |
| TES | triethylsilane |
| TMOB | 2,4,6-trimethoxybenzyl |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofurane |
| UV | ultraviolet |
| VIS | visual |

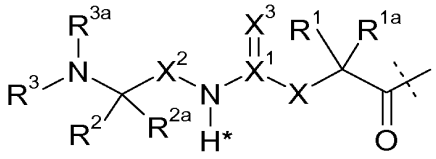
**Patent Claims**

1. A prodrug or a pharmaceutically acceptable salt thereof comprising a drug linker conjugate D-L, wherein

-D is a nitrogen containing biologically active moiety; and

-L is a

non-biologically active linker moiety -$L^1$ represented by formula (I),



(I)

,

wherein the dashed line indicates the attachment to the nitrogen of the biologically active moiety by forming an amide bond;

X is $C(R^4R^{4a})$; $N(R^4)$; O; $C(R^4R^{4a})$-$C(R^5R^{5a})$; $C(R^5R^{5a})$-$C(R^4R^{4a})$; $C(R^4R^{4a})$-$N(R^6)$; $N(R^6)$-$C(R^4R^{4a})$; $C(R^4R^{4a})$-O; or O-$C(R^4R^{4a})$;

$X^1$ is C; or S(O);

$X^2$ is $C(R^7, R^{7a})$; or $C(R^7, R^{7a})$-$C(R^8, R^{8a})$;

$X^3$ is O; S; or N-CN;

$R^1$, $R^{1a}$, $R^2$, $R^{2a}$, $R^3$, $R^{3a}$, $R^4$, $R^{4a}$, $R^5$, $R^{5a}$, $R^6$, $R^7$, $R^{7a}$, $R^8$, $R^{8a}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl;

Optionally, one or more of the pairs $R^{1a}/R^{4a}$, $R^{1a}/R^{5a}$, $R^{4a}/R^{5a}$, $R^{7a}/R^{8a}$ form a chemical bond;

Optionally, one or more of the pairs $R^1/R^{1a}$, $R^2/R^{2a}$, $R^4/R^{4a}$, $R^5/R^{5a}$, $R^7/R^{7a}$, $R^8/R^{8a}$ are joined together with the atom to which they are attached to form a $C_{3-7}$ cycloalkyl; or 4 to 7 membered heterocyclyl;

Optionally, one or more of the pairs $R^1/R^4$, $R^1/R^5$, $R^1/R^6$, $R^4/R^5$, $R^4/R^6$, $R^7/R^8$, $R^2/R^3$ are joined together with the atoms to which they are attached to form a ring A;

Optionally, $R^3/R^{3a}$ are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle;

A is selected from the group consisting of phenyl; naphthyl; indenyl; indanyl; tetralinyl; $C_{3-10}$ cycloalkyl; 4 to 7 membered heterocyclyl; and 9 to 11 membered heterobicyclyl; and

wherein $L^1$ is substituted with one to four groups $L^2$-Z and optionally further substituted, provided that the hydrogen marked with the asterisk in formula (I) is not replaced by a substituent; wherein

$L^2$ is a single chemical bond or a spacer; and

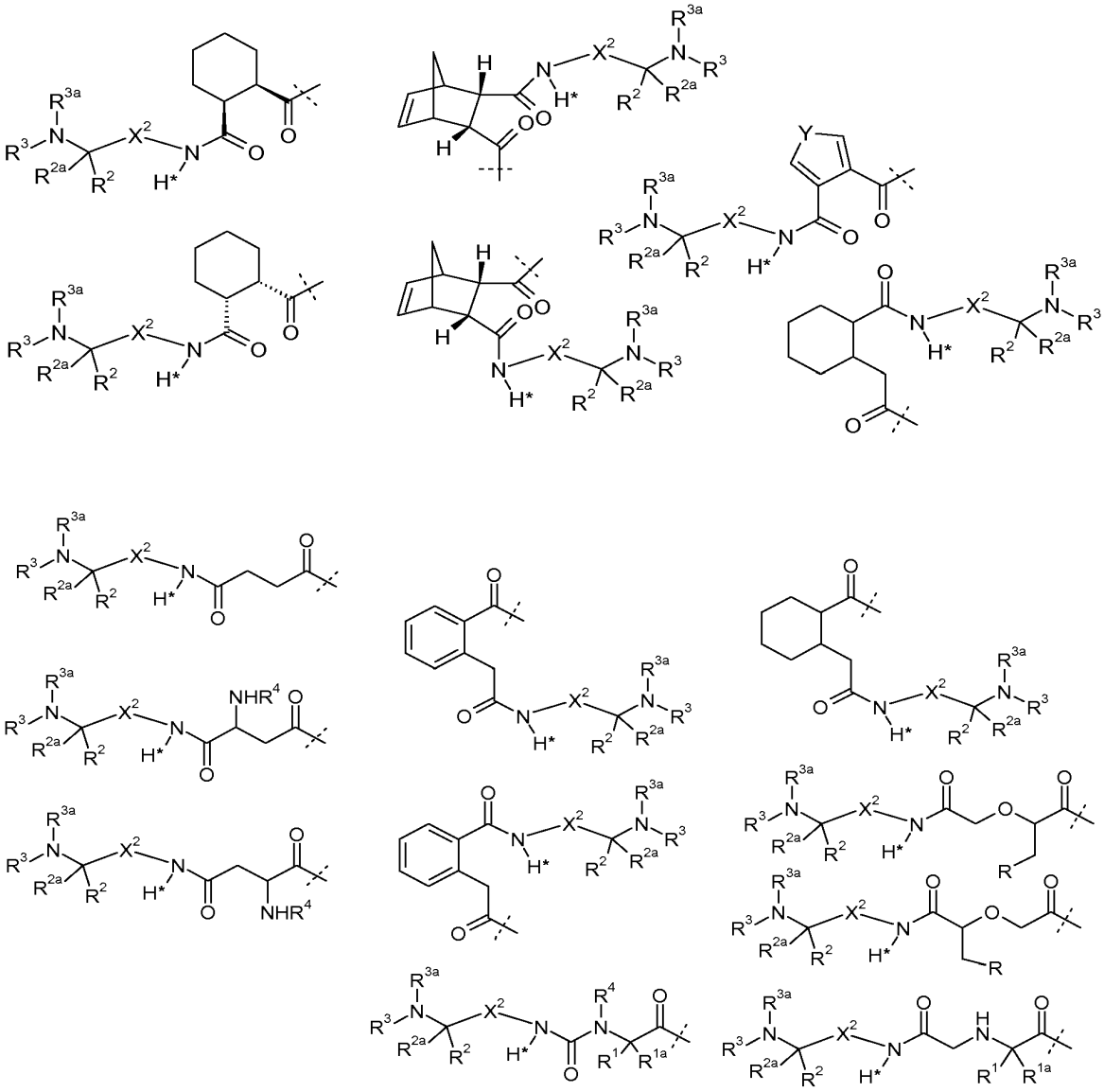
Z is a carrier group.

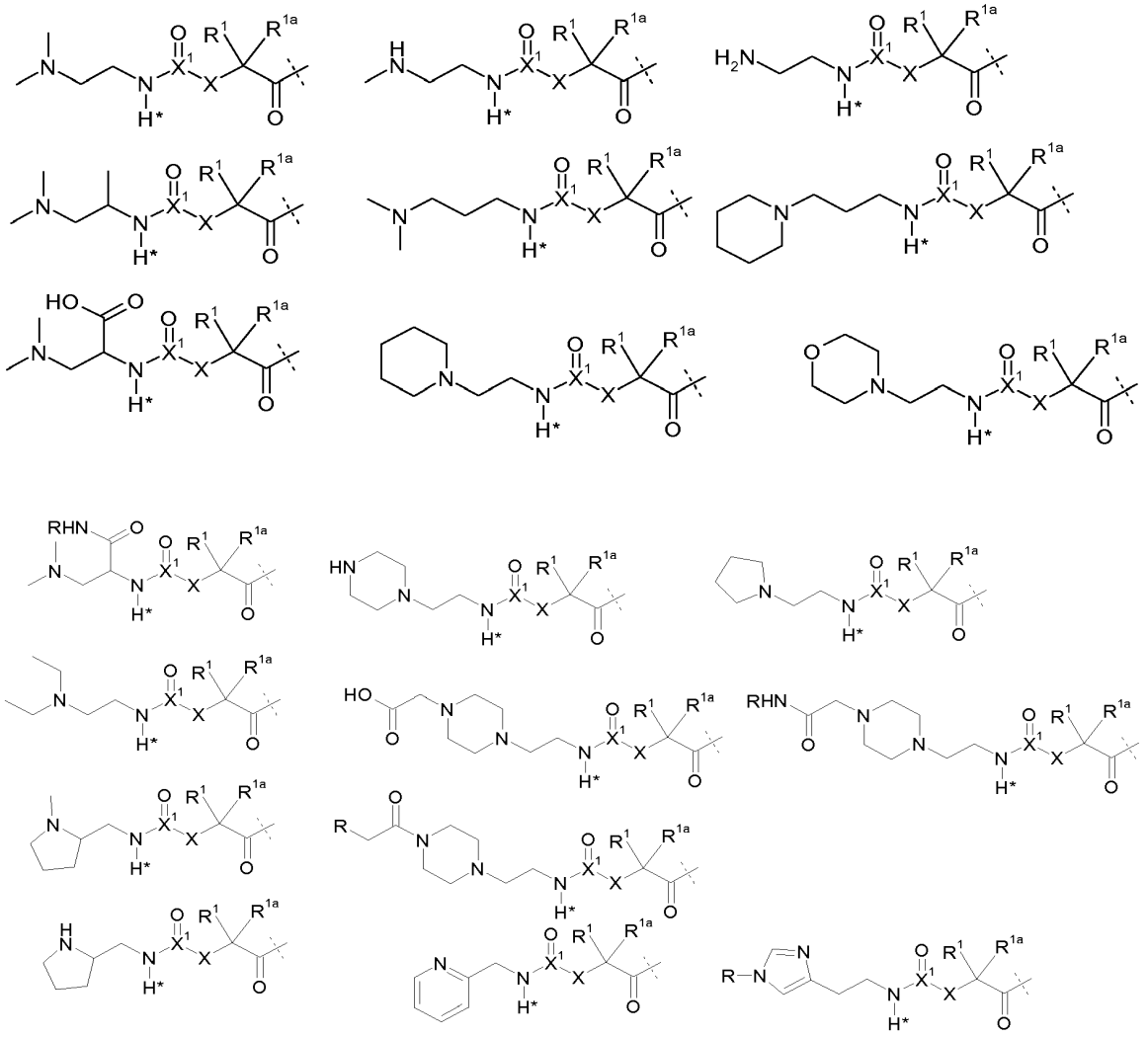
2. The prodrug of claim 1, wherein $X^3$ is O.

3. The prodrug of claim 1 or 2, wherein X is $N(R^4)$, $X^1$ is C and $X^3$ is O.

4. The prodrug of any of claims 1 to 3, wherein $X^2$ is $C(R^7R^{7a})$.

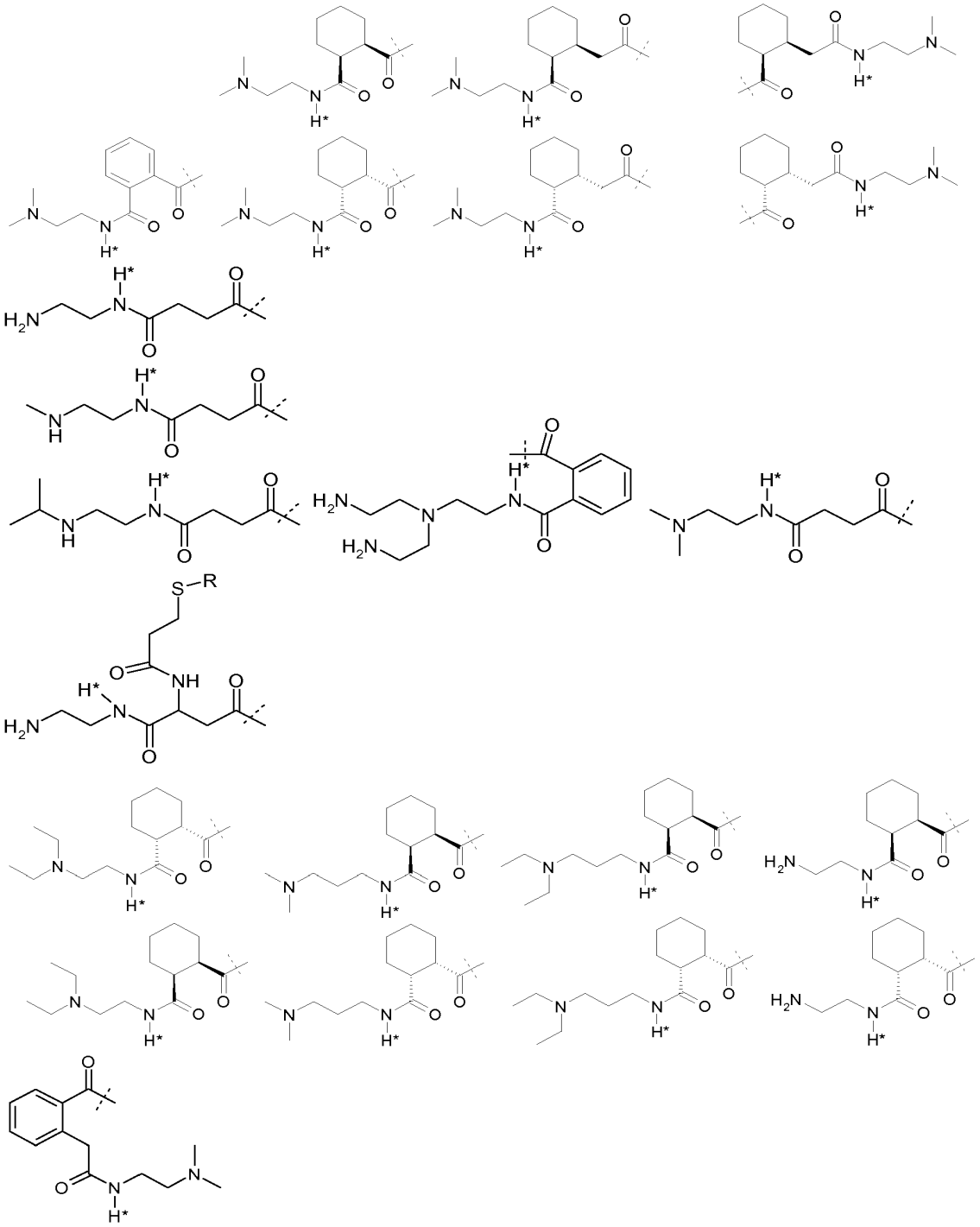
5. The prodrug of any of claims 1 to 4, wherein $L^1$ is selected from the group consisting of

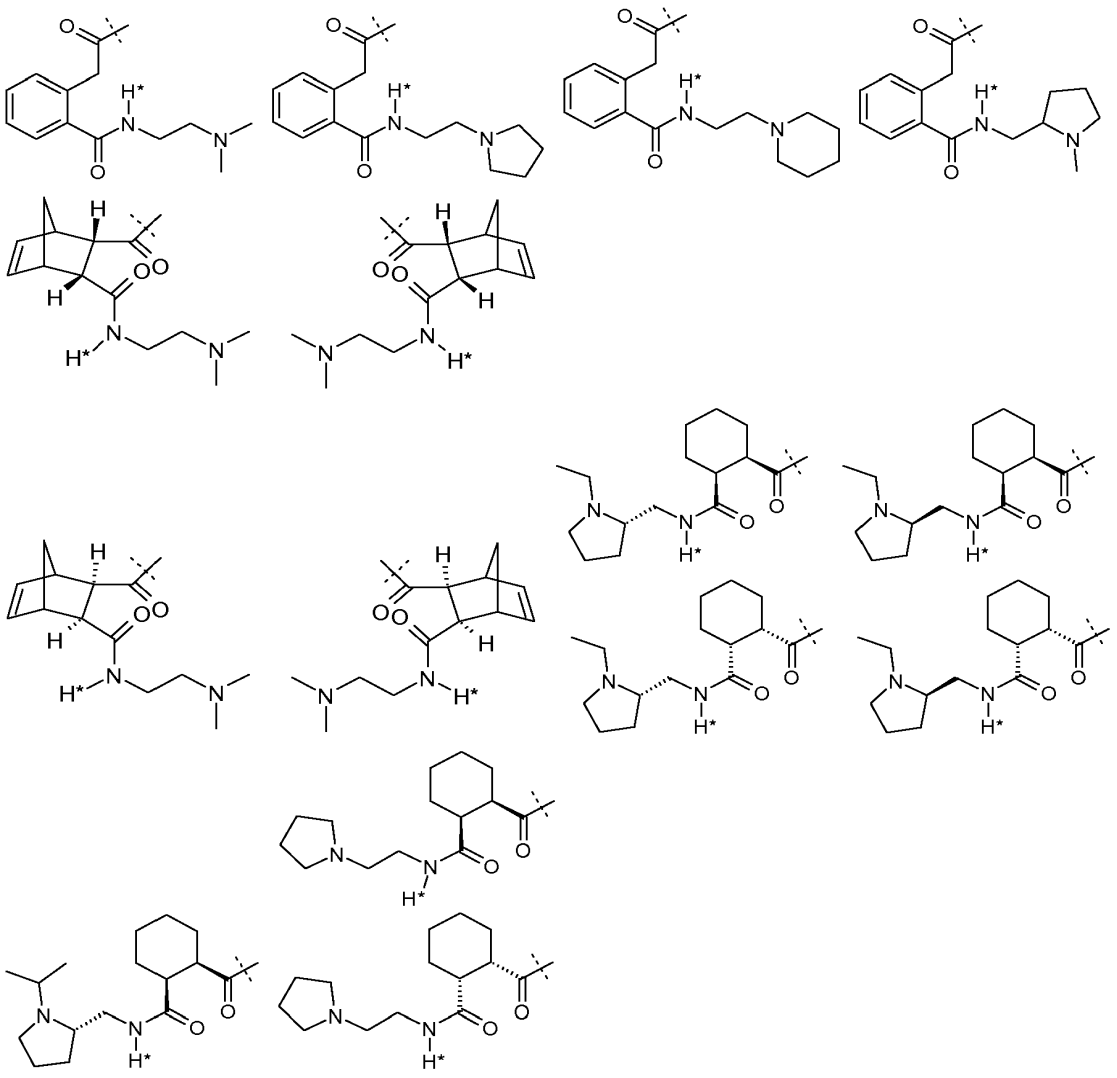


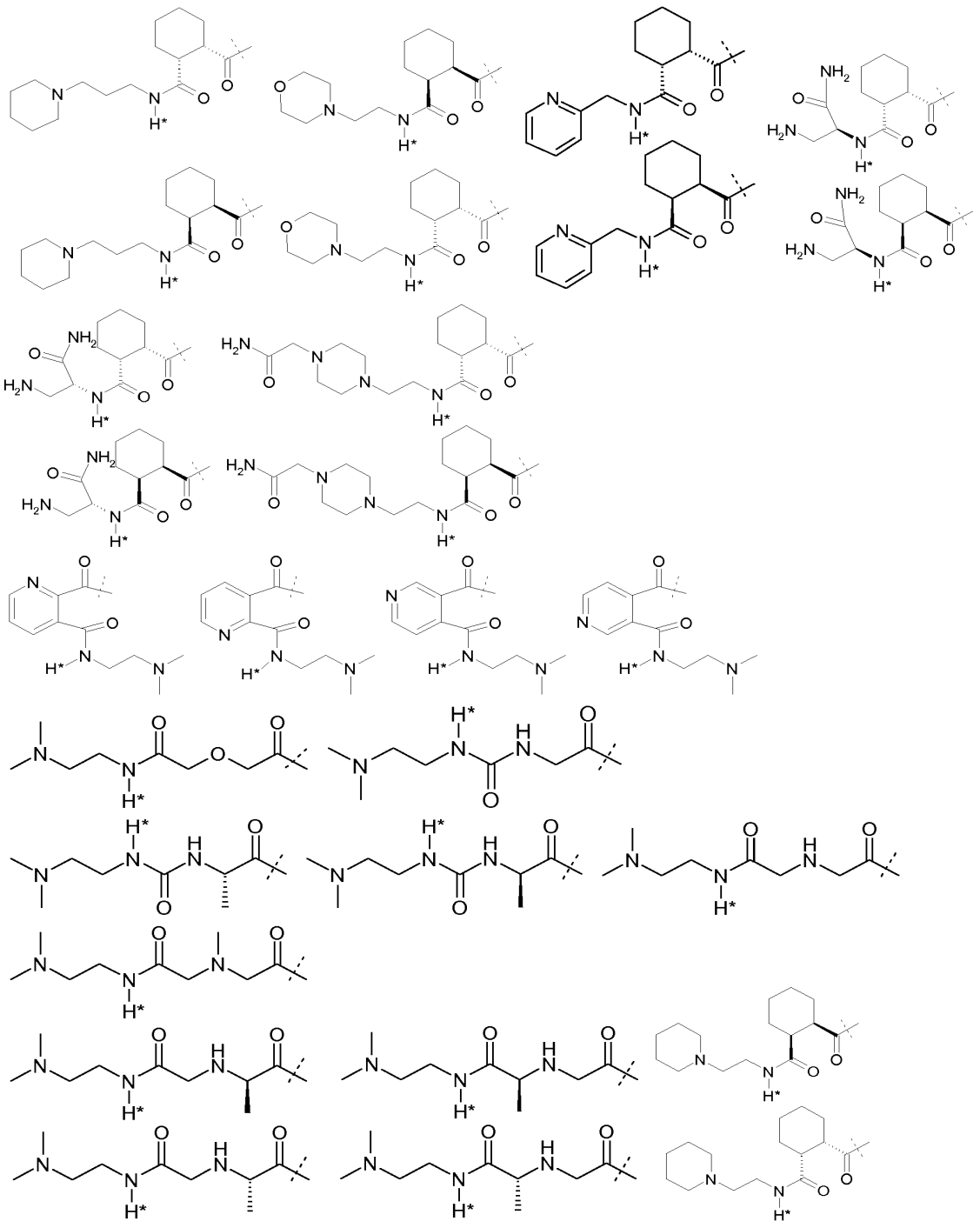


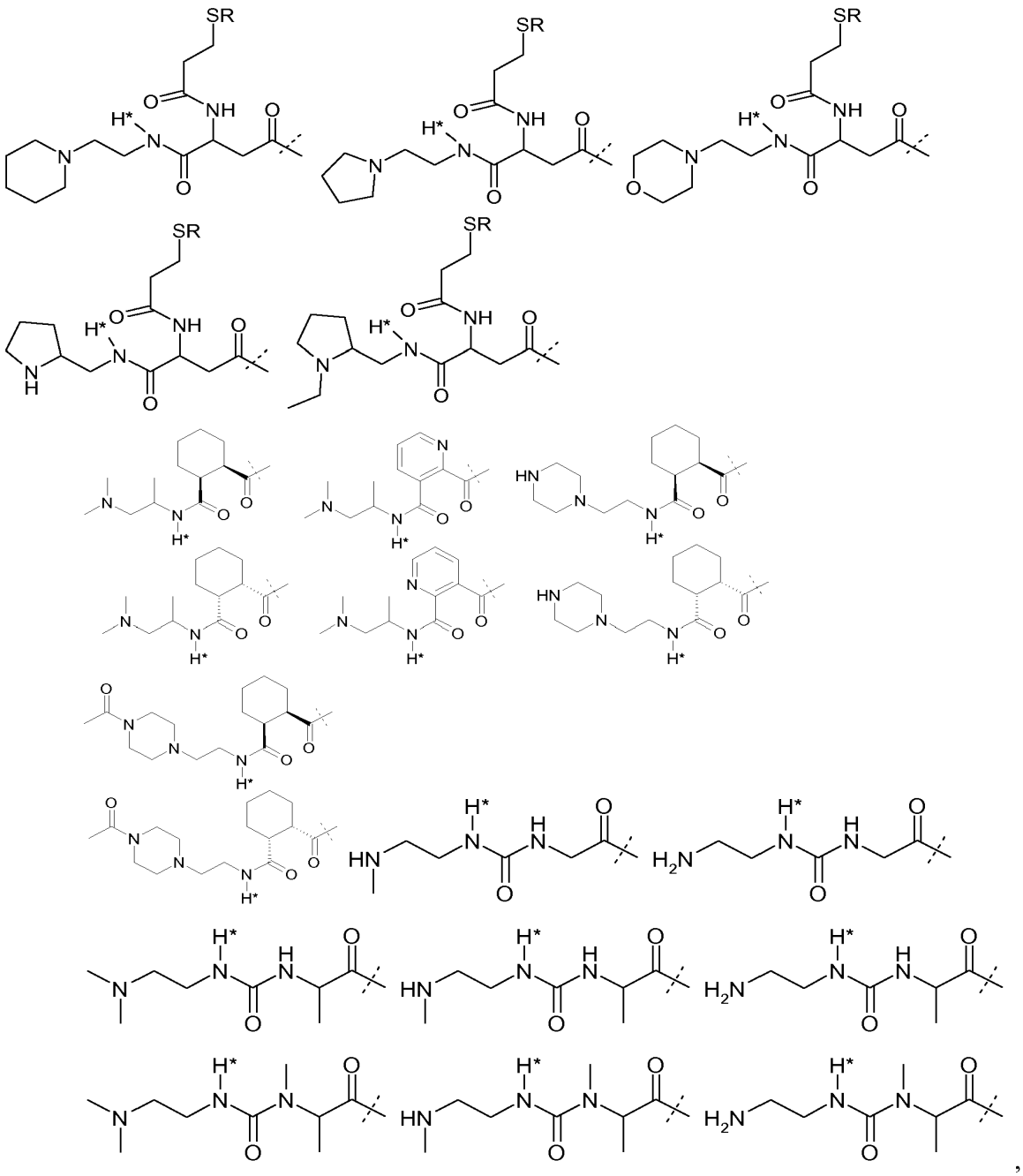
wherein R is H; or $C_{1-4}$ alkyl; Y is NH; O; or S; and $R^1$, $R^{1a}$, $R^2$, $R^{2a}$, $R^3$, $R^{3a}$, $R^4$, X, $X^1$, $X^2$ have the meaning as indicated in claim 1.

6. The prodrug of any of claims 1 to 5, wherein $L^1$ is selected from the group consisting of









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wherein R has the meaning as indicated in claim 5.

7. The prodrug of any of claims 1 to 6, wherein $L^2$ is a single chemical bond; or $L^2$-Z is $COOR^9$; $OR^9$; $C(O)R^9$; $C(O)N(R^9R^{9a})$; $S(O)_2N(R^9R^{9a})$; $S(O)N(R^9R^{9a})$; $S(O)_2R^9$; $S(O)R^9$; $N(R^9)S(O)_2N(R^{9a}R^{9b})$; $SR^9$; $N(R^9R^{9a})$; $OC(O)R^9$; $N(R^9)C(O)R^{9a}$; $N(R^9)S(O)_2R^{9a}$; $N(R^9)S(O)R^{9a}$; $N(R^9)C(O)OR^{9a}$; $N(R^9)C(O)N(R^{9a}R^{9b})$;

$OC(O)N(R^9R^{9a})$; T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally interrupted by one or more groups selected from the group consisting of -T-, -C(O)O-; -O-; -C(O)-; $-C(O)N(R^{11})-$; $-S(O)_2N(R^{11})-$; $-S(O)N(R^{11})-$; $-S(O)_2-$; -S(O)-; $-N(R^{11})S(O)_2N(R^{11a})-$; -S-; $-N(R^{11})-$; $-OC(O)R^{11}$; $-N(R^{11})C(O)-$; $-N(R^{11})S(O)_2-$; $-N(R^{11})S(O)-$; $-N(R^{11})C(O)O-$; $-N(R^{11})C(O)N(R^{11a})-$; and $-OC(O)N(R^{11}R^{11a})$;

$R^9$, $R^{9a}$, $R^{9b}$ are independently selected from the group consisting of H; Z; T; and $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; or $C_{2-50}$ alkynyl, wherein T; $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally substituted with one or more $R^{10}$, which are the same or different and wherein $C_{1-50}$ alkyl; $C_{2-50}$ alkenyl; and $C_{2-50}$ alkynyl are optionally interrupted by one or more groups selected from the group consisting of T, -C(O)O-; -O-; -C(O)-; $-C(O)N(R^{11})-$; $-S(O)_2N(R^{11})-$; $-S(O)N(R^{11})-$; $-S(O)_2-$; -S(O)-; $-N(R^{11})S(O)_2N(R^{11a})-$; -S-; $-N(R^{11})-$; $-OC(O)R^{11}$; $-N(R^{11})C(O)-$; $-N(R^{11})S(O)_2-$; $-N(R^{11})S(O)-$; $-N(R^{11})C(O)O-$; $-N(R^{11})C(O)N(R^{11a})-$; and $-OC(O)N(R^{11}R^{11a})$;

T is selected from the group consisting of phenyl; naphthyl; indenyl; indanyl; tetralinyl; $C_{3-10}$ cycloalkyl; 4 to 7 membered heterocyclyl; or 9 to 11 membered heterobicyclyl, wherein t is optionally substituted with one or more $R^{10}$, which are the same or different;

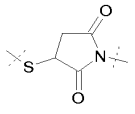
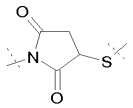
$R^{10}$ is Z; halogen; CN; oxo (=O); $COOR^{12}$; $OR^{12}$; $C(O)R^{12}$; $C(O)N(R^{12}R^{12a})$; $S(O)_2N(R^{12}R^{12a})$; $S(O)N(R^{12}R^{12a})$; $S(O)_2R^{12}$; $S(O)R^{12}$; $N(R^{12})S(O)_2N(R^{12a}R^{12b})$; $SR^{12}$; $N(R^{12}R^{12a})$; $NO_2$; $OC(O)R^{12}$; $N(R^{12})C(O)R^{12a}$; $N(R^{12})S(O)_2R^{12a}$; $N(R^{12})S(O)R^{12a}$; $N(R^{12})C(O)OR^{12a}$; $N(R^{12})C(O)N(R^{12a}R^{12b})$; $OC(O)N(R^{12}R^{12a})$; or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different;

$R^{11}$, $R^{11a}$, $R^{12}$, $R^{12a}$, $R^{12b}$ are independently selected from the group consisting of H; Z; or $C_{1-6}$ alkyl, wherein $C_{1-6}$ alkyl is optionally substituted with one or more halogen, which are the same or different;

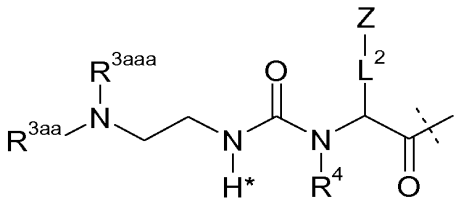
provided that one of $R^9$, $R^{9a}$, $R^{9b}$, $R^{10}$, $R^{11}$, $R^{11a}$, $R^{12}$, $R^{12a}$, $R^{12b}$ is Z.

8. The prodrug of any of claims 1 to 7, wherein $L^2$ is a $C_{1-20}$ alkyl chain, which is optionally interrupted by one or more groups independently selected from -O-; and $C(O)N(R^{3aa})$; optionally substituted with one or more groups independently selected from OH; and $C(O)N(R^{3aa}R^{3aaa})$; and wherein $R^{3aa}$, $R^{3aaa}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl.

9. The prodrug of any of caims 1 to 8, wherein $L^2$ has a molecular weight in the range of from 14 g/mol to 750 g/mol.

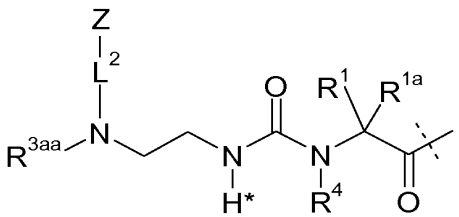
10. The prodrug of any of claims 1 to 9, wherein $L^2$ is attached to Z via a terminal group selected from  ; and  .

11. The prodrug of any of claims 1 to 10, wherein L is represented by formula (Ia)


(Ia)
,

wherein $R^4$, $L^2$, and Z have the meaning as indicated in claim 1, and wherein $R^{3aa}$, $R^{3aaa}$ are independently selected from the group consisting of H; and $C_{1-4}$ alkyl; or are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle.

12. The prodrug of any of claims 1 to 10, wherein L is represented by formula (Ib)


(Ib)
,

wherein $R^1$, $R^{1a}$, $R^4$, $L^2$ and Z have the meaning as indicated in claim 1, and wherein $R^{3aa}$ is H; or $C_{1-4}$ alkyl.

13. The prodrug of any of claims 1 to 10, wherein $R^1$ in formula (I) is $L^2$-Z.

14. The prodrug of any of claims 1 to 10, wherein $R^3$ in formula (I) is $L^2$-Z.

15. The prodrug of any of claims 1 to 10, wherein $R^3$, $R^{3a}$ in formula (I) are joined together with the nitrogen atom to which they are attached to form a 4 to 7 membered heterocycle and wherein the heterocycle is substituted with $L^2$-Z.

16. The prodrug of any of claims 1 to 15, wherein D-H is a small molecule bioactive agent or a biopolymer.

17. The prodrug of any of claims 1 to 16, wherein D-H is a biopolymer selected from the group of biopolymers consisting of proteins, polypeptides, oligonucleotides, and peptide nucleic acids.

18. The prodrug of any of claims 1 to 17, wherein D-H is a polypeptide selected from the group of polypeptides consisting of ACTH, adenosine deaminase, agalsidase, alfa-1 antitrypsin (AAT), alfa-1 proteinase inhibitor (API), alteplase, amylins (amylin, symlin), anistreplase, ancrod serine protease, antibodies (monoclonal or polyclonal, and fragments or fusions), antithrombin III, antitrypsins, aprotinin, asparaginases, atosiban, biphalin, bivalirudin, bone-morphogenic proteins, bovine pancreatic trypsin inhibitor (BPTI), cadherin fragments, calcitonin (salmon), collagenase, complement C1 esterase inhibitor, conotoxins, cytokine receptor fragments, DNase, dynorphine A, endorphins, enfuvirtide, enkephalins, erythropoietins, exendins, factor VII, factor VIIa, factor VIII, factor VIIIa, factor IX, fibrinolysin, fibroblast growth factor (FGF), growth hormone releasing peptide 2 (GHRP2), fusion proteins, follicle-stimulating hormones, gramicidin, ghrelin, desacyl-ghrelin, granulocyte colony stimulating factor (G-CSF), galactosidase, glucagon, glucagon-like peptides, glucocerebrosidase, granulocyte macrophage colony stimulating factor (GM-CSF), human heat shock proteins (HSP), phospholipase-activating protein (PLAP), gonadotropin chorionic (hCG), hemoglobins, hepatitis B vaccines, hirudin, human serine protease inhibitor,

hyaluronidases, idurnonidase, immune globulins, influenza vaccines, interleukins (1 alfa, 1 beta, 2, 3, 4, 6, 10, 11, 12, 13, 21), IL-1 receptor antagonist (rhIL-1ra), insulins, insulin like growth factors, insulin-like growth factor binding protein (rhIGFBP), interferons (alfa 2a, alfa 2b, alfa 2c, beta 1a, beta 1b, gamma 1a, gamma 1b), intracellular adhesion molecule, keratinocyte growth factor (KGF), P-selectin glycoprotein ligand (PSGL), transforming growth factors, lactase, leptin, leuprolide, levothyroxine, luteinizing hormone, lyme vaccine, natriuretic peptides (ANP, BNP, CNP and fragments), neuropeptide Y, pancrelipase, pancreatic polypeptide, papain, parathyroid hormone, PDGF, pepsin, peptide YY, platelet activating factor acetylhydrolase (PAF-AH), prolactin, protein C, thymalfasin, octreotide, secretin, sermorelin, soluble tumor necorsis factor receptor (TNFR), superoxide dismutase (SOD), somatropins (growth hormone), somatoprim, somatostatin, streptokinase, sucrase, terlipressin, tetanus toxin fragment, tilactase, thrombins, thymosin, thyroid stimulating hormone, thyrotropin, tumor necrosis factor (TNF), TNF receptor-IgG Fc, tissue plasminogen activator (tPA), TSH, urodilatin, urate oxidase, urokinase, vaccines, vascular endothelial growth factor (VEGF), vasoactive intestinal peptide, vasopressin, ziconotide, lectin and ricin.

19. The prodrug of any of claims 1 to 18, wherein D-H is a protein prepared by recombinant DNA technologies.

20. The prodrug of any of claims 1 to 19, wherein D-H is a protein selected from the group of proteins consisting of antibody fragments, single chain antigen binding proteins, catalytic antibodies and fusion proteins.

21. The prodrug of any of claims 1 to 16, wherein D-H is a small molecule bioactive agent selected from the group of agents consisting of central nervous system-active agents, anti-infective, anti-allergic, immunomodulating, anti-obesity, anticoagulants, antidiabetic, anti-neoplastic, antibacterial, anti-fungal, analgesic, contraceptive, anti-inflammatory, steroidal, vasodilating, vasoconstricting, and cardiovascular agents with at least one primary or secondary amino group.

22. The prodrug of any of claims 1 to 16 and 21, wherein D-H is a small molecule bioactive agent selected from the group of agents consisting of acarbose, alaproclate,

alendronate, amantadine, amikacin, amineptine, aminoglutethimide, amisulpride, amlodipine, amotosalen, amoxapine, amoxicillin, amphetamine, amphotericin B, ampicillin, amprenavir, amrinone, anileridine, apraclonidine, apramycin, articaine, atenolol, atomoxetine, avizafone, baclofen, benazepril, benserazide, benzocaine, betaxolol, bleomycin, bromfenac, brofaromine, carvedilol, cathine, cathinone, carbutamid, cefalexine, clinafloxacin, ciprofloxacin, deferoxamine, delavirdine, desipramine, daunorubicin, dexmethylphenidate, dexmethylphenidate, diaphenylsulfon, dizocilpine, dopamin, dobutamin, dorzolamide, doxorubicin, duloxetine, eflornithine, enalapril, epinephrine, epirubicin, ergoline, ertapenem, esmolol, enoxacin, ethambutol, fenfluramine, fenoldopam, fenoterol, fingolimod, flecainide, fluvoxamine, fosamprenavir, frovatriptan, furosemide, fluoexetine, gabapentin, gatifloxacin, gemiflocacin, gentamicin, grepafloxacin, hexylcaine, hydralazine, hydrochlorothiazide, icofungipen, idarubicin, imiquimod, inversine, isoproterenol, isradipine, kanamycin A, ketamin, labetalol, lamivudine, levobunolol, levodopa, levothyroxine, lisinopril, lomefloxacin, loracarbef, maprotiline, mefloquine, melphalan, memantine, meropenem, mesalazine, mescaline, methyldopa, methylenedioxymethamphetamine, metoprolol, milnacipran, mitoxantron, moxifloxacin, norepinephrine, norfloxacin, nortriptyline, neomycin B, nystatin, oseltamivir, pamidronic acid, paroxetine, pazufloxacin, pemetrexed, perindopril, phenmetrazine, phenelzine, pregabalin, procaine, pseudoephedrine, protriptyline, reboxetine, ritodrine, sabarubicin, salbutamol, serotonin, sertraline, sitagliptin, sotalol, spectinomycin, sulfadiazin, sulfamerazin, sertraline, sprectinomycin, sulfalen, sulfamethoxazol, tacrine, tamsulosin, terbutaline, timolol, tirofiban, tobramycin, tocainide, tosufloxacin, trandolapril, tranexamic acid, tranylcypromine, trimerexate, trovafloxacin, valaciclovir, valganciclovir, vancomycin, viomycin, viloxazine, and zalcitabine.

23. The prodrug of any of claims 1 to 22, wherein Z is a polymer of at least 500 Da or a $C_{8-18}$ alkyl group.

24. The prodrug of any of claims 1 to 23, wherein Z is selected from the group of optionally crosslinked polymers consisting of poly(propylene glycol), poly(ethylene glycol), dextran, chitosan, hyaluronic acid, alginate, xylan, mannan, carrageenan, agarose, cellulose, starch, hydroxyalkyl starch (HAS), poly(vinyl alcohols),

poly(oxazolines), poly(anhydrides), poly(ortho esters), poly(carbonates), poly(urethanes), poly(acrylic acids), poly(acrylamides), poly(acrylates), poly(methacrylates), poly(organophosphazenes), polyoxazoline, poly(siloxanes), poly(amides), poly(vinylpyrrolidone), poly(cyanoacrylates), poly(esters), poly(iminocarbonates), poly(amino acids), collagen, gelatin, hydrogel, or a blood plasma protein, and copolymers thereof.

25. The prodrug of any of claims 1 to 24, wherein Z is a protein.

26. The prodrug of any of claims 1 to 25, wherein Z is a protein selected from the group consisting of albumin, transferrin, immunoglobulin.

27. The prodrug of any of claims 1 to 23, wherein Z is a linear or branched poly(ethylene glycol) with a molecular weight from 2,000 Da to 150,000 Da.

28. The prodrug of any of claims 1 to 27, wherein D-H is a GLP-1 receptor agonist; L is $L^1$ represented by formula (I) as indicated in claim 1; and Z is a hydrogel.

29. The prodrug of claim 28, wherein the GLP-1 receptor agonist is Exendin-4.

30. The prodrug of claim 28 or 29, wherein in formula (I) X is $N(R^4)$, $X^1$ is C and $X^3$ is O.

31. The prodrug of any of claims 28 to 30, wherein L is represented by formula (Ia) as indicated in claim 11 or L is represented by formula (Ib) as indicated in claim 12.

32. A prodrug precursor of formula Act-L, wherein L has the meaning as indicated in claim 1 and Act is a leaving group.

33. The prodrug precursor of claim 32, wherein Act is chloride, bromide, fluoride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy, 2-thiooxo-thiazolidinyl, or N-hydroxysulfosuccinimidyl.

34. A pharmaceutical composition comprising a prodrug of any of claims 1 to 31 or a pharmaceutical salt thereof together with a pharmaceutically acceptable excipient.

35. A prodrug of any of claims 1 to 31 or a pharmaceutical composition of claim 30 for use as a medicament.

36. A method of treating, controlling, delaying or preventing in a mammalian patient in need of the treatment of one or more conditions comprising administering to said patient a therapeutically effective amount of a prodrug of any of claims 1 to 31 or a pharmaceutical composition of claim 34 or a pharmaceutically acceptable salt thereof.

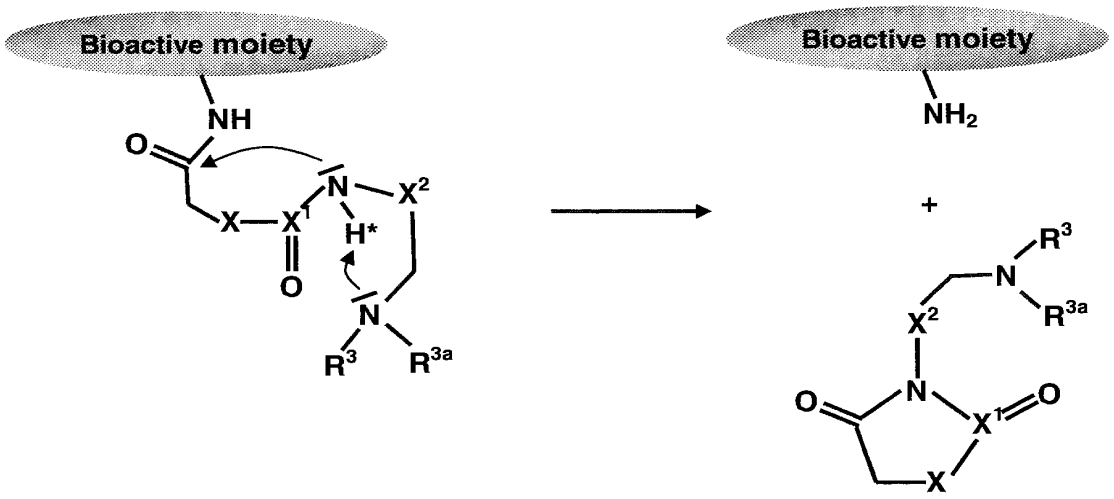

Bioactive moiety
NH
O
X
X1
O
N
X2
H*
N
R3
R3a
Bioactive moiety
NH2
+
R3
N
R3a
X2
N
O
O
X1
X

Fig. 1

5

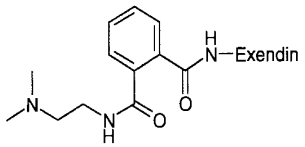
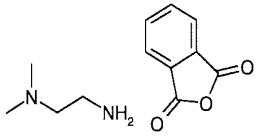
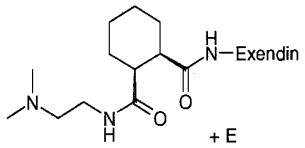
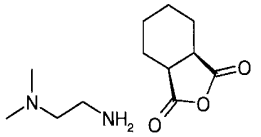
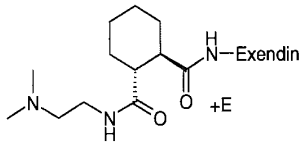
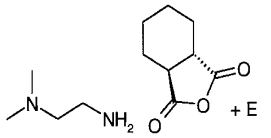
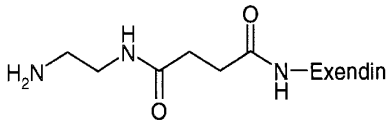
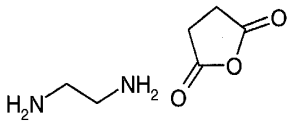
| Compound | Structure | Starting materials | Synthesis method | MW (calc.) (g/mol | m/z (ESI-MS) $[M+3H]^{3+}$ |
|---|---|---|---|---|---|
| **38a** |  |  | A | 4404 | 1469.4 |
| **38b** |  |  | A | 4410 | 1471.5 |
| **38c** |  |  | A | 4410 | 1471.5 |
| **38d** |  |  | A | 4328 | 1443.8 |

**Fig. 2**

| | | | | |
|---|---|---|---|---|
| 38e |  | A | 4356 | 1453 |
| 38f |  | A | 4438 | 1481.4 |
| 38g |  | A | 4424 | 1475.6 |
| 38h |  | A | 4452 | 1485.9 |
| 38i |  | A | 4382 | 1462.8 |

**Fig. 2 (continued)**

| | | | | |
|---|---|---|---|---|
| 38j |  | C | 4418 | 1474.1 |
| 38k |  | A | 4420 | 1475.2 |
| 38l |  | A | 4450 | 1484.9 |
| 38m |  | A | 4436 | 1480.2 |

**Fig. 2 (continued)**

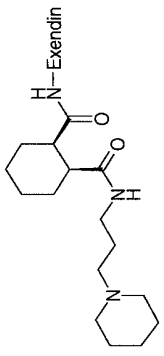
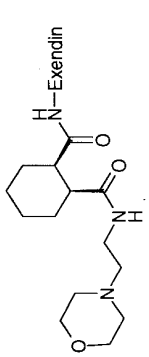
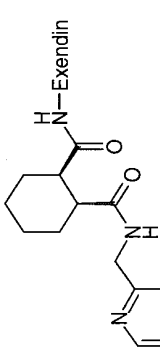
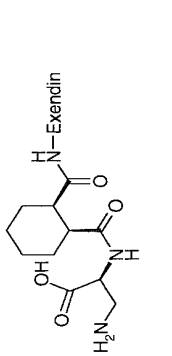
| | | | | |
|---|---|---|---|---|
| 38n |  | A | 4464 | 1490.0 |
| 38o |  | A | 4452 | 1485.2 |
| 38p |  | A | 4430 | 1478.2 |
| 38q |  | D | 4426 | 1476.3 |

**Fig. 2 (continued)**

| | | | | | |
|---|---|---|---|---|---|
| 38r |  | D | 4509 | 1504.1 | |
| 38s |  | A | 4421 | 1476.1 | |
| 38t |  | A | 4421 | 1475.4 | |
| 38u |  | A | 4388 | 1464.4 | |
| 38v |  | E | 4357 | 1453.9 | |
| 38w |  | E | 4371 | 1458.7 | |

Fig. 2 (continued)

| | | | | |
|---|---|---|---|---|
| 38x |  | B | 4371 | 1458.7 |
| 38y |  | A | 4450 | 1484.1 |
| 38z |  | A | 4424 | 1475.0 |
| 38aa |  | A | 4451 | 1484.6 |
| 38ab |  | F | 4493 | 1497.7 |

Fig. 2 (continued)

**Fig. 2 (continued)**

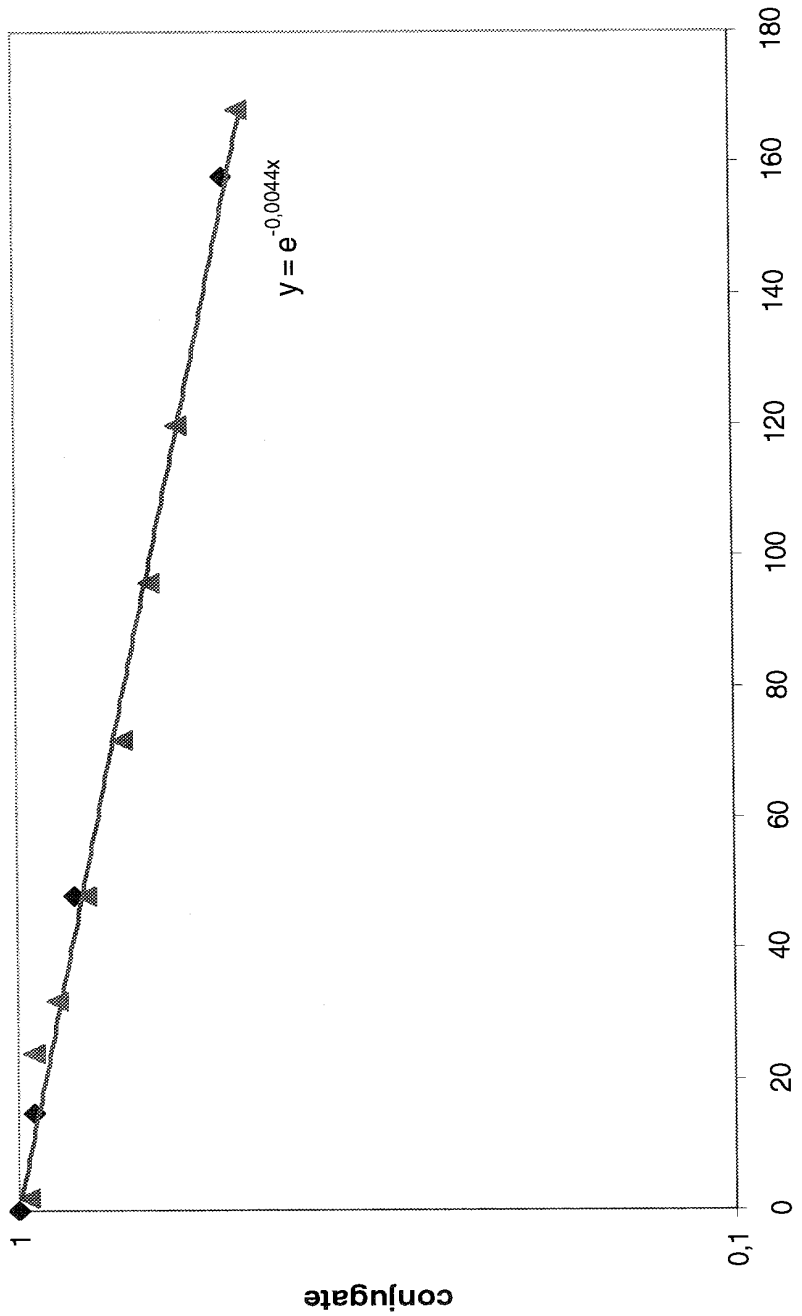



Fig. 3

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(71) Applicants *(for all designated States except US)*: **BOEHRINGER INGELHEIM INTERNATIONAL GMBH** [DE/DE]; Binger Strasse 173, 55216 Ingelheim (DE). **SYNTONIX PHARMACEUTICALS, INC.** [US/US]; 9 Fourth Avenue, Waltham, Massachusetts 02451 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CANADA, Keith** [US/US]; 900 Ridgebury Road, Ridgefield, Connecticut 06877 (US). **SCHOENBECK, Uwe** [DE/US]; 900 Ridgebury Road, Ridgefield, Connecticut 06877 (US). **HICKEY, Eugene R.** [US/US]; 900 Ridgebury Road, Ridgefield, Connecticut 06877 (US). **MEZO, Adam** [CA/US]; 15 Montclair Avenue, Waltham, Massachusetts 02451 (US). **MCDONNELL, Kevin** [CA/US]; 305 Dale Street, Waltham, Massachusetts 02451 (US).

(74) Agents: **BETTI, Christopher J.** et al.; Bell, Boyd & Lloyd LLP, P.O. Box 1135, Chicago, Illinois 60690-1135 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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(54) Title: NATRIURETIC FUSION PROTEINS

(57) Abstract: Natriuretic peptide fusion proteins comprising natriuretic peptides linked to antibody Fc domains, nucleic acid molecules encoding the fusion proteins disclosed herein, expression vectors expressing said fusion proteins, pharmaceutical compositions comprising said fusion proteins, and methods for their therapeutic use are disclosed.

TITLE OF THE INVENTION

# NATRIURETIC FUSION PROTEINS

## STATEMENT REGARDING JOINT RESEARCH AGREEMENT

[0001] One or more inventions contained in this application were developed under a joint research agreement as defined in the Cooperative Research and Technology Enhancement Act of 2004 between Boehringer Ingelheim International, GmbH and Syntonix Pharmaceuticals, Inc.

## FIELD OF THE INVENTION

[0002] This invention relates to fusion proteins having diuretic, natriuretic, and vasodilatory activity, specifically natriuretic peptides linked to antibody Fc domains. The fusion proteins of this invention exhibit extended plasma half-life compared to wild type natriuretic peptides, which serve as ligands to membrane guanylyl cyclases. The invention is also directed to nucleic acid molecules encoding the fusion proteins disclosed herein, expression vectors expressing said proteins, pharmaceutical compositions comprising the fusion proteins and/or nucleic acid molecules of the present invention. Compositions according to this invention may be administered by injection. The invention also relates to methods of treating or ameliorating pathological conditions in which activation of the NPRA receptor by binding of the fusion proteins of the present invention confers a therapeutic benefit on the patient.

## BACKGROUND OF THE INVENTION

[0003] Natriuretic peptides are involved in numerous biological functions including the regulation of intravascular volume, blood pressure, natriuresis, diuresis and long bone growth. These peptides include, for example, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), both of which are believed to exert their cellular effects upon binding to a cell surface glycoprotein receptor, natriuretic peptide receptor A (NPRA), which is a membrane guanylyl cyclase that catalyzes the synthesis of cGMP upon ligand binding. Among their biological functions, ANP and BNP are believed to have an effect on the cardiovascular system and may be particularly effective therapeutics for the treatment of various heart conditions including acute heart failure as well as chronic congestive heart failure.

[0004] Unfortunately, despite this promising clinical application, the therapeutic usefulness of ANP and BNP is sorely limited as endopeptidase degradation, as well as natriuretic peptide clearance receptor (NPR-C) mediated internalization, causes these proteins to have a fairly short half-life *in vivo*. For example, the plasma half life of ANP in humans is approximately two minutes and that of BNP is estimated to be approximately 20 minutes (Potter et al., Endocrine Reviews (2006) 27(1):42-72). Thus, previous therapeutic administration of these peptides has been limited to time consuming intravenous infusion, typically in a hospital or other medical care facility. As such, there is a need for alternative therapies, which would allow medical science to more fully realize the clinical benefits of natriuretic peptides such as ANP and BNP.

## SUMMARY OF THE INVENTION

[0005] This invention relates to novel, biologically active fusion proteins comprised of one or more natriuretic peptides linked to an Fc region of IgG or other antibody.

[0006] Also provided herein are nucleic acid molecules that encode natriuretic fusion proteins of the present invention, and expression vectors that comprise polynucleotide sequences encoding natriuretic fusion proteins, for uses that include treatment or amelioration of pathological conditions in which activation of the NPRA receptor confers a therapeutic benefit on the patient, including but not limited to diseases associated with abnormal diuretic, natriuretic and vasodilatory activity. Fusion proteins or nucleic acid molecules according to the invention may be present in compositions that include pharmaceutically acceptable excipients, carriers or diluents.

[0007] In one aspect, the present invention is directed to fusion proteins that comprise one or more natriuretic peptides bound to an Fc domain by a glycine succinate linker. As contemplated herein, when a glycine succinate linker is used to link a natriuretic peptide and a Fc domain, the glycine residue of the linker is linked to the N- terminus of the Fc domain and the succinate moeity is linked to the C- terminus of the natriuretic peptide. and/or an amino acid linker of various length and sequence. In relation to the linker, the length and composition are necessary to achieve prolonged efficacy of the natriuretic peptide. As contemplated herein, the peptide may be linked to the Fc domain in different orientations. In one orientation, the C-terminus of peptide is linked to the N-terminus of the Fc domain and in another orientation, the N-terminus of the peptide is linked to the N-terminus of the Fc domain. The Fc domain exists as a homodimer of the hinge, CH2 and CH3 regions of an IgG

molecule, with the Fc domain beginning at the first N-terminal cysteine residue within the IgG hinge region and the homodimer is held together by two disulfide bonds in the hinge from the cysteine residues of CysProProCysPro (SEQ ID NO: 1).

[0008] In a further aspect, the invention comprises pharmaceutical compositions comprising pharmaceutically acceptable excipients, carriers or diluents and any of the fusion peptides described herein.

[0009] In an additional aspect, the invention is also directed to nucleic acid molecules encoding the fusion proteins disclosed herein and expression vectors expressing said proteins.

[0010] In another aspect, the invention relates to methods to treat or ameliorate pathological conditions in which activation of the NPRA receptor confers a therapeutic benefit on the patient, including, but not limited to, diseases associated with abnormal diruretic, natriuretic and vasodilatory activity and/or in which it is desirable to induce naturesis, diuresis, vasodilation or to modulate the renin-angiotensin II and aldosterone systems. These conditions include those that may be characterized by an excess in extraceullar fluid, including, but not limited to, pulmonary edema. In a particularly preferred embodiment, the invention includes methods to treat or ameliorate pathological conditions of the cardiovascular system including, but not limited to, chronic heart failure (non-ischemic), post-MI heart failure (ischemic CHF), acute MI, reperfusion injury, left ventricular dysfunction (LVD), cardiac fibrosis, diastolic heart failure, and hypertrophic cardiomyopathy. In addition, hypertensive disorders including, but not limited to hypertension, *e.g.*, pulmonary hypertension, systolic hypertension, resistant hypertension and other cardiovascular related diseases such as diabetic nephropathy may be treated or ameliorated by the methods of the present invention. It is also contemplated herein that the fusion proteins and pharmaceutical compositions of the present invention may provide therapeutic benefit for patients undergoing coronary artery bypass graft surgery (CABG).

[0011] It is also contemplated herein that the invention includes the use of the fusion proteins of the present invention in the manufacture of a medicament for the treatment or amelioration of any of the pathological conditions provided above.

[0012] These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

## BRIEF DESCRIPTION OF THE FIGURES

[0013] Figure 1A-B: DNA and protein sequences of 4 recombinantly-produced ANP-Fc fusion proteins. The bolded mouse IgG kappa light chain signal sequence is cleaved off and not part of the final protein product. hANP28 is underlined. The $(GGS)_xGG$ linker is italicized.

[0014] Figure 2: Representative dose response curve for cGMP assay. Recombinantly-produced fusion proteins are assayed for cGMP production in rat NPRA expressing 293T cells.

[0015] Figure 3: ANP or Fusion Protein Induced cGMP Production in hNPRA 293 Cells +/- NEP.

## DETAILED DESCRIPTION OF THE INVENTION

[0016] The present disclosure provides fusion proteins that comprise a natriuretic peptide and an antibody Fc domain, wherein said natriuretic peptide is conjugated to the Fc domain directly or through a linker. The natriuretic peptide and Fc region of the fusion proteins serve two distinct biological roles that contribute to efficacy of the fusion proteins. Surprisingly, linker length also influences efficacy of the fusion proteins.

[0017] The present disclosure also provides fusion proteins that comprise at least two natriuretic peptides separated from each other by an antibody Fc domain, wherein said natriuretic peptides are conjugated to the Fc domain directly or through a linker.

[0018] In some embodiments, the fusion protein comprises the following formula:

$X\text{-}L_a\text{-}F\text{:}F\text{-}\ L_a\text{-}X$ or $X\text{-}L_a\text{-}F\text{:}F$,

wherein,

X is a natriuretic peptide;

L is a linker comprising "a" amino acid residues;

a is an integer of at least 0;

: is a chemical association or crosslink; and

F is at least a portion of an immunoglobulin Fc domain comprising an FcRn binding site.

[0019] In some embodiments, the fusion protein comprises the following formula:

$X\text{-}L_a\text{-}F\text{:}F\text{-}La\text{-}X$,

wherein

X is one or more natriuretic peptides;

L is a linker comprising amino acid residues;

a is an integer of at least 0;

: is a chemical association or crosslink; and

F is at least a portion of an immunoglobulin Fc domain comprising an FcRn binding site.

[0020] In some embodiments, the natriuretic peptide is selected from the group consisting of ANP, BNP, Urodilatin, DNP or a biologically active sequence variant thereof. In some embodiments, the natriuretic peptide is ANP or BNP.

[0021] In some embodiments, the fusion protein comprises at least two natriuretic peptides. In some embodiments, x may be more than one natriuretic peptide. In some embodiments, both natriuretic peptides are ANP. In some embodiments, both natriuretic peptides are BNP.

[0022] In certain embodiments, the chemical association, *i.e.*, (:) is a covalent bond. In other embodiments, the chemical association, *i.e.*, (:) is a non-covalent interaction, *e.g.*, an ionic interaction, a hydrophobic interaction, a hydrophilic interaction, a Van der Waals interaction or a hydrogen bond.

[0023] In some embodiments, the fusion protein comprises at least two Fc domains.

[0024] In some embodiments, the linker is at least 2, 4, 6, 9, 11, 16 or 20 amino acids in length. In other embodiments, the linker is at least 0, 1, 5, 7, 8, 10, 12, 13, 14, 15, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids, but may optionally be longer, *e.g.*, between 30 and 40 amino acids in length or between 40 and 50 amino acids in length. In some embodiments, the linker is selected from 6 to 11 amino acids in length, 11 to 16 amino acids in length, 9 to 20 amino acids in length, 16 to 20 amino acids in length, 16 to 25 amino acids in length, 20 to 30 amino acids in length, 25 to 35 amino acids in length, 30 to 50 amino acids in length, 30 to 40 amino acids in length or 35 to 45 amino acids in length. In some embodiments, the linker is more than 10, more than 15, more than 20, more than 25, or more than 30 amino acids in length. In some embodiments, the linker is selected from a glycine succinate linker (L1), an amino acid linker or combination thereof. In some embodiments, the amino acid linker is GlyGly (L2), $Gly(SerGlyGly)_2SerGly$ (L3) (SEQ ID NO. 2), $(GlyGlySer)_3GlyGly$ (L4) (SEQ ID NO. 3), $(GlyGlySer)_4GlyGly$ (SEQ ID NO. 4), $(GlySerGly)_5Gly$ (L5a) (SEQ ID NO: 5), $(GlyGlySer)_5Gly$ (L5) (SEQ ID NO. 6) or $(GlyGlySer)_6GlyGly$ (L6) (SEQ ID NO. 7).

[0025] In some embodiments, the fusion protein is more resistant to proteolytic degradation than a corresponding wild type natriuretic protein. In some embodiments, the fusion protein displays a longer half-life than a corresponding wild type natriuretic protein. In some embodiments, the fusion protein is made by recombinant techniques, synthetic chemistry or semi-synthetic chemistry.

[0026] The present disclosure provides natriuretic fusion proteins that comprise any one of SEQ ID NOS. 8-11 (coded by SEQ ID NOS: 33-36, respectively). The present disclosure also provides natriuretic fusion proteins that comprise any one of SEQ ID NOS. 12-13.

[0027] The present disclosure also provides isolated polypeptides that exhibit at least 90% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

[0028] The present disclosure also provides isolated polypeptides that exhibit at least 90% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

[0029] The present disclosure also provides isolated polypeptides that exhibit at least 95% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

[0030] The present disclosure also provides isolated polypeptides that exhibit at least 95% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

[0031] The present disclosure also provides isolated polypeptides that exhibit at least 99% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

[0032] The present disclosure also provides isolated polypeptides that exhibit at least 99% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

[0033] The present disclosure provides pharmaceutical compositions comprising a natriuretic fusion protein as described herein. In some embodiments, the fusion protein is adapted for intravenous, subcutaneous or oral administration.

[0034] The present disclosure provides isolated nucleic acid molecules that encode a polypeptide selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11. The present disclosure also provides isolated nucleic acid

molecules that encode a polypeptide selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13. In some embodiments, the fusion protein is recombinantly produced by employing mammalian, prokaryotic, yeast, plant, or transgenic expression systems.

[0035] The present disclosure provides methods for treating or ameliorating a condition characterized by an excessive level of extracellular fluid by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0036] The present disclosure provides methods for treating or ameliorating a pathological condition in which activation of the NPRA receptor confers a therapeutic benefit by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0037] The present disclosure provides methods for treating or ameliorating a disease associated with abnormal diruretic, natriuretic and vasodilatory activity by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0038] The present disclosure provides methods for treating or ameliorating a disease in which it is desirable to induce naturesis, diuresis, vasodilation or to modulate the renin-angiotensin II and aldosterone systems by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0039] The present disclosure provides methods for treating or ameliorating a pathological condition of the cardiovascular system selected from the group consisting of chronic heart failure (non-ischemic), reperfusion injury, left ventricular dysfunction (LVD), cardiac fibrosis, diastolic heart failure, and hypertrophic cardiomyopathy by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0040] The present disclosure provides methods for treating or ameliorating a hypertensive disorder selected from the group consisting of hypertension, pulmonary hypertension, systolic hypertension and resistant hypertension by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

[0041] The present disclosure provides methods for treating or ameliorating diabetic nephropathy by administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition that comprises one or more of the natriuretic fusion peptides as described herein.

**A. Definitions**

[0042] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. Headers are used for the convenience of the reader and are also not intended to be limiting of the invention. All publications, patent applications, patents, and other references mentioned herein are incorporated herein by reference in their entirety, as are the package inserts of any branded drugs referred to herein by their brand names.

[0043] As used in the specification and the embodiments set forth herein, the following terms have the meanings indicated.

[0044] Singular forms "a", "an", and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0045] A "fusion protein", *e.g.* a "natriuretic fusion protein" as the term is used herein, refers to a protein having at least two polypeptides covalently linked, in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from another protein sequence or domain. Generally, the polypeptides of a fusion protein may be linked either directly or via a covalent linker. The term ("linker") refers to an amino acid linker, such as a polyglycine linker, or another type of chemical linker, *e.g.*, a glycine succinate linker, a carbohydrate linker, a lipid linker, a fatty acid linker, a polyether linker, etc. The linker may consist of at least 2, 4, 6, 9, 11, 16 or 20 amino acids in length. Alternatively, the linker may consist of at least 0, 1, 5, 7, 8, 10, 12, 13, 14, 15, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids, but may optionally be longer, *e.g.*, between 30 and 40 amino acids in length or between 40 and 50 amino acids in length. Amino acids are selected from the 20 naturally occurring amino acids, of either isomeric form $_{D}$ or $_{L}$, for example, glycine, alanine, proline, asparagine, glutamine, and lysine. A linker may be made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. The linker may comprise a range of lengths amino acid residues, such as for example, 6 to 11

amino acids in length, 11 to 16 amino acids in length, 16 to 20 amino acids in length, 16 to 25 amino acids in length, 20 to 30 amino acids in length, 30 to 35 amino acids in length, 35 to 40 amino acids in length, 40 to 45 amino acids in length or 45 to 50 amino acids in length. Some of these amino acids may be glycosylated. Non-peptide linkers are also possible. For example, alkyl linkers such as --NH--$(CH_2)_s$ --C(O)--, wherein s=2-20 could be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (*e.g.*, $C_1$ --$C_6$) lower acyl, halogen (*e.g.*, Cl, Br), CN, $NH_2$, phenyl, etc. An exemplary non-peptide linker is a PEG linker, wherein the linker has a molecular weight of 100 to 5000 kD, preferably 100 to 500 kD. The peptide linkers may be altered to form derivatives in the same manner as described above. As described in the examples herein, the preferred linker of the fusion proteins of the present invention is a stretch of amino acids with the basic repeat $(GGS)_x$ or $(GGS)_xGG$. For example, x may be an integer from 0 to 16. Although specific orientations are described in detail herein, polypeptides forming fusion proteins may be linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus and the polypeptides of the fusion protein can be in any order. It is also contemplated herein that the fusion proteins of the present invention may contain two peptide fusions. For example, the fusion protein may comprise one peptide flanked by two Fc domains, *e.g.*, Fc-Natriuretic Peptide-Fc, where the natriuretic peptide is conjugated to the Fc domains directly or through a linker. As contemplated herein, when a glycine succinate linker is used to link a natriuretic peptide and a Fc domain, the glycine residue of the linker is linked to the N- terminus of the Fc domain and the succinate moeity is linked to the C- terminus of the natriuretic peptide.

[0046] The term "fusion protein" also refers to conservatively modified variants, polymorphic variants, alleles, mutant, subsequences and interspecies homologues of the polypeptides that make up the fusion protein. Fusion proteins may be produced by covalently linking a chain of amino acids from one protein sequence to a chain of amino acids from another protein sequence, *e.g.*, by preparing a recombinant polynucleotide contiguously encoding the fusion protein or by synthetic means familiar to one of skill in the art.

[0047] The term "protein" is used herein interchangeably with "polypeptide" and "peptide."

[0048] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are

synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Such analogs are familiar to one of skill in the art and include, e.g, phosphorothioates and phosphoramidates. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. As used herein, the term "nucleic acid" may also be referred to as "gene", "cDNA", "mRNA", "oligonucleotide", and "polynucleotide".

[0049] As contemplated herein, a polynucleotide sequence comprising a fusion protein of the present invention hybridizes under stringent conditions to each of the nucleotide sequences encoding each individual polypeptide of the fusion protein. The polynucleotide sequences encoding the individual polypeptides of the fusion polypeptide therefore include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs.

[0050] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0051] The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 25% sequence identity. Alternatively, percent identity can be any integer from 25% to 100%. More preferred embodiments include at least: 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% or higher, compared to a reference sequence using the programs familiar to one of skill in the art; preferably BLAST using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. "Substantial identity" of amino acid sequences for these purposes normally means sequence identity of at least 40%.

Preferred percent identity of polypeptides can be any integer from 40% to 100%. More preferred embodiments include at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. Polypeptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

[0052] Optimal alignment of sequences for comparison may be conducted according to conventional methods, *e.g.*, by the local identity algorithm of Smith and Waterman (1981) Add. APL. Math. 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, or by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis.).

[0053] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) Nuc. Acids Res. 25:3389 3402 and Altschul et al. (1990) J. Mol. Biol. 215:403 410, respectively and are familiar to one of skill in the art.

[0054] Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other, or to a third nucleic acid, under moderately, and preferably highly, stringent conditions. Generally, hybridization conditions of high, moderate and low stringency are familiar to one of skill in the art. For example, stringent conditions are selected to be about 5 to 10°C below the temperature under defined ionic strength and pH at which 50% of the target sequence hybridizes to a perfectly matched probe ($T_m$); medium stringency is about 20-29°C below the $T_m$ and low stringency conditions are characterized by temperature that is about 40-48 °C below the $T_m$. An example of stringent hybridization conditions may be considered to be 50% formamide, 5 X SSC, and 1% SDS, incubating at 42°C, or 5 X SSC, 1% SDS, incubating at 65°C and washing in 0.2 X SSC, and 0.1% SDS at 65°. See, e.g, Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993); Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, 1987, Berger and Kimmel, eds.).

[0055] "Amino acid" is defined herein as any naturally occurring, artificial, or synthetic amino acid in either its $_L$ or $_D$ stereoisomeric forms, unless otherwise specified. The

term "residue" is used interchangeably with the term "amino acid," and is often designated as having a particular position in a given sequence of amino acids.

[0056] "Biologically active" refers to an agent having therapeutic or pharmacologic activity, such as an agonist, partial agonist or antagonist.

[0057] "Effective amount" as provided herein refers to a nontoxic but sufficient amount to provide the desired therapeutic effect. As will be pointed out below, the exact amount required will vary from subject to subject, depending on age, general condition of the subject, the severity of the condition being treated, the particular biologically active agent administered, and the like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art by reference to the pertinent texts and literature and/or by using routine experimentation.

[0058] As used herein, the term "Fc domain" refers to that part of an antibody derived from the stem of the "Y," and is composed of two heavy chains that each contribute two to three constant domains (depending on the class of the antibody). The Fc region binds to various cell receptors and complement proteins, and mediates different physiological effects of antibodies. As contemplated herein, the Fc domain of any antibody which displays minimal to no effector function may be used with the present invention. These include, but are not limited to, IgG1, IgG2, IgG4 but may also include any Fc domain of any antibody, the sequence of which has been altered, according to methods familiar to one of skill in the art, to possess minimal effector activity.

[0059] Alternatively, the term "Fc domain" may be described as an IgG heavy chain comprising hinge, CH2 and CH3 regions, wherein the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc domain at the first and second N-terminal cysteine residues.

[0060] The term "Fc" is also used to describe part of the fusion proteins. In this context, Fc is an IgG heavy chain comprising hinge, CH2 and CH3 domains, the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc at the first and second N-terminal cysteine residues. In other words, the N-terminal amino acid sequence of each chain of the Fc homodimer begins with CysProProCysPro (SEQ ID NO: 1) of the IgG hinge region and both Cys residues are disulfide bonded.

[0061] As used herein, a peptide is said to be "isolated" or "purified" when it is substantially free of cellular material or free of chemical precursors or other chemicals. The

fusion peptides of the present invention can be purified to homogeneity or other degrees of purity. The level of purification will be based on the intended use. The critical feature is that the preparation allows for the desired function of the peptide, even if in the presence of considerable amounts of other components.

[0062] "Natriuretic peptides" as referred to herein include the mammalian natriuretic factors (ANP, BNP, CNP), as well as Salmon cardiac peptide (Tervonen et al., Endocrinology 139(9):4021-4025 (1998)) and Dendroaspis natriuretic peptide (DNP) (Schweitz et al. J. Biol. Chem., 267(20):13928-13932 (1992)), urodilatins and peptides analogous thereto, and analogs, active fragments, degradation products, salts, variants, derivatives and combinations thereof. Specifically, human ANP and BNP include "hANP28" and "hBNP32" as disclosed in Kangawa et al., Biochem. Biophys. Res. Comm., 118(1):131-139 (1984), Sudoh,et al. Nature 332(6159):78-81 (1988), and Kambayashi et al. FEBS Lett., Jan 1; 259(2):341-5 (1990).

[0063] "Natriuretic peptides" include peptides that exhibit natriuretic activity, including, for example, peptides that are allelic variants, orthologs, splice variants, and/or species homologues of a natriuretic peptide. Procedures known in the art can be used to obtain full-length genes and cDNAs, allelic variants, splice variants, full-length coding portions, orthologs, and/or species homologues of genes and cDNAs corresponding to a nucleotide sequence coding for a natriuretic peptide. For example, allelic variants, orthologs and/or species homologues may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source for allelic variants and/or the desired homologue using any technique known to those skilled in the art.

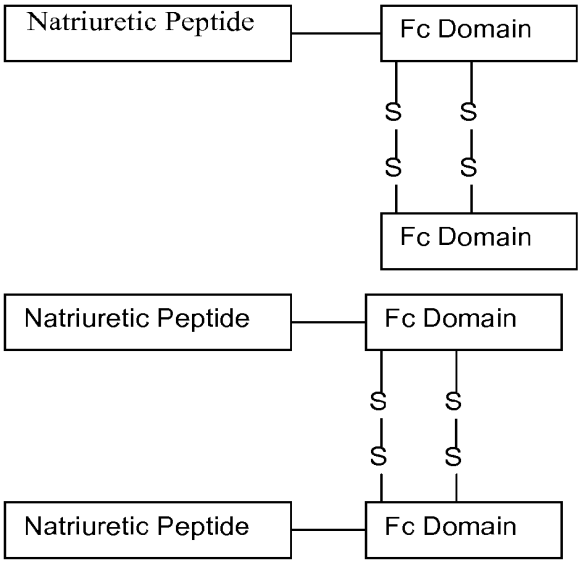
[0064] Without being limited by any particular mode of action, as referred to herein, the pharmacokinetics of a "sustained-release, or depot formulation" may be characterized as displaying an increase in bioavailability, due to FcRn binding and recycling of FcRn-bound molecules from within acidic lysosomes back to the general circulation (V. Ghetie and E.S. Ward, Annual Rev. Immunol., 18, 739-766, (2000)).

[0065] The term "semi-synthetic" as used herein refers to a process to synthesize the fusion proteins of the present invention comprising the use of both synthetic chemistry and recombinant techniques. For example, the Fc domain of the fusion proteins disclosed herein may be made recombinantly, while the natriuretic peptide and linker may be made synthetically.

**B. Peptide Molecules**

[0066] This invention relates to novel, biologically active fusion proteins comprised of one or more natriuretic peptides linked to an Fc region of IgG or other antibody for uses that include treatment or amelioration of pathological conditions in which activation of the NPRA receptor confers a therapeutic benefit on the patient, including but not limited to diseases associated with abnormal diruretic, natriuretic and vasodilatory activity. Fusion proteins according to the invention may be present in compositions that include pharmaceutically acceptable excipients, carriers or diluents.

[0067] The invention relates to fusion proteins as described herein that may have one of the following general formulas, A or B,




wherein,

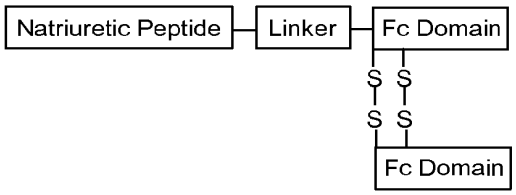
the natriuretic peptide (i) is selected from the group consisting of one or more ANP, BNP, urodilatin, DNP and a biologically active sequence variant thereof and (ii) may be in an orientation of N' to C' of the amino acid sequence, C' to N' of the amino acid sequence or in the case of more than one natriuretic peptide, N' to C', C' to N' or a mixture of N' to C' and C' to N'; and

the Fc domain is an IgG heavy chain comprising hinge, CH2 and CH3 regions, wherein the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc domain at the first and second N-terminal

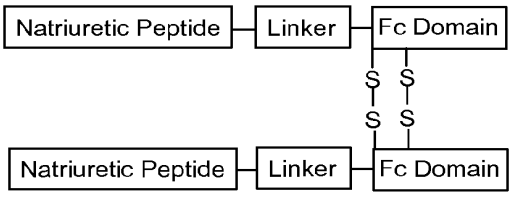
cysteine residues (*e.g.*, SEQ ID NO: 14 or SEQ ID NO: 17), and wherein the Fc domain is denoted by $Fc_{AB}$ or $Fc_{BA}$, wherein AB is an orientation of N' to C' of both Fc domains and BA is an orientation of C' to N' of both Fc domains.

[0068] As contemplated herein, in one aspect this invention embodies fusion proteins comprising at least one natriuretic peptide conjugated to the Fc domain of an antibody by way of a linker. The fusion protein may actually comprise one natriuretic peptide or two natriuretic peptides conjugated to an antibody Fc domain. As described in detail below, the sequence and length of the linker employed to conjugate the peptide with the Fc domain may vary depending on whether the fusion protein comprises one or two natriuretic peptides.

[0069] One aspect of the invention relates to a fusion protein having one of the following general formulas, 1 or 2,




1,




2,

wherein,

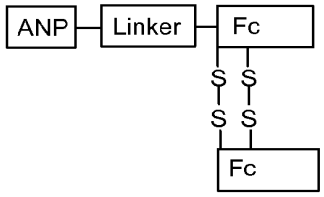
the natriuretic peptide (i) is selected from the group consisting of one or more ANP, BNP, urodilatin, DNP and a biologically active sequence variant thereof and (ii) may be in an orientation of N' to C' of the amino acid sequence, C' to N' of the amino acid sequence or in the case of more than one natriuretic peptide, N' to C', C' to N' or a mixture of N' to C' and C' to N';

the linker is one or more linkers selected from the group consisting of a succinate-glycine linker (L1), a GlyGly linker (L2), a $Gly(SerGlyGly)_2SerGly$ linker (L3) (SEQ ID NO. 2) a $(GlyGlySer)_yGlyGly$ linker, wherein y is 3 to 6 (*e.g.*, SEQ ID NOS. 3 (L4), 4 and 7 (L6))

$(GlyGlySer)_5Gly$ (L5) (SEQ ID NO. 6) and a $(GlySerGly)_5Gly$ linker (L5a) (SEQ ID NO: 5); and

the Fc domain is an IgG heavy chain comprising hinge, CH2 and CH3 regions, wherein the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc domain at the first and second N-terminal cysteine residues (*e.g.*, SEQ ID NO: 14 or SEQ ID NO: 17), and wherein the Fc domain is denoted by $Fc_{AB}$ or $Fc_{BA}$, wherein AB is an orientation of N' to C' of both Fc domains and BA is an orientation of C' to N' of both Fc domains.

[0070] In another aspect of this invention, the fusion protein has the following formula 3,




3,

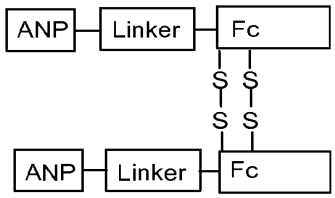
wherein,

ANP is in an orientation of N' to C' ($ANP_{XY}$) of the amino acid sequence of ANP (SEQ ID NO. 15) or in an orientation of C' to N' ($ANP_{YX}$) of the amino acid sequence of ANP;

the linker is one or more linkers selected from the group consisting of L1, L2, L3, L4, L5, L5a and L6, wherein L1 is a glycine succinate linker as described herein, L2 is a GlyGly linker, L3 is a $Gly(SerGlyGly)_2SerGly$ linker (SEQ ID NO. 2), L4 is a $(GlyGlySer)_3GlyGly$ linker (SEQ ID NO. 3), L5 is a $(GlyGlySer)_5Gly$ linker (SEQ ID NO. 6), L5a is a $(SerGlyGly)_5Gly$ (SEQ ID NO. 5) and L6 is a $(GlyGlySer)_6GlyGly$ linker (SEQ ID NO. 7); and

Fc is (i) an IgG heavy chain comprising hinge, CH2 and CH3 regions, wherein the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc at the first and second N-terminal cysteine residues (*e.g.*, SEQ ID NO: 14 and SEQ ID NO: 17), and (ii) is denoted by $Fc1_{AB}$, $Fc1_{BA}$, $Fc2_{AB}$ or $Fc2_{BA}$, wherein Fc1 is derived from an IgG1 molecule, Fc2 is derived from a IgG2 molecule, AB is an orientation of N' to C' of the Fc and BA is an orientation of C' to N' of the Fc.

[0071] In yet another aspect of this invention, the fusion protein has the following formula 4,




4,

wherein,

ANP is in an orientation of N' to C' ($ANP_{XY}$) of the amino acid sequence of ANP (SEQ ID NO. 15) or in an orientation of C' to N' ($ANP_{YX}$) of the amino acid sequence of ANP;

Linker is one or more linkers selected from the group consisting of L1, L2, L3, L4, L5, L5a, and L6 wherein L1 is a glycine succinate linker as described herein, L2 is a GlyGly linker, L3 is a $Gly(SerGlyGly)_2SerGly$ linker (SEQ ID NO. 2), L4 is a $(GlyGlySer)_3GlyGly$ linker (SEQ ID NO. 3), L5 is a $(GlyGlySer)_5Gly$ linker (SEQ ID NO. 6), L5a is a $(SerGlyGly)_5Gly$ (SEQ ID NO. 5) and L6 is a $(GlyGlySer)_6GlyGly$ linker (SEQ ID NO. 7); and

Fc is (i) an IgG heavy chain comprising hinge, CH2 and CH3 regions, wherein the IgG heavy chain begins at the first N-terminal cysteine residue within the hinge region and forms a homodimer with another Fc at the first and second N-terminal cysteine residues (*e.g.*, SEQ ID NO: 14 or SEQ ID NO: 17), and (ii) is denoted by $Fc1_{AB}$, $Fc1_{BA}$, $Fc2_{AB}$ or $Fc2_{BA}$, wherein Fc1 is derived from an IgG1 molecule, Fc2 is derived from a IgG2 molecule, AB is an orientation of N' to C' of the Fc and BA is an orientation of C' to N' of the Fc.

[0072] The fusion proteins of the present invention are biologically active molecules, *e.g.*, they are able to catalyze cGMP, but are more useful for therapeutic purposes as they possess much longer half-lives and are also less susceptible to proteolytic degradation. In addition, by exploiting FcRn mediated transport, the therapeutic fusion proteins disclosed herein may be administered by bolus injection but may display pharmacokinetic properties resembling that of a slow-release depot formulation.

[0073] The fusion proteins of the present invention are natriuretic peptides that are conjugated to a Fc region of an antibody, such as IgG, directly or through a linker. By conjugating the peptide to the Fc region of an antibody, these fusion proteins exhibit much longer half-lives than the unconjugated peptides. Without being limited by any particular

mode of action, the fusion proteins of the present invention may be pinocytosed and sequestered upon binding of the Fc region to the neonatal constant region fragment receptor (FcRn) and by exploiting the FcRn active carrier system, (the FcRn pathway transports maternal antibodies (IgG) across the intestinal epithelium of a newborn animal), levels of the fusion proteins disclosed herein can be protected from intracellular lysozomal degredation as well as have reduced exposure to neutral endopeptidase (NEP) or the NPR clearance receptor. The fusion protein may be recycled and represented to the circulation upon normal release from the cell. In this way, activation of the NPRA receptor all at once, such as typical after a bolus dose of ligand, may be avoided. The bioavailability of the fusion proteins of the present invention may more closely resemble a slow-release depot preparation.

[0074] The FcRn receptor is expressed on the surface of endothelial cells in several different types of tissue in adult humans, including lung, kidney and intestine. Without being limited by any particular mode of action, the normal function of the FcRn receptor may be exploited as a means to administer bioactive ANP-Fc and BNP-Fc fusion proteins in a human for a myriad of clinical uses. For example, in addition to methods to treat or ameliorate pathological conditions of the cardiovascular system, the fusion proteins of the present invention may be used in methods to treat diseases associated with abnormal diuretic, natriuretic and vasodilatory activity in which activation of the NPRA receptor confers a therapeutic benefit on the patient.

[0075] The fusion proteins of the present invention may comprise any natriuretic peptide, but are preferably ANP or BNP. Human versions of these proteins are known as, *e.g.*, hANP28 (Kangawa et al. Biochem. Biophys. Res. Comm. 118(1):131-139 (1984) of SEQ ID NO. 15 (NCBI database accession number NM_006172) and hBNP32 (Kambayashi et al. FEBS Lett 1990 Jan 1; 259(2):341-5) of SEQ ID NO. 16 (NCBI database accession number NM_002521). In addition to various structural and sequence forms of the wild type natriuretic peptides, it is understood that the present invention contemplates any and all possible biologically active sequence variants, whether naturally occurring or synthesized by design. It is further understood that peptides from any species are included within the scope of the present invention, although human peptides are preferred. In addition, the fusion proteins of the present invention may comprise any possible combination between human and non-human polypeptides, *e.g.*, from animals such as, rat, mouse, guinea pig, horse, cow, pig, sheep, dog, etc.

[0076] It is also contemplated that in addition to sequence variants, fragments of said peptides are included within the scope of the invention disclosed herein, where such fragments are of sufficient size to be therapeutically effective in the methods of the present invention. One of skill in the art can determine alterations in peptide length and sequence variety that does not impact biological activity and/or therapeutic effectiveness without undue experimentation. The proteins may be in the form of acidic or basic salts, or may be in a neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

[0077] Other variants within the scope of the invention include fusion proteins in which the primary amino acid structure is modified by forming covalent or aggregative conjugates with other peptides or polypeptides, or chemical moieties such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives may be prepared, for example, by linking particular functional groups to amino acid side chains or at the N- or C-terminus.

[0078] The fusion proteins of the present invention may or may not be glycosylated. Fusion proteins expressed in yeast or mammalian expression systems may be similar to, or slightly different in molecular weight and glycosylation pattern from the native molecules, depending upon the expression system; expression of DNA encoding polypeptides in bacteria such as *E. coli* provides non-glycosylated molecules.

[0079] In certain embodiments, dimer constructs of the present invention have been found to possess increased *in vitro* potency when compared to monomer constructs. The increased potency of dimer constructs of natriuretic peptides linked to antibody Fc domains as described herein is particularly surprising in view of the monomeric interaction between the natriuretic peptide ligands (*e.g.*, ANP, BNP, etc.) and their cell surface receptors. It would be expected that dimeric constructs as described herein would be sterically hindered from interacting with the cells and/or receptors and thus, little and/or no activity of such dimeric constructs would be predicted.

[0080] In certain embodiments, monomer constructs of the present invention have been found to possess increased *in vivo* serum concentrations (Cmax) when compared to dimer constructs. The increased Cmax of monomer constructs of natriuretic peptides linked to antibody Fc domains as described herein is surprising in view of the previous results from intravenous administration of monomeric EPO-Fc constructs that showed a lower Cmax as compared to intravenous administration of dimeric EPO-Fc constructs (see, *e.g.*, Table 4 of U.S. Patent Application Publication No. 2007/0172928).

[0081] The Fc domain conjugated to the natriuretic peptide(s) is preferably the Fc domain of IgG, including, but not limited to, IgG1, IgG2 or IgG4 (see, *e.g.*, SEQ ID NO: 14 or SEQ ID NO: 17). However, Fc domains of other antibodies may be used if modified to possess minimal or no effector function. Human antibody Fc domains are preferred, but other species types, wild-type forms as well as sequence variants, may be used, e.g, a recombinant Fc molecule is described in the Examples provided herein. In one aspect of this invention, the Fc domain is made up of two Fc heavy chains from IgG1 or IgG2 isotypes with the hinge residues removed down to the CPPCP sequence on each chain to allow for interchain disulfide bonding of the cysteine residues. Ideally, the natriuretic fusion proteins of the present invention comprise an Fc domain that is able to bind to the FcRn receptor, trigger the active carrier function of this receptor and cause delivery of the fusion protein into the cell. Once inside the cell, pH changes result in the release of the fusion protein from the FcRn receptor, and the fusion protein may ultimately be released from the cell back into the circulation. (Roopenian D.C. et. al. (2003) J. Immunology 170:3528-3533; Lencer, W.I. et al, Trends in Cell Biology 15(1):5 – 9 (2005)).

[0082] As shown herein, the length and sequence of the amino acid linker used to conjugate the natriuretic peptide with the Fc domain vary. As described in the Examples provided below, structural modeling of NPRA with ANP bound in relation to an Fc domain was used to predict the minimum linker distance required to allow insertion of the Fc-fused ANP into the NPRA active site. As contemplated herein, desirable linker length is one that minimizes steric and electrostatic repulsions between the natriuretic peptide and the Fc domain. For example, the desired minimal distance from the C-terminus of ANP is 12 Å from the closest N-terminus of the Fc homodimer and 17 Å from the other N-terminus of the Fc homodimer. Furthermore, if the Fc homodimer has only one ANP fused, (*e.g.*, monomer) a 4 to 6 amino acid minimum linker length would be preferred. With two ANP peptides bound to the Fc homodimer (*e.g.*, dimer), and with only one ANP bound to NPRA receptor (*i.e.*, in a 1:1 Fc dimer:NPRA ratio) then a minimum linker length of 9 amino acids for each linker is preferred. For both ANPs to bind to NPRA (*e.g.*, to two adjacent receptors or to 1 NPRA receptor in nonstatic, alternating fashion) then linkers with a minimum length of 12 amino acids would be preferred. Surprisingly, it has been discovered that increasing linker length enabled the fusion proteins to approach the potency of the fused natriuretic peptide. For example, it was found that longer linker lengths increased potency of the natriuretic

peptide (as measured by ability to induce cGMP in vitro), particularly those fusion proteins having a linker length of 20 amino acids.

[0083] Unexpectedly, it has been discovered that increasing linker length enabled the fusion proteins to approach the potency of the natriuretic peptide. Those fusion proteins having a linker length longer than predicted showed the most improved potency, particularly those fusion proteins having a linker length of, *e.g.*, 20 amino acids (see, *e.g.*, Examples 3 and 6). In fact, the potency of the fusion proteins with linker lengths longer than predicted by modeling approached the potency of native ANP. In embodiments of this invention, the potency of the fusion protein is improved at a linker length of between 16 and 50 amino acids, preferably between 16 and 40 amino acids and most preferably between 16 and 30 amino acids. Additionally, it would have been expected that increasing linker length would increase the fusion protein's susceptibility to proteolytic degradation by NEP. In contrast, experiments unexpectedly demonstrate that longer linker lengths are not affected by NEP (see, *e.g.*, Example 5).

[0084] Linker sequences employed in the present invention comprising (GGS)x repeats (*e.g.*, where x is an integer from 0 to 16), may be made according to conventional synthetic, semi-synthetic, or recombinant methods (see, *e.g.*, Evers T.H. et. al. (2006) Biochemistry, 45:13183-13192). With regard to the actual amino acid sequence of the linkers employed, typically glycines and serines are preferred, as the presence of glycines in the linker provide flexibility and serines provide solubility. A preferred linker sequence is made up of a series of repeats of these amino acids, *e.g.*, (GGS)x-GG, for example, where x is an integer from 0 to 16, such as GGSGGSGGSGG (SEQ ID NO. 3) or GGSGGSGGSGGSGGSGGSGG (SEQ ID NO. 7).

[0085] As mentioned above, the orientation of conjugation of Fc domain and natriuretic peptide may vary. For example, the carboxy terminus of the peptide may be linked to the amino terminus of the Fc domain by a normal peptide bond (see Table 2, may also be referred to as Orientation #1). Alternatively, the amino terminus of the peptide may be linked to the amino terminus of the Fc domain (see Table 2, may also be referred to as Orientation #2). In the latter case, the chemistry leaves a succinate moiety in place of one amino acid of the fusion. As such, one of skill in the art will recognize that fusion proteins of the latter case may not be made recombinantly as normal peptide bonding does not take place between two amino termini. Data gathered indicate that orientation does not seem to effect the potency of a given fusion protein (see, *e.g.*, Example 2).

[0086] ANP-Fc fusion constructs of the present invention may comprise one or more ANP, one or more linkers and one or more Fc. The following ANP-Fc fusion constructs are contemplated by the present invention.

[0087] An exemplary fusion construct (Construct 2), which comprises $ANP_{XY}$ (Construct 1) (SEQ ID NO: 15), is represented by:

$$ANP_{XY}\text{-}L4\text{-}Fc1_{AB}$$

The construct comprises $ANP_{XY}$-L4-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L4 is SEQ ID NO: 3 and $Fc1_{AB}$ is SEQ ID NO: 14. $ANP_{XY}$-L4-$Fc1_{AB}$ is represented by SEQ ID NO: 8. When the $ANP_{XY}$-L4-$Fc1_{AB}$ construct is made recombinantly, a homodimer may be produced, for example, the $ANP_{XY}$-L4-$Fc1_{AB}$ may be linked to a second $ANP_{XY}$-L4-$Fc1_{AB}$ construct via a disulfide linkage.

[0088] An exemplary fusion construct (Construct 3) is represented by:

$$ANP_{XY}\text{-}L6\text{-}Fc1_{AB}$$

The construct comprises $ANP_{XY}$-L6-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L6 is SEQ ID NO: 7 and $Fc1_{AB}$ is SEQ ID NO: 14. $ANP_{XY}$-L6-$Fc1_{AB}$ is represented by SEQ ID NO: 9. When the $ANP_{XY}$-L6-$Fc1_{AB}$ construct is made recombinantly, a homodimer may be produced, for example, the $ANP_{XY}$-L6-$Fc1_{AB}$ may be linked to a second $ANP_{XY}$-L6-$Fc1_{AB}$ construct via a disulfide linkage.

[0089] An exemplary fusion construct (Construct 4) is represented by:

$$ANP_{XY}\text{-}L4\text{-}Fc2_{AB}$$

The construct comprises $ANP_{XY}$-L4-$Fc2_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L4 is SEQ ID NO: 3 and $Fc2_{AB}$ is SEQ ID NO: 17. $ANP_{XY}$-L4-$Fc2_{AB}$ is represented by SEQ ID NO: 10. When the $ANP_{XY}$-L4-$Fc2_{AB}$ construct is made recombinantly, a homodimer may be produced, for example, the $ANP_{XY}$-L4-$Fc2_{AB}$ may be linked to a second $ANP_{XY}$-L4-$Fc2_{AB}$ construct via a disulfide linkage.

[0090] An exemplary fusion construct (Construct 5) is represented by:

$$ANP_{XY}\text{-}L6\text{-}Fc2_{AB}$$

The construct comprises $ANP_{XY}$-L6-$Fc2_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L6 is SEQ ID NO: 7 and $Fc2_{AB}$ is SEQ ID NO: 17. $ANP_{XY}$-L6-$Fc2_{AB}$ is represented by SEQ ID NO: 11.

When the $ANP_{XY}$-L6-$Fc2_{AB}$ construct is made recombinantly, a homodimer may be produced, for example, the $ANP_{XY}$-L6-$Fc2_{AB}$ may be linked to a second $ANP_{XY}$-L6-$Fc2_{AB}$ construct via a disulfide linkage.

[0091] An exemplary fusion construct (Construct 6) is represented by:

$$\begin{array}{c} ANP_{YX}\text{-}L1\text{-}Fc1_{AB} \\ \vdots \\ Fc1_{AB} \end{array}$$

The construct comprises $ANP_{YX}$-L1-$Fc1_{AB}$, wherein $ANP_{YX}$ is SEQ ID NO: 15 inverted in orientation from its C' to N' terminus, L1 is Succinate-Gly and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{YX}$-L1-$Fc1_{AB}$ may be linked to a second $Fc1_{AB}$ via a disulfide linkage.

[0092] An exemplary fusion construct (Construct 7) is represented by:

$$\begin{array}{c} ANP_{YX}\text{-}L1\text{-}Fc1_{AB} \\ \vdots \\ ANP_{YX}\text{-}L1\text{-}Fc1_{AB} \end{array}$$

The construct comprises $ANP_{YX}$-L1-$Fc1_{AB}$, wherein $ANP_{YX}$ is SEQ ID NO: 15 inverted in orientation from its C' to N' terminus, L1 is Succinate-Gly and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{YX}$-L1-$Fc1_{AB}$ may be linked to a second $ANP_{YX}$-L1-$Fc1_{AB}$ construct via a disulfide linkage.

[0093] An exemplary fusion construct (Construct 8) is represented by:

$$\begin{array}{c} ANP_{XY}\text{-}L2\text{-}Fc1_{AB} \\ \vdots \\ Fc1_{AB} \end{array}$$

The construct comprises $ANP_{XY}$-L2-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L2 is GlyGly and $Fc1_{AB}$ is SEQ ID NO: 14. $ANP_{XY}$-L2-$Fc1_{AB}$ is represented by SEQ ID NO: 13. The $ANP_{XY}$-L2-$Fc1_{AB}$ may be linked to a second $Fc1_{AB}$ via a disulfide linkage.

[0094] An exemplary fusion construct (Construct 9) is represented by:

$$\begin{array}{c} ANP_{XY}\text{-}L2\text{-}Fc1_{AB} \\ \vdots \\ ANP_{XY}\text{-}L2\text{-}Fc1_{AB} \end{array}$$

The construct comprises $ANP_{XY}$-L2-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L2 is GlyGly and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{XY}$-L2-$Fc1_{AB}$ may be linked to a second $ANP_{XY}$-L2-$Fc1_{AB}$ construct via a disulfide linkage.

[0095] An exemplary fusion construct (Construct 10) is represented by:

$$\begin{array}{c} ANP_{XY}\text{-L4-}Fc1_{AB} \\ \vdots \\ Fc1_{AB} \end{array}$$

The construct comprises $ANP_{XY}$-L4-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L4 is SEQ ID NO: 3 and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{XY}$-L4-$Fc1_{AB}$ is represented by SEQ ID NO: 13. The $ANP_{XY}$-L4-$Fc1_{AB}$ may be linked to a second $Fc1_{AB}$ via a disulfide linkage.

[0096] An exemplary fusion construct (Construct 11) is represented by:

$$\begin{array}{c} ANP_{XY}\text{-L4-}Fc1_{AB} \\ \vdots \\ ANP_{XY}\text{-L4-}Fc1_{AB} \end{array}$$

The construct comprises $ANP_{XY}$-L4-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L4 is SEQ ID NO: 3 and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{XY}$-L4-$Fc1_{AB}$ may be linked to a second $ANP_{XY}$-L4-$Fc1_{AB}$ construct via a disulfide linkage.

[0097] An exemplary fusion construct (Construct 12) is represented by:

$$\begin{array}{c} ANP_{YX}\text{-L3-L1-}Fc1_{AB} \\ \vdots \\ Fc1_{AB} \end{array}$$

The construct comprises $ANP_{YX}$-L3-L1-$Fc1_{AB}$, wherein $ANP_{YX}$ is SEQ ID NO: 15 inverted in orientation from its C' to N' terminus, L3 is SEQ ID NO: 2, L1 is Succinate-Gly and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{YX}$-L3-L1-$Fc1_{AB}$ may be linked to a second $Fc1_{AB}$ via a disulfide linkage.

[0098] An exemplary fusion construct (Construct 13) is represented by:

$$\begin{array}{c} ANP_{YX}\text{-L3-L1-}Fc1_{AB} \\ \vdots \\ ANP_{YX}\text{-L3-L1-}Fc1_{AB} \end{array}$$

The construct comprises $ANP_{YX}$-L3-L1-$Fc1_{AB}$, wherein $ANP_{YX}$ is SEQ ID NO: 15 inverted in orientation from its C' to N' terminus, L3 is SEQ ID NO: 2, L1 is Succinate-Gly and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{YX}$-L3-L1-$Fc1_{AB}$ may be linked to a second $ANP_{YX}$-L3-L1-$Fc1_{AB}$ construct through a disulfide linkage.

[0099] An exemplary fusion construct (Construct 14) is represented by:

$$\begin{matrix} ANP_{XY}\text{-L5a-}Fc1_{AB} \\ \vdots \\ ANP_{XY}\text{-L5a-}Fc1_{AB} \end{matrix}$$

The construct comprises $ANP_{XY}$-L5a-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L5a is SEQ ID NO: 5 and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{XY}$-L5a-$Fc1_{AB}$ may be linked to a second $ANP_{XY}$-L5a-$Fc1_{AB}$ construct through a disulfide linkage.

[00100] An exemplary fusion construct (Construct 15) is represented by:

$$\begin{matrix} ANP_{XY}\text{-L5a-}Fc1_{AB} \\ \vdots \\ Fc1_{AB} \end{matrix}$$

The construct comprises $ANP_{XY}$-L5a-$Fc1_{AB}$, wherein $ANP_{XY}$ is SEQ ID NO: 15, L5a is SEQ ID NO: 5 and $Fc1_{AB}$ is SEQ ID NO: 14. The $ANP_{XY}$-L5a-$Fc1_{AB}$ may be linked to a second $Fc1_{AB}$ via a disulfide linkage.

**C. Synthesis of the fusion proteins**

[00101] Unless otherwise indicated herein, the fusion proteins of the present invention may be made by any of a number of techniques of protein chemistry or molecular biology familiar to one of skill in the art. (See, *e.g.*, Dawson et al., Ann. Rev. Biochem., 69:923-960, 2000.) Possible synthesis scenarios are described in the Examples provided herein and include synthetic and semi-synthetic chemical synthesis as well as recombinant methods.

[00102] Fusion proteins may be produced using chemical methods in whole or in part and using classical or nonclassical amino acids or chemical amino acid analogs as appropriate. Techniques include solid phase chemistry (Merrifield, J. Am. Chem. Soc., 85:2149,1964; Houghten, Proc. Natl. Acal. Sci. USA 82:5132, 1985) and equipment for such automated synthesis of polypeptides is commercially available (*e.g.*, Applied Biosystems, Foster City, Calif.). Synthesized peptides can be purified using conventional methods such as high performance liquid chromatography. The composition of the synthetic fusion polypeptides may be confirmed by amino acid analysis or sequencing using techniques

familiar to one of skill in the art. Further treatment of a synthesized protein under oxidizing conditions may also be utilized to obtain the proper native conformation. See, *e.g.* Kelley, R. F. & Winkler, M. E. in Genetic Engineering Principles and Methods, Setlow, J. K., ed., Plenum Press, N.Y., vol. 12, pp 1-19, 1990; Stewart, J. M. & Young, J. D. Solid Phase Peptide Synthesis Pierce Chemical Co., Rockford, Ill.,1984).

[00103] The fusion proteins disclosed herein may also be made by recombinant techniques involving gene synthesis, cloning and expression methodologies. These techniques are well known and are explained in, for example, Current Protocols in Molecular Biology, Volumes I, II, and III, 1997 (F. M. Ausubel ed.); Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; DNA Cloning: A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.); A Practical Guide to Molecular Cloning; the series, Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); and Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

[00104] Briefly, the fusion proteins of the present invention may be made recombinantly by isolating or synthesizing nucleic acid sequences encoding any of the amino acid sequences described herein by conventional cloning or chemical synthesis methods. For example, DNA fragments coding for the different fusion protein sequences may be ligated together in-frame in accordance with conventional techniques or synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence. The recombinant nucleic acids can further comprise other nucleotide sequences such as sequences that encode affinity tags to facilitate protein purification protocol.

[00105] The nucleic acid sequence encoding a fusion protein of the present invention may be ligated into a suitable expression vector capable of expressing the nucleic acid sequence in a suitable host, followed by transforming the host with the expression vector into which the nucleic acid sequence has been ligated, culturing the host under conditions suitable for expression of the nucleic acid sequence, whereby the protein encoded by the selected nucleic acid sequence is expressed by the host and purifying the protein produced. In this process, the ligating step may further contemplate ligating the nucleic acid into a suitable

expression vector such that the nucleic acid is operably linked to a suitable secretory signal, whereby the amino acid sequence is secreted by the host. Suitable secretory signals for use with the present invention include but are not limited to, the mouse IgG kappa light chain signal sequence (Ho et. al. PNAS (2006) 103(25): 9637-9642).

[00106] As described above, a nucleic acid sequence encoding a fusion protein described herein may be inserted into an appropriate plasmid or expression vector that may be used to transform a host cell. In general, plasmid vectors containing replication and control sequences that are derived from species compatible with the host cell are used in connection with those hosts. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. For example, E. coli may be transformed using pBR322, a plasmid derived from an E. coli species (Mandel, M. et al., J. Mol. Biol. 53:154,1970). Plasmid pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides easy means for selection. Other vectors include different features such as different promoters, which are often important in expression. The vectors used for mammalian expression often contain the constitutive CMV promoter that leads to high recombinant protein expression. These vectors also contain selection sequence genes that are used for the generation of stable expressing cell lines.

[00107] Host cells may be prokaryotic or eukaryotic. Prokaryotes are preferred for cloning and expressing DNA sequences to produce parent polypeptides, segment substituted polypeptides, residue-substituted polypeptides and polypeptide variants. Such prokaryotic cells familiar to one skilled in the art include, but are not limited to, *E. coli, B subtillus,* and *P. aeruginosa* cell strains. In addition to prokaryotes, eukaryotic organisms, such as yeast cultures, or cells derived from multicellular organisms may be used. Vertebrate cells may also be used as useful host cell lines. Useful cells and cell lines are familiar to one of skill in the art and include, but are not limited to, HEK293 cells, HeLa cells, Chinese Hamster Ovary (CHO) cell lines, W138, 293, BHK, COS-7 and MDCK cell lines.

[00108] The invention also relates to isolated or purified polynucleotides that encode the natriuretic fusion proteins of the present invention. As discussed above, the polynucleotides of the invention which encode a fusion protein, fragments thereof, or functional equivalents thereof may be used to generate recombinant nucleic acid molecules that direct the expression of the fusion protein, fragments thereof, or functional equivalents

thereof, in appropriate host cells. The fusion polypeptide products encoded by such polynucleotides may be altered by molecular manipulation of the coding sequence.

[00109] Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the expression of the fusion polypeptides. Such DNA sequences include those which are capable of hybridizing to the coding sequences or their complements disclosed herein under low, moderate or high stringency conditions described herein.

[00110] Altered nucleotide sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues, which result in a silent change.

[00111] The nucleotide sequences of the invention may be engineered in order to alter the fusion protein coding sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, *e.g.*, to insert or delete restriction sites, to alter glycosylation patterns, phosphorylation, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions, to facilitate further in vitro modification, etc. One of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include, *e.g.*, site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to chemical mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques.

[00112] Purified fusion proteins may be prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts. For example, supernatants from systems which secrete recombinant polypeptide into culture media may be first concentrated using a commercially available protein concentration filter, such as, *e.g.*, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. Affinity chromatography or

reverse-phase high performance liquid chromatography (RP-HPLC) may also be used to purify the fusion proteins of the present invention.

[00113] Monoclonal or polyclonal antibodies to a purified fusion protein of the present invention may be produced according to conventional methods. For example, described herein are methods for the production of antibodies capable of specifically recognizing one or more differentially expressed gene epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

[00114] For the production of antibodies to the fusion proteins discussed herein, various host animals may be immunized by injection with the polypeptides, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice, and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

[00115] Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as target gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with the polypeptides, or a portion thereof, supplemented with adjuvants as also described above.

[00116] Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this

invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

[00117] In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable or hypervariable region derived from a murine mAb and a human immunoglobulin constant region.

[00118] Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce differentially expressed gene-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

[00119] Most preferably, techniques useful for the production of "humanized antibodies" can be adapted to produce antibodies to the polypeptides, fragments, derivatives, and functional equivalents disclosed herein. Such techniques are disclosed in U.S. Patent Nos. 5,932, 448; 5,693,762; 5,693,761; 5,585,089; 5,530,101; 5,910,771; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,545,580; 5,661,016; and 5,770,429, the disclosures of all of which are incorporated by reference herein in their entirety.

[00120] Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

**D. Pharmaceutical Compositions**

[00121] The natriuretic fusion proteins of the present invention can be administered as pharmaceutical compositions for use in methods to treat or ameliorate pathological conditions

in which activation of the NPRA receptor confers a therapeutic benefit on the patient, including but not limitcd to discascs associatcd with abnormal dirurctic, natriurctic and vasodilatory activity and/or in which it is desirable to induce naturesis, diuresis, vasodilation or to modulate the renin-angiotensis II and aldosterone systems. Such pathological conditions include disorders of the cardiovascular system such as described in detail above. These conditions include those that may be characterized by an excess in extracellular fluid, including but not limited to Chronic Heart Failure (CHF) and pulmonary edema. In a particularly preferred embodiment, the invention includes methods to treat or ameliorate pathological conditions of the cardiovascular system including but not limited to, chronic heart failure (non-ischemic), post-MI heart failure (ischemic CHF), acute MI, reperfusion injury, left ventricular dysfunction (LVD), cardiac fibrosis, diastolic heart failure, and hypertrophic cardiomyopathy. In addition, hypertensive disorders including, but not limited to hypertension, *e.g.*, pulmonary hypertension, systolic hypertension, resistant hypertension and other cardiovascular related diseases such as diabetic nephropathy may be treated or ameliorated by the methods of the present invention. It is also contemplated herein that the fusion proteins and pharmaceutical compositions of the present invention may be provide therapeutic benefit for patients undergoing coronary artery bypass graft procedures.

[00122] Pharmaceutical compositions for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients for administration by various means, for example, by inhalation or insufflation (either through the mouth or the nose) or topical, oral, buccal, parenteral or rectal administration. For example, parenteral may include, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal and epidural administration. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.* oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In a preferred embodiment, it may be desirable to introduce the pharmaceutical compositions of the invention into the affected tissues by any suitable route. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[00123] The pharmaceutical compositions may further comprise a vehicle or carrier, including a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a

state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin, which is herein incorporated by reference in its entirety. The formulation should suit the mode of administration.

[00124] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats);

emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

[00125] Where appropriate, preparations for oral administration may be suitably formulated to give controlled release of the active compound.

[00126] Where appropriate, for buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

[00127] In another embodiment, the fusion proteins of the present invention are for administration by inhalation or insufflation (either through the mouth or the nose). As such, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[00128] In a particularly preferred embodiment, the pharmaceutical compositions of the present invention may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. In addition, as contemplated herein, the fusion proteins and pharmaceutical compositions of the present invention may be suitable for self-injection by a patient in need thereof, *e.g.* long term treatment of CHF. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[00129] Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use. For example, lyophilized protein compositions may be inhaled or reconstituted then injected in a suitable vehicle.

[00130] The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[00131] In addition to the formulations described previously, which may exhibit pharmacokinctics similar to a slow rclcasc formulation, thc compounds may also bc formulated as an actual depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[00132] The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[00133] Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art. For example, for any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, *e.g.*, of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the $IC_{50}$ (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms). Such information can then be used to determine useful doses and routes for administration in humans.

[00134] A therapeutically effective dose or "effective amount" refers to that amount of active ingredient that is nontoxic but sufficient to provide the desired therapeutic effect. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no

toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

[00135] The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

[00136] Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc. Pharmaceutical formulations suitable for oral administration of proteins are described, *e.g.*, in U.S. Patents 5,008,114; 5,505,962; 5,641,515; 5,681,811; 5,700,486; 5,766,633; 5,792,451; 5,853,748; 5,972,387; 5,976,569; and 6,051,561.

[00137] The present invention further provides kits for use with any of the above methods. Such kits typically comprise two or more components necessary for performing a method described herein. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a pharmaceutical composition comprising fusion proteins of the present invention. One or more additional containers may enclose elements, such as reagents or buffers, or equipment to be used in a method to administer the pharmaceutical composition.

[00138] It is also contemplated herein that the fusion proteins and pharmaceutical compositions of the present invention may be administered alone or in combination with other compounds or substances that may be used to treat any of the pathological conditions described herein. Such compounds or formulations that may be used in combination therapy with the present invention include, for example, diuretics, beta blockers, and Ang II receptor blockers.

[00139] It is contemplated that the invention described herein is not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention in any way.

[00140] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices and materials are now described. All references described herein are expressly incorporated by reference.

## EXAMPLES

[00141] The materials and methods relevant to the examples described herein are provided immediately below:

*Structural Modeling of the ANP-Fc Fusion/NPRA Interactions:*

[00142] Monomer Fc Fusion (1 ANP linked, 1 ANP bound): The linker requirements for the ANP and Fc fusions were determined through structure-based modeling using the NPRA and Fc crystal structures (RCSB PDB database 3D crystal structure numbers, 1T34 (co-structure with ANP) and 1HZH, respectively, H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne: The Protein Data Bank. Nucleic Acids Research, 28 pp. 235-242 (2000)). These structures are positioned in space (a) with the C-terminus of ANP and the N-terminus of Fc proximal to one another, yet with enough space between them to minimize steric and electrostatic repulsions, and (b) distal to the two F425 residues (F457 in human full-length NPRA sequence) of the dimeric NPRA to minimize possible interactions with the residues that lead toward NPRA's transmembrane region, which is predicted to start with V474. Once the position of the two structures was finalized, the distance between the C-terminus of ANP and the N-terminus of Fc was measured; this value was divided by 3.5 and rounded up to the nearest whole number to give the minimum number of amino acids (aa) predicted to be needed for the "Fc-linker-hANP28" or "hANP28-linker-Fc" (*i.e.*, Orientation #1 or Orientation #2, respectively, *supra* pp. 19) fusion to be a viable ligand of NPRA (example: distance = 12 Å. Minimum linker length = 12 / 3.5 = 3.4 => 4 aa linker). The 3.5 value represents the average amino acid span in β-sheets (the distance from a nitrogen atom in one amino acid to the nitrogen atom in the adjacent amino acid).

[00143] Dimer Fc Fusion (2 ANP linked, 1 ANP bound): The procedure outlined for the Monomer Fc Fusion was followed with the added challenge of growing a second linker from the second N-terminus on the Fc toward one ANP-NPRA trimeric complex and one ANP peptide in its binding conformation as defined in 1T34, RCSB PDB Database 3D Structure (H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne: The Protein Data Bank. Nucleic Acids Research, 28 pp. 235-242 (2000)).

[00144] Dimer Fc Fusion (2 ANP linked, 2 ANP bound): The procedure outlined for the Monomer Fc Fusion was followed with the added challenge of growing a second linker from the second N-terminus on the Fc toward two ANP-NPRA trimeric complexes.

*Cloning, Expression and Purification of CysFc:*

[00145] The vector pEE12,4 was licensed from Lonza group (Basel, Switzerland). The human Fc sequence from lgG1 was obtained from a human leukocyte cDNA library with the mouse $lg_K$ signal sequence (in bold below) using standard PCR techniques (pSYNFc-001, pSYNFc-007). The cysFc sequence with the mouse $lg_K$ signal sequence was obtained from pSYNFc-007 (pSYNcysFc-002). The CysFc sequence was then cloned into pEE12.4 through an intermediate cloning step (pSYNcysFc-003) to create pSYNcysFc-004. CysFc-004 has the following amino acid sequence:

**METDTLLLWVLLLWVPGSTG**CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCVKGFYPSD IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNFSCSVMHEALH NHYTQKSLSLSPGK (SEQ ID NO: 18).

[00146] Lonza CHOK1Sv suspension cells ($1x10^7$ cells) were transfected with 40 μg of PstI linearized pSYNcysFc-004 by electroporation according to the manufacturer's instructions. Cells were plated in 40 x 96 well plates at a seeding density of 2500 cells/well. Twenty four hours later selection was initiated with 25 μM MSZ and cells were incubated at 37°C/5% $CO_2$ for three weeks. Transfected cells were expanded from 96 well plates to 24 well plates to T25 and T75 flasks before adapting to serum-free suspension culture. CysFc-004 (CysFc) expression levels were monitored during growth curves in serum-free suspension using a protein A immunoprecipitation procedure followed by non-reducing SDS-PAGE analysis. Based on these growth curves, the cell line 9F4 was chosen for production.

[00147] Cys-Fc-004 (CysFc) was produced from 20 L of 9F4 cells seeded at $5x10^5$ cells/ml in 3 L Fernbach flasks (1L cells/flask). At the end of the production run, cells were removed by centrifugation and the conditioned medium concentrated 4-fold by tangential flow filtration. CysFc-004 was purified by protein A chromatography, the column washed with Dulbeccos PBS (DPBS) followed by DPBS containing 0.9 M NaCl. The protein was eluted with 0.1 M sodium citrate containing 0.15 M sodium chloride, pH 3.4. The protein was then diafiltered into DPBS and stored at 4°C until use.

*Synthesis of C-terminal ANP Thioesters:*

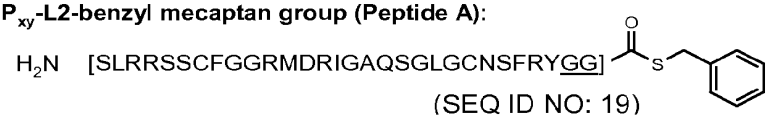
[00148] ANP peptide (0.1 mmol) was synthesized on an Advanced ChemTech 3960 synthesiser on Fmoc-Gly-NovaSyn® TGT (Novabiochem) by standard Fmoc-solid phase peptide synthesis using HBTU as the coupling agent, N,N-diisopropylethylamine (DIEA) as the base and N,N,-dimethylformamide (DMF) as the solvent. After automated synthesis, the N-terminus was protected by manually treating the resin with 0.218g (10 eq.) of the di-t-butyl dicarbonate ($Boc_2O$) and 174 μL (20 eq.) of DIEA in 10 mL DMF, and allowed to mix overnight at room temperature. The reaction was vacuum-filtered, and the resin was washed with DMF and dichloromethane (DCM). The fully protected peptide was cleaved from the resin by mild acid cleavage in DCM with 1, 1, 1, 3, 3, 3-hexafluoro-2 propanal (HFIP), in 50 mL of 3:7 HFIP:DCM for two hours. The resin was filtered into a pre-tared round bottom flask, and the filtrate was concentrated in vacuo over-night, yielding the protected peptide with a free C-terminus. To generate the thioester, this material was treated with 1.5 eq. of glycine benzyl thioester hydrochloride salt (Gly(SBn) HCl), 1.5 eq. of PyBOP (Novabiochem, and 4.5 eq. of DIEA in 10 mL DMF and stirred for 18 hours. The reaction mixture was again concentrated in vacuo for 18 hours. The protected peptide thioester was deprotected by treatment with 25 mL of cleavage cocktail (23.75 mL trifluoroacetic acid (TFA), 0.625 mL triisopropysilane (TIS), 0.625 mL ethanedithiol (EDT)) for two hours and 45 minutes, after which 0.625 mL bromotrimethysilane (TMSBr) was added and allowed to mix for 15 minutes. The reaction mixture was concentrated, and the crude peptide thioester was precipitated with cold diethyl ether ($Et_2O$). Peptide was centrifuged, supernatant decanted, and crude peptide was triturated two more times with cold $Et_2O$. The crude peptide was then purified by reverse phase prepatory HPLC (Phenomenex Jupiter 5u C4 300 A column, 250 x 21.20 mm); HPLC method parameters are listed below. Fractions were analyzed by LC/MS (Applied Biosystems, Mariner) for identity and fractions containing

>90% peptide were pooled, flash frozen, and lyophilized yielding the pure ANP C-terminal thioester.

*Synthesis of N-terminal ANP Thioesters:*

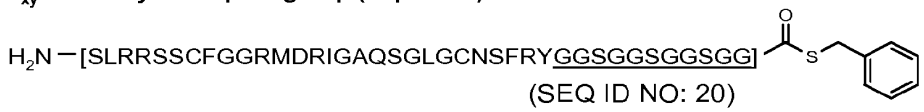
[00149] ANP peptide (0.1 mmol) was synthesised on an Advanced ChemTech 396Ω synthesiser on Fmoc-Tyr(tBu)-NovaSyn® TGT (Novabiochem) by standard Fmoc-solid phase peptide synthesis using HBTU as the coupling agent DIEA as the base and DMF as the solvent. After automated synthesis, the *N*-terminus was converted to a free carboxylic acid by manually treating the resin with 0.100 g succinic anhydride (10 eq.) and 174 μL (10 eq.) DIEA in 10 mL DMF for two hours at room temperature. Reaction contents were removed via vacuum filtration, and the resin was washed with DMF and DCM respectively. To generate the thioester, this material was treated with 0.109 g (5 eq.) of Gly(SBn)·HCl, 0.260 g (5 eq.) of PyBOP (Novabiochem), and 261 μL (15 eq.) of DIEA in 10 mL DMF and stirred for 18 hours at room temperature. The reaction contents were then removed via vacuum filtration, and the resin was washed with DMF and DCM. The protected peptide thioester was cleaved from the resin and deprotected by treatment with 25 mL of cleavage cocktail (23.75 mL TFA, 0.625 mL TIS, 0.625 mL EDT) for two hours and 45 minutes, after which 0.625 mL TMSBr was added and allowed to mix for 15 minutes. The reaction contents were filtered, conserving the filtrate. The filtrate was concentrated, and crude peptide thioester was precipitated with cold diethyl ether ($Et_2O$). The peptide was centrifuged, the supernatant was decanted, and crude peptide was triturated two more times with cold $Et_2O$. The crude peptide was then purified by reverse phase preparatory HPLC, as previously described. Fractions were analyzed by LC/MS (Applied Biosystems, Mariner) for identity and fractions containing >90% peptide were pooled, flash frozen, and lyophilized yielding the pure ANP *C*-terminal thioester. Structures/sequences and mass spectral data are shown below and in Table 1.

**$ANP_{xy}$-L2-benzyl mecaptan group (Peptide A):**



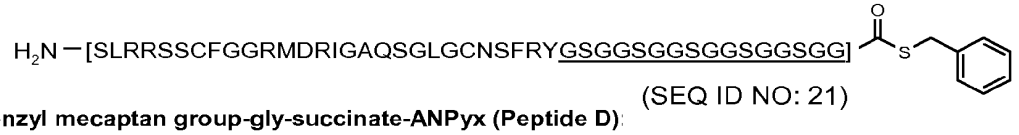

(SEQ ID NO: 19)

**$ANP_{xy}$-L4-benzyl mecaptan group (Peptide B):**




(SEQ ID NO: 20)

**$ANP_{xy}$-L5a-benzyl mecaptan group (Peptide C):**




(SEQ ID NO: 21)

**Benzyl mecaptan group-gly-succinate-ANPyx (Peptide D):**

(SEQ ID NO: 22)

**Benzyl mecaptan group-gly-succinate-L3-ANPyx (Peptide E):**

(SEQ ID NO: 23)

**Table 1:** Mass spectral data.

| Peptide | $MW_{calculated}$ (Da) | $MW_{observed}$* (Da) |
|---|---|---|
| **D** | 3345.84 | 3345.28 (M+1) |
| **A** | 3302.82 | 3302.29 (M+1) |
| | | 3417.12 (M+TFA) |
| **B** | 3906.37 | 3905.31 (M+1) |
| | | 4020.14 (M+TFA) |
| **E** | 3949.39 | 3950.14 (M+1) |
| **C** | 4251.68 | 4251.58 (M+1) |
| | | 4365.54 (M+TFA) |

* As determined by LCMS from a single ion conversion process of the multiply-charged peptide ions

*Conjugation of ANP Thioester Peptides to Cys-Fc:*

[00150] ANP peptide S-benzyl thioesters were conjugated to the N-terminus of Cys-Fc. Cys-Fc (5 mg/mL in PBS, pH 7.4) was treated with 2-mercaptoethanesulfonic acid, sodium salt (MESNA, Sigma-Aldrich) such that the final concentration of MESNA was 20 mM. Peptide thioester was added to the reaction mixture (2-6 mol equivalents depending on the peptide) and allowed to gently mix for 18 hours at room temperature. The actual number of equivalents of peptide was selected based on pilot scale reactions such that equal percentages of monomer (1 ANP peptide per Fc) and dimer (2 ANP peptides per Fc) conjugate were formed. The crude reaction mixture was diluted to 1 mg/mL in PBS and dialyzed exhaustively against PBS (pH 7.4) (7 changes over 24 hours). NuPage SDS-PAGE gels (Invitrogen) were used to determine the extent of reaction. Prior to protein purification, the conjugate was dialyzed into 50 mM Tris-HCl buffer pH 7.2.

*Purification of the Semi-Synthetic ANP-Fc Conjugates:*

[00151] The dialyzed ANP-Fc conjugation reaction mixture was adjusted to a final concentration of 50 mM Sodium Acetate, pH 5.0 with 1M Sodium Acetate pH 5.0 then filtered through a 0.8/0.2 μM syringe filter. This clarified solution was loaded onto a 1 x 10 cm column packed with Fractoprep $SO_3$-650(M) cation exchange resin (CEX) equilibrated with 50 mM Sodium Acetate, pH 5.5. After loading, the column was washed with 3 column volumes (CV) of equilibration buffer. The protein was eluted with a linear gradient from 0M-0.5M Sodium Chloride (in 50 mM Sodium Acetate pH 5.5) over 30 CV. Fractions were analyzed using NuPage SDS-PAGE gels (Invitrogen) and the fractions containing the majority of the monomer conjugate were pooled together, while separately the fractions containing the majority of the dimer conjugate were pooled together. The CEX pools were first adjusted to 0.1M Tris pH 7.2 using a 1M Tris pH 7.2 stock solution, then adjusted to 1M Ammonium Sulfate using a 3M Ammonium Sulfate stock solution. After filtration with a 0.8/0.2 μM syringe filter this clarified protein solution was loaded onto a 1 X 10 cm column packed with EMD Fractogel TA 650(S) thio-affinity resin pre-equilibrated with 50 mM Tris, 1 M Ammonium Sulfate, pH 7.2. After washing the column with 3CV of equilibration buffer the protein was eluted with a decreasing linear gradient from 1.0M to 0.33M Ammonium Sulfate over 20 CV. This was followed by a step gradient from 0.33M to 0M Ammonium Sulfate. The peak fractions containing highly purified conjugate were identified using SDS-PAGE gels then pooled together. The final pool was dialyzed against 1x Dulbecco's

Phosphate Buffered Saline (1 Liter x 2 Changes) (Invitrogen). Protein concentration was determined using UV-280nm analysis. If needed, the final protein was concentrated using Amicon Ultra-15 Centrifugal Concentration Unit(s) (Millipore). The final protein was aseptically filtered through a 0.2 μM filter then aliquots are stored at –80°C.

*Size-Exclusion Chromatography of the Semi-Synthetic ANP-Fc Fusions:*

[00152] The aggregation profile of the semi-synthetic ANP-Fc fusion proteins was assessed using analytical size exclusion chromatography (SEC-HPLC). The TSKgel Super SW2000 4.6 mm (Tosoh Biosciences) SEC-HPLC column was equilibrated with PBS, pH 7.4 + 250mM NaCl. Area under the curve integration was done to estimate the percent of total protein in each peak.

[00153] For example, a TSKgel Super SW2000 4.6 mm (Tosoh Biosciences) SEC-HPLC column was run at a flow rate of 0.4 mL/min using PBS, pH 7.4 and 250mM NaCl as the eluent. 5 μL of a 1.0 mg/mL sample was injected for each analysis. BioRad MW standards (cat# 151-1901) were injected prior and after each set of injections to ensure the integrity of the column.

*Characterization of "Free" Peptide in the Semi-Synthetic ANP-Fc Fusions:*

[00154] The "free" unconjugated ANP peptide contaminant levels in the purified semi-synthetic ANP-Fc fusions preps was determined using reversed-phase chromatography. A Protein C4 reversed phase column (Grace Vydac) was injected with 100 μg of the purified semi-synthetic Fc fusion protein. The two mobile phases were A) 0.1%TFA in water and B) 0.1% TFA in Acetonitrile. The reversed phase chromatography was run using the following gradient profile over a total run time of 50 minutes (min): 0.5 min at 5% B, 5-35 min ramp up to 95%B, 35-40 min hold at 95%B, 40-42 min ramp down to 5% B, 42-50 min hold at 5%B. Alternate runs were spiked with 0.1μg of "free" ANP peptide (0.1 μg spike is equivalent to 1.3 mole %) to visualize expected positioning and peak heights of potential contamination. The lower limits of "free" ANP detection of this method are to 1% molar ANP contamination levels.

*Characterization of Peptide Disulfide Linkages in the Semi-Synthetic ANP-Fc Fusions:*

[00155] Trypsin digestion of ANP-Fc fusions was performed at pH 5.25, 37°C for 18 hours, preserving the disulfide (Cys-Cys) linkages. The tryptic digest was split into two

aliquots, one of which was reduced by DTT and capped with 2-iodoacetamide. The reduced sample (0.5-1.0 pmol) was loaded onto a self-packed C18 capillary column (10cm) hooked to a nano-LC/MS/MS. Peptide sequencing was performed using Sequest or *de novo*. Free peptide (oxidized form) was treated the same way (reduced and non-reduced) and used as a positive control.

[00156] An Agilent 1100 HPLC binary system and a Thermo LCQ-Advantage ion trap mass spectrometer was used for the LC/MS/MS analysis. The unconjugated ANP peptide analog (including GS linker region) of the ANP-Fc fusions was used as the positive control for the identification experiment. 3 μg of peptide-Fc fusion (1 μg of free peptide in oxidized form) was diluted to a final volume of 30 μL with 100 mM ammonium acetate (pH 5.25). 10μL of a 0.01 μg/mL trypsin solution (trypsin gold, MS grade, Promega) was added to the protein/peptide solution and the digestion is carried out at 37°C for 18 hours. The digested sample was split into two aliquots of 20 μL. One aliquot of the digest (non-reduced condition) is frozen immediately and stored at -20°C until analysis. The 2nd aliquot is dried down *in vacuo* and redissolved with 20 μL of 50 mM Tris-HCl and 1 mM EDTA (pH 8.0). One microliter of DTT (0.2 M) is added and the solution is incubated at 56°C for 30 minutes. Subsequently, one microliter of 2-iodoacetamide (0.5 M) is added and the reaction proceeded for 30 minutes in the dark to generate the thiol-capped digestion sample. The unconjugated peptide digest is diluted five-fold with water. 0.5 μL of digested peptide-Fc fusion (or 1 μL of digested peptide) is loaded onto a self-packed C18 capillary LC column (YMC C18, 5 μm, 10 cm long) for nano-LC/MS/MS analysis. The HPLC pump flowrate of 0.2 mL/min is split to obtain 1 μL/min for the capillary LC. The mobile phases used is 0.05 M acetic acid in water (A) and 0.05 M acetic acid in acetonitrile (B). The LC/MS data (non-reduced and reduced) are compared to theoretical masses corresponding to possible disulfide folding linkages for the digested fragments to determine whether or not the proper disulfides within the ANP peptide(s) are formed. The reduced peptide fragment sequence is confirmed by SEQUEST database searching of MS/MS data.

*Characterization of the FcRn Binding Properties of the Semi-Synthetic ANP-Fc Fusions:*

[00157] Purified soluble human FcRn was bound to Biacore chip surfaces at pH=6.0. The semi-synthetic ANP-Fc fusions were flowed over the chip surfaces in a series of different concentrations. The equilibrium responses ($R_{eq}$) were fit by non-linear regression to a model with two classes of non-interacting binding sites using the Biacore BiaEval software. This

modeling assumes there are two classes of Fc binding sites on the FcRn coated sensor chip ($K_{D1}$, $K_{D2}$) that contribute to the overall observed binding.

[00158] For example, soluble human FcRn were cross-linked to the dextran surface of a CM5 sensor chip by amine coupling using 1-ethyl-(3-dimethylaminopropyl)- carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as recommended by Biacore (BIAapplications Handbook, version AB, section 4.2). For immobilization, the FcRn was diluted to 10 μg/mL in 10mM sodium acetate, pH 4.5. Residual sites on the dextran were blocked with 1 M ethanolamine hydrochloride pH 8.5. FcRn was immobilized to one flow cell on the sensor chip, while a control flow cell was blocked with ethanolamine for reference subtraction. For analysis of ANP-Fc conjugates, FcRn was coated to a final density of 400 to 600 response units (RU). This level of coating was used to ensure that the sensor chip was fully saturated by the injected Fc protein within the allowable injection time while still giving a reasonable signal. Experiments were performed at pH 6 using 50 mM sodium phosphate, 100 mM sodium chloride, 0.01% surfactant P20 (Biacore AB). Eleven - 2 fold serial dilutions between 1 μM and 1 nM of the ANP-Fc or control proteins were injected over the FcRn-CM5 chip at 30 μL/min for 5 minutes. Bound protein was then dissociated from the chip for 2.5 minutes with running buffer. Any remaining protein was removed from the chip with a 27 second injection of pH 7.4 HBS-P buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20) at 30 μL/min.

[00159] Next, sensorgrams were analyzed using BiaEval software version 3.1 (Biacore AB) and were baseline corrected using a buffer blank, then baseline averaged. When a given concentration of the protein was injected onto the chip, the signal gradually reached a maximal plateau. This was presumed to be the extent of binding at equilibrium and the observed signal was termed Req (Response Units at Equilibrium). Req values were plotted against concentration and the equilibrium affinity constants ($K_D$'s) were then derived by fitting the data to a heterogeneous ligand model using BiaEval software. This model assumes that there are two classes of non-interacting binding sites of FcRn on the sensor chip. Thus, the $K_D$ values were reported as two separate $K_D$'s that contribute some fractional % to the theoretical maximal observed Req value (Rmax).

*Recombinant ANP-Fc Fusion Protein Production and Characterization:*

[00160] ANP-Fc Fusion Construct Generation: Four DNA sequences were synthesized according to conventional oligonucleotide synthesis techniques, and subcloned

into the vector pJ13 (DNA2.0 Inc.). Each construct contained the same basic order of a *Hind*III restriction enzyme (RE) site followed by a Kozak sequence, mouse IgG kappa light chain signal sequence, human ANP28 sequence, a linker sequence, a human Fc gamma sequence followed by another RE site. The Kozak sequence and the mouse IgG kappa light chain signal sequence were identical to the sequence in the vector pSYN-Fc-015 (pcDNA3.1/Igk sig seq -hFc). The pSYN-Fc-015 vector expresses the Fc protein of an IgG1 isotype protein that matches the sequence in NCBI database for accession number Y_14735. The human ANP28 sequence is from the NCBI database with the accession number NM_006172 (SEQ ID NO. 15). The linker sequence encodes for either an 11 or 20 aa peptide consisting of repeats of glycine and serine residues, $(GGS)_3GG$ (SEQ ID NO: 3) for the 11 aa linker (L4) and $(GGS)_6GG$ (SEQ ID NO: 7) for the 20 aa linker (L6). Constructs pJ13-ANP28-11aa-hFc1 and pJ13-ANP28-20aa-hFc1 contain sequence identical to the first 39bp of the human Fc gamma 1 sequence in pSYN-Fc-015 which includes a *Bsp*EI RE site. Constructs pJ13-ANP28-11aa-hFc2 and pJ13-ANP28-20aa-hFc2 are the same as the two previously described constructs through the linker sequence except the entire human Fc gamma 1 sequence is replaced with a human Fc gamma 2 sequence. Following the human Fc2 sequence an *Eco*RI restriction site is present.

[00161] The expression vector pSYN-Fc-015 was digested with *Hind*III and *Eco*RI and the fragment containing the Kozak sequence, mouse IgG kappa light chain signal sequence and the entire Fc gamma 1 sequence was removed. The 5.4kb vector backbone was isolated by standard molecular biology techniques. Constructs pJ13-ANP28-11aa-hFc2 and pJ13-ANP28-20aa-hFc2 were each digested with *Hind*III and *Eco*RI; an 858bp fragment was isolated from pJ13-ANP28-11aa-hFc2 and an 885bp fragment was isolated from pJ13-ANP28-20aa-hFc2 using standard molecular biology techniques. Each contained the Kozak sequence, mouse IgG kappa light chain signal sequence and either an 11aa linker (L4) or a 20aa linker (L6) followed by the entire Fc gamma 2 sequence. The 5.4kb vector was ligated to either the 858bp fragment or the 885bp fragment overnight with T4 DNA ligase at 4°C. The ligated DNAs were transformed into the bacterial strain DH5α, plated onto LB + ampicillin plates and incubated at 37°C overnight. The following morning the plates were removed from the incubator and several single colonies from each ligation/transformation plate were each picked into 15ml of LB media containing ampicillin and grown overnight at 37°C in a shaking incubator. The next day the bacterial cells were harvested and plasmid

DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen Inc.). Aliquots were sent out for sequencing to confirm the DNA sequence (Agencourt).

[00162] Vector pSYN-Fc-015 was digested with *Hind*III and *Bsp*EI and the 6kb vector backbone was isolated. This removed the Kozak sequence, mouse IgG kappa light chain signal sequence and the first 37bp of the Fc gamma 1 sequence. Constructs pJ13-ANP28-11aa-hFc1 and pJ13-ANP28-20aa-hFc1 were digested with *Hind*III and *Bsp*EI; a 209bp fragment was isolated from pJ13-ANP28-11aa-hFc1 and a 236bp fragment was isolated from pJ13-ANP28-20aa-hFc1. Each fragment contained the Kozak sequence, mouse IgG kappa light chain signal sequence and either an 11aa linker (L4) or a 20aa linker (L6) as well as the first 37bp of the Fc gamma 1 sequence. The 6kb vector was ligated to either the 209bp fragment or the 236bp fragment; transformations, DNA plasmid preps and sequencing were performed as previously described.

[00163] Mammalian Expression of the ANP-Fc Fusions: The initial expression of the ANP fusion constructs was done in HEK293 cell lines using a transient transfection protocol on a 1L scale. HEK293 cells were grown in FreeStyle 293 medium and transfected using the 293Fectin reagents and protocols as described by the manufacturer (Invitrogen). Transiently transfected cells were grown in a spinner flask with agitation of 90 rpm at 37°C and 5% $CO_2$. The cells were monitored daily for cell density, viability, and diameter, level of glucose, lactate, glutamine and pH. The conditioned media was harvested 72 hours after the transfection by centrifugation at 3600 rpm for 15 minutes and was delivered fresh to purification. Expression levels were determined by SDS-Page and Western-blotting analysis.

[00164] Once the expression levels were determined for each construct, various scale-up methods, including 20L transient transfection and stable pool generation, were performed to generate the protein required. The 20L transient transfection was carried out using the PEI-25 reagent in a 50L wave bioreactor. A 25 mg aliquot of DNA was diluted into 250 ml of FreeStyle 293 media, and 75 mg aliquot of PEI-25 was diluted into 250 ml of the same media. The DNA sample was then mixed with PEI solution. After a 15 minute incubation at room temperature, the DNA:PEI mixture was loaded into the wave bioreactor with 10L of freshly loaded HEK293 cells at the cell density of 2 million/ml. The wave bioreactor was set at a rocking angle of 8 degrees and a rocking speed of 22 rpm at 37°C and 5% $CO_2$. Five hours after the transfection, 10L of FreeStyle 293 medium was loaded into the wave bioreactor. The cells were monitored daily for cell density, viability, and diameter, level of glucose, lactate, glutamine and pH. The conditioned media was harvested 120 hours after the

transfection by centrifugation at 3600 rpm for 15 minutes and was delivered fresh to purification. Expression levels were determined by SDS-Page and Western-blotting analysis. The stable pools generation started in a flask where stable cells were selected with 400 μg/ml of G418 and then transferred into spinner. Once the cells stabilized under these conditions, they were expanded and 4-5L runs were carried out either in spinners or a wave bioreactor.

[00165] Purification of the Recombinant ANP-Fc Fusion Proteins: The purifications were performed at 4°C using an AKTA system (GE Healthcare) or BioOptix10 (ISCO). Five milliliters of Protease Inhibitor Cocktail (Sigma) were added per liter of conditioned media. A column was packed with 3-4 mL Protein A Ceramic HyperDF® resin (Pall) per liter of conditioned media and equilibrated with 10CV of Equilibration Buffer (1x Dulbecco's PBS without Mg/Ca, pH 7.3, Invitrogen) at 1 mL/min. Conditioned media was loaded onto the column overnight at 1-5 mL/min depending upon the volume of media. The resin was then washed, in 10CV sequences, with Wash Buffer 1 (DPBS without Mg/Ca, pH 7.3), Wash Buffer 2 (DPBS without Mg/Ca pH 7.3 + 1M NaCl), and then Wash Buffer 1 again. The bound protein was eluted with 10CV Elution Buffer (0.1 M Glycine/HCl, pH 2.5 in $dH_2O$) at 2.5 ml/min. The 3 mL fractions were neutralized immediately with 300 μL (10% of fraction) of Neutralization Buffer (1M Tris-HCl pH 8.0, Invitrogen). The fraction pools were dialyzed against PBS (Hyclone), 2x5L for total of 22 hours, using Slide-A-Lyzer cassettes (Pierce) and concentrated using Amicon Ultra-15 10kDa MWCO concentrators (Millipore) to 1-2 mg/mL. Aliquots were flash-frozen in liquid nitrogen and then stored at -80°C. Final products were characterized by SDS-PAGE (non-reduced and reduced), analytical ultracentrifugation and mass spectroscopic analysis before being submitted for biological function assays. Endotoxin contaminant levels were determined using the gel-clot LAL reagents (The Associates of Cape Cod).

[00166] Analytical Ultracentrifugation of the Recombinant ANP-Fc Fusion Proteins: Sedimentation velocity experiments were conducted to assess the purity and aggregate content of the purified ANP-Fc fusions. The ANP-Fc fusions were evaluated by sedimentation velocity in a phosphate buffered saline buffer containing 10 mM Sodium Phosphate, 150 mM NaCl, pH 7.3. Samples were loaded into centrifuge cells containing double-sector charcoal-epon centerpieces and quartz windows. Data was collected using a Beckman XLI analytical ultracentrifuge at 280 nm, 50,000 rpm and 20°C. Solution densities and viscosities were measured using an automated Anton-Paar AMVn/SP3-V viscometer and

DMA4500/DMA5000 densitometer at 20°C. The sedimentation data were analyzed using the program SEDFIT version 9.3b (2).

[00167] Mass Spectroscopy Analysis of the Recombinantly Produced ANP-Fc Fusions: Mass spectrometric experiments were conducted to measure intact molecular weight, to perform partial sequence validation via enzymatic digestion and peptide mapping, and to identify degradation products of the ANP-Fc fusions.

[00168] To evaluate intact molecular weight 5 µl of fusion protein was injected onto a Jupiter C4 column (5u, 300A, 1.0 x 150mm) at a flow rate of 40 µl/min. The protein is eluted with a gradient from 0% to 100%A in 40 minutes [Mobile phase A is 98/2/0.06 (Water/Acetonitrile/TFA) and Mobile phase B is 95/5/0.052 (Acetonitrile/Water/TFA)]. The effluent was flowed into a Micromass Quattro Ultima mass spectrometer scanning from 900-1500amu with a 2 second scan time. Data was deconvoluted using the program MaxEnt1 to yield molecular weight(s).

[00169] The fusion proteins were digested with trypsin or Endo-LysC (8010-108) for peptide mapping and degradation analysis. Briefly, 25 µl of each sample was denatured, reduced, and alkylated. Trypsin or Endo-LysC was added, and the sample incubated overnight in a 37°C water bath. Analysis was performed via HPLC/FTMS. The instrument generated multiple scans during the chromatographic separation at approximately 50,000 resolution. The mass of each eluting peptide was determined with an accuracy of 0.0005% relative error. Data was processed with the ProQual program (3) to yield the peptide map. Peptides predicted based on enzyme specificity were matched if the theoretical value was within 0.0005% of the experimentally determined value. The Endo-LysC data was also searched for *N*-terminal truncations by looking for the masses corresponding to possible amino acid deletions. Unlike trypsin, the Endo-LysC experiment allows for observation of the intact amino terminal region.

*Generation of Stable Cell Lines for in vitro NPRA Cell Assays*:

[00170] Full-length human NPRA, human NPRB, and human GUCY2C (NPRC) sequence containing plasmids were purchased from OriGene Technologies, Inc. (Rockville, MD) then sub-cloned into pcDNA3.1 directional mammalian expression vector (Invitrogen). Insert orientation and nucleotide sequence of each construct was verified by an outside vendor (SeqWright, Inc.). The pcDNA3.1 NPR clones were transfected using Lipofectamine (Invitrogen) into HEK293 cells where stable cell lines expressing human NPRA, human

NPRB, or human NPRC are selected using G418. Clones were screened using the natriuretic peptide induced cGMP assay described below. (NPRA clones were treated with human ANP while NPRB clones were treated with human CNP (Sigma)). High cGMP producing clones were expanded. NPRC clones were screened using a $^{125}$I ANP binding assay. $^{125}$I ANP high binders were expanded. Cell lines were grown in DMEM containing, 100 µg/ml penicillin/streptomycin, L-glutamine, 400 µg/ml of G418, and 10% FBS (Hyclone)). HEK293T-GCA (rat NPRA expressing cells) (Lincoln Potter, Dept. Biochemistry, Molecular Biology and Biophysics University of Minnesota, Minneapolis/St. Paul, MN) were grown in DMEM containing 100 µg/ml penicillin/streptomycin, L-glutamine, Hygromycin B and 10% FBS.

*Natriuretic Peptide Fusion Protein Induced cGMP Assay*:

[00171] Natriuretic peptides of >95% HPLC purity were obtained from Sigma. HEK293 NPRA cells grown to 90% confluence were harvested using HANKS based Cell Dissociation Medium (GIBCO). Cells were washed and resuspended at 3.3 x $10^5$ cells per mL in pre warmed Dulbecco's PBS, pH 7.4, 25 mM HEPES, 0.1% BSA, 500 µM 3-Isobutyl-1-methylxanthine (IBMX) [Assay Buffer]. Assays were performed in Optiplate-96 White Opaque 96-well Microplates (Perkin Elmer). 15 µL of cell suspension is added to 15 µL of 2X fusion protein in Assay Buffer in triplicate and incubated for 20 minutes at 37°C. The cGMP concentration was measured using the HitHunter™ cGMP Assay Kit (DiscoveRx Corporation). cGMP production dose response curves were generated with a four parameter logistic equation fitted using the Levenburg Marquardt algorithm in XLfit4.2 data analysis software (ID Business Solutions, Ltd.).

*$^{125}$I ANP Binding Assay:*

[00172] Millipore Multiscreen glass fiber filter microtiter plates were coated with 0.2% polyethylenimine (PEI) (Sigma) and washed with binding buffer (RPMI, 0.1 % BSA) by vacuum filtration. 100 ml of 1*$10^6$ HEK293-NPRC cell clone suspension in binding buffer were added to each well. 100 ml of 2X $^{125}$I-ANP +/- 1000 x cold ANP (non-specific binding control) were added to the appropriate wells. The plates were incubated on an orbital shaker at room temperature for two hours. Following the incubation, the plates were washed 5X with 200 µl of cold binding buffer followed by a 200 µl final wash with Wash Buffer (10 mM Tris, 200 mM NaCl, 0.02% BSA) using a vacuum manifold. The filters were allowed to

dry at room temperature. 30 µl of Microscint 20 was added to each well. The plates were read on a PerkinElmer Topcount NTX Liquid Scintillation Counter.

*Pharamacokinetics of ANP-Fc in Rats:*

[00173] Femal Wistar rats (~100g; 4 rats/protein) were dosed intravenously with 0.5 mg/kg monomer (Construct 14) and dimer (Construct 15) or monomer (Construct 10) and dimer Construct 11) in PBS. Blood was collected from each rat by tail nick at 1, 2, 4, 8, 24, 48, and 72 hours (Construct 15) or 0.25, 1, 2, 4, 8, 24, 48, and 72 hours (Constructs 10, 11 and 14) after dosing. Blood (2 x 60 µl aliquots) was collected into microcapillary tubes (Fisher Scientific, cat #22-362-574) containing 6 µl 3.2% sodium citrate and plasma generated by centrifugation. Plasma was stored at -20°C until analysis by ELISA.

*Oral Uptake of ANP-Fc in Neonatal Rats:*

[00174] Ten day old neonatal Wistar rats (4 rats/group) were orally dosed with 0.5 mg/kg ANP-Fc dimer (Construct 14) in PBS supplemented with 5 mg/ml soybean trypsin inhibtor (Calbiochem; catalog #650357). Blood was collected from a group of rats by cardiac puncture at 1, 2, 4, 8, 24, 48, and 72 hours after dosing. Blood (700 µl) was collected into 0.1 volume 3.2% sodium citrate and plasma generated by centrifugation. Plasma was stored at -20°C until analysis by ELISA.

*ANP/Fc ELISA :*

[00175] 96-well plates (Costar, catalog #369) were coated with 5 µg/ml (50 µl/well) mouse anti-human ANP (US Biologicals, Cat #A4150) in 50 mM of carbonate/bicarbonate buffer pH 9.6 at 4°C overnight. Plates were blocked with 300 µl/well of PBS containing 5% bovine serum albumin (PBS/5% BSA) (Jackson ImmunoResearch#001-000-162) for 2 hours at room temperature (RT). Standards and samples (100 µl/well) were diluted in PBS/5% BSA and incubated for 2 hours at RT. Standard curves ranged from 0.039 ng/ml to 10 ng/ml. Plates were washed three times with 300 µl/well of PBS containing 0.05% Tween-20 (PBST) in a Tecan plate washer. Plates were then incubated with 100 µl.well of goat anti-human (Fc specific) horseradish peroxidase (HRP) conjugated antibody (Pierce Biotechnology, cat#31414) diluted 1:7,500 in PBS/5% BSA for 3 hours at room temperature. Plates were washed four times with 300 µl/well of PBST before development with 100 µl/well of TMB supersensitive subtrate (BioFx Laboratories, cat#TMBS) at RT for approximately 6 minutes.

Reactions were stopped by addition of 0.25M of sulfuric acid (100 µl/well). Plates were read at 450 (-600) nm in a Spectromax plate reader.

**Example 1: Structural modeling to predict minimum linker distance**

[00176] Structural modeling was done to determine the linker requirements for the Fc and ANP fusions. With the both molecules oriented to minimize distance as well as minimize steric and electrostatic repulsions, a minimal distance from the *C*-terminus of ANP is 12 Å from the closest *N*-terminus of the Fc dimer and 17 Å from the other *N*-terminus of the Fc dimer. If the Fc dimer has only one ANP fused, a 4 to 6 aa minimum linker length would be suggested. With two ANP peptides bound to the Fc dimer, if only one ANP bound (in a 1:1 Fc dimer:NPRA ratio) then a minimum linker length of 9 aa for each linker would be suggested. For both ANP's to bind in a 1:2 Fc dimer:NPRA ratio, linkers with a minimum length of 12 aa would be suggested.

**Example 2: Synthesis and characterization of synthetic ANP fusion proteins**

[00177] Synthetic chemistry was used to generate hANP28 peptides with linkers fused to human Fc of IgG1 isotype. The resulting semi-synthetic ANP-Fc fusion molecules generated are shown in Table 2. The linkers tested were glycine succinate (L1), GlyGly (L2), $(GlyGlySer)_3GlyGly$ (L4) (SEQ ID NO: 3) and $Gly(SerGlyGly)_2SerGly$ (L3) (SEQ ID NO: 2). The glycines (G) were used to add flexibility, while the polar serines (S) were built in to add solubility. The hANP28 peptides with linkers were linked to recombinantly produced Cys-Fc in two orientations: 1) *C*-terminus of hANP28+Linker fused to *N*-terminus of Cys-Fc [Orientation #1], and 2) *N*-terminus of Linker+hANP28 fused to *N*-terminus of Cys-Fc [Orientation #2]. The synthetic chemistry of the first orientation resulted in a complete peptide bonded structure while the chemistry of the second orientation left a succinate moiety in place of one amino acid of the fusion.

[00178] The semi-synthetic ANP-Fc conjugates were purified using a two-step purification protocol consisting of cation exchange chromatography followed by thio-affinity chromatography. The cation exchange step separated the Cys-Fc, the monomer conjugate (1 ANP peptide per Fc), and dimer conjugate (2 ANP peptides per Fc), while also removing the protein aggregates. The individual ANP-Fc conjugate cation exchange pools were independently put through a thio-affinity chromatography purification step to remove the unconjugated free ANP peptide and "polish" the monomer and dimer conjugate species.

[00179] The purified semi-synthetic ANP28-Fc fusions were extensively characterized using SEC-HPLC, reverse-phase chromatography, mass spectroscopy, and

molecular binding analysis. The SEC-HPLC, used to assess aggregation showed that the purified preps contained >90% of the expected MW species. A reverse phase chromatography method was established to monitor the "free" unconjugated ANP peptide levels in the final pools. Synthesized preps were verified to have less than 1% molar contamination of "free" ANP peptide. Mass spectroscopy techniques were used to assess the quality of the disulfide bond formation. The results of the mass spectroscopy indicated that the semi-synthetic constructs with longer linkers showed a small percentage of disulfide bond mixing. As the percentage with mixed disulfides was estimated to be very small fraction of the total, the synthesized preps were considered good for further analysis. Finally, Biacore analysis was performed to monitor binding of the semi-synthetic ANP-Fc fusion proteins to immobilized soluble human FcRn. This analysis was done to verify that the synthetic chemistry does not alter the FcRn binding capabilities of the Fc domain. No significant changes in FcRn binding were observed.

[00180] Once the quality of the semi-synthetic ANP-Fc fusion constructs was confirmed, their potency was tested in the human NPRA cGMP induction assay by two different laboratories, and the data processed using two different methods (Table 2: Labs 1 & 2 and Results 1 & 2). Results 1 from both Labs 1 and 2 were generated by interpreting the raw Relative Luminescence Units (RLU). Results 2 from Lab 1 were generated by comparing the RLU to a standard curve made up of known concentrations of cGMP in the same assay. The EC50 values generated in both analyses showed the same trends. One trend seen is that the dimer constructs are more potent in this assay than the respective monomer constructs. It is also clear that the ANP-Fc fusions with short (2 aa) linkers are at least 2-fold weaker relative to those with longer (11 aa) linkers.

**Table 2**: *Semi-synthetic ANP-Fc fusions and cGMP Induction Response from Human NPRA Transfected Cells*

| Construct Number | Construct Formula | cGMP $EC_{50}$ (nM) Lab 1 (Results 1) | cGMP $EC_{50}$ (nM) Lab 1 (Results 2) | cGMP $EC_{50}$ (nM) Lab 2 (Results1) |
|---|---|---|---|---|
| 6 | $ANP_{YX}$-L1-$Fc1_{AB}$ : $Fc1_{AB}$ | 68 ± 23 (n=4) | 150 ± 49 (n=4) | NA |
| 7 | $ANP_{YX}$-L1-$Fc1_{AB}$ : $ANP_{YX}$-L1-$Fc1_{AB}$ | 45 ± 22 (n=3) | 114 ± 40 (n=3) | NA |
| 8 | $ANP_{XY}$-L2-$Fc1_{AB}$ : $Fc1_{AB}$ | 234 ± 199 (n=2) | 329 ± 57 (n=2) | 282 ± 123 (n=3) |
| 9 | $ANP_{XY}$-L2-$Fc1_{AB}$ : $ANP_{XY}$-L2-$Fc1_{AB}$ | 128 ± 161 (n=4) | 118 ± 55 (n=3) | 88 ± 21 (n=3) |
| 10 | $ANP_{XY}$-L4-$Fc1_{AB}$ : $Fc1_{AB}$ | 85 ± 16 (n=2) | 327 ± 65 (n=2) | 100 ± 39 (n=4) |
| 11 | $ANP_{XY}$-L4-$Fc1_{AB}$ : $ANP_{XY}$-L4-$Fc1_{AB}$ | 19 ± 4 (n=2) | 68 ± 23 (n=2) | 26 ± 11 (n=4) |

| | | | | |
|---|---|---|---|---|
| 12 | $ANP_{YX}$-L3-L1-$Fc1_{AB}$ : $Fc1_{AB}$ | 47 ± 6 (n=4) | 158 ± 101 (n=4) | 77 ± 20 (n=3) |
| 13 | $ANP_{YX}$-L3-L1-$Fc1_{AB}$ : $ANP_{YX}$-L3-L1-$Fc1_{AB}$ | 19 ± 11 (n=4) | 66 ± 55 (n=4) | 24 ± 2 (n=3) |
| 14 | $ANP_{XY}$-L5a-$Fc1_{AB}$ : $ANP_{XY}$-L5a-$Fc1_{AB}$ | NA | | 14 ± 1 (n=2) |
| 15 | $ANP_{XY}$-L5a-$Fc1_{AB}$ : $Fc1_{AB}$ | NA | | 83 ± 51 (n=3) |

[00181] In order to evaluate the correlation between linker length and potency, an ANP-Fc Fusion with a 16 aa linker (L5a) was generated (refer to Construct 14 and Construct 15). To evaluate even longer linkers, a recombinant 20 aa (L6) linked ANP-Fc fusion protein was generated. In both cases the composition of the longer linkers was kept consistent with the (GGS)x repeat pattern, where x is an integer from 0 to 16. Both the monomeric and dimeric ANP-Fc constructs with a 16 amino acid linker (L5a), shown in Table 2, demonstrated enhanced in vitro activity over the 11 amino acid linker (L4) constructs. To confirm that the semi-synthetic ANP-Fc fusion proteins generated were identical to recombinantly produced ANP-Fc fusions, recombinant constructs that mimic Construct 11 were also made as both IgG1 and IgG2 Fc fusions.

[00182] Human ANP28 peptide was assayed for cGMP production in species specific NPRA transfected 293 or 293T cells. Values for the concentration of fusion protein resulting in half-maximal stimulation of cGMP are listed in Table 3. Dose response curves for cGMP production stimulated by human ANP28 peptide in rat NPRA (rNPRA) expressing 293T cells and human NPRA (hNPRA) and canine NPRA (caNPRA) expressing 293 cells are shown in Figure 2.

**Table 3**: *Assay results for cGMP production in species specific NPRA transfected 293 or 293T cells.*

| Ligand | Assay Type | Mean EC50 nM | StdDev | N |
|---|---|---|---|---|
| ANP | cGMP-hNPRA 293 | 0.82 | 0.51 | 15 |
| ANP | cGMP-rNPRA 293 | 0.33 | 0.06 | 2 |
| ANP | cGMP-hNPRA 293 | 1.00 | 0.57 | 16 |
| ANP | cGMP-rNPRA 293 | 0.67 | 0.19 | 4 |
| ANP | cGMP-caNPRA 293 | 1.34 | 1.43 | 3 |

**Example 3: Recombinant ANP fusion proteins**

[00183] A production platform for the rapid generation of recombinant ANP-Fc fusion proteins was generated. The process starts with "base" Fc fusion vectors that allows DNA cassettes to be rapidly and seamlessly inserted onto the *N*-terminus of either IgG1 or IgG2 Fc. The Fc fusions to both IgG1 and IgG2 isotypes were generated in such a way that the hinge region is chopped down to the same CPPCP hinge residues thus ensuring that the linker extension and ANP fusion would be equally extended on the two isotypes. The respective DNA and protein sequences of the four recombinant ANP-Fc fusion proteins generated are represented by SEQ ID NOs: 24-31 (see, *e.g.*, Figure 1A-B). These fusion proteins each comprise a N-terminal mouse IgG kappa light chain signal sequence METDTLLLWVPGSTG (SEQ ID NO: 32) that is cleaved off and not part of the final protein product. The bolded The ANP-Fc fusion constructs were initially produced using 1L transient mammalian expression followed by affinity chromatography. This production process typically yields 1-3 mg/L of >90% pure protein (by SDS-PAGE). To meet the increasing protein demands, two other mammalian expression technologies, large-scale (20L) transient transfection and stable pool generation, were both successfully implemented. Both of these methodologies yielded at least 3-fold more protein per liter than the initial 1L

transient transfection. Overall these processes allowed for the production of more than 50 mg of each recombinant construct to be used for *in vitro* and *in vivo* analysis. The predicted MW of the non-reduced ANP-Fc fusion is 57.5 kDa, while the predicted MW of the reduced ANP-Fc fusion is 28.7 kDa.

[00184] To complement the recombinant production platform a characterization platform was put in place to ensure the quality of the ANP-Fc fusions being tested. As hANP28 has such high potency (see Table 4), and its sequence contains many critical amino acids and a disulfide necessary for functionality, mass spectroscopy (intact mass and tryptic digests) was utilized to monitor both "free" hANP28 peptide concentrations and cleavage products in the lots generated. A mass spectroscopy Endo-LysC digest protocol was also implemented to evaluate the *N*-terminal end of the ANP-Fc fusion peptides. Only lots with >90% intact N-termini were selected for further analysis. The level of aggregation was tested by sedimentation velocity experiments in an analytical ultracentrifuge where the lots were found to contain >85% monomer.

**Table 4**: *Recombinant ANP-Fc fusions and cGMP Induction Response Generated on Various NPRA Cell Types*

| **Construct Number** | **Construct Formula[1,2]** | **cGMP EC50 (nM) Human NPRA** | **cGMP EC50 (nM) Rat NPRA** | **cGMP EC50 (nM) Dog NPRA** | **cGMP EC50 (nM) Monkey NPRA** |
|---|---|---|---|---|---|
| 1 | $ANP_{XY}$ | 1 +/- 0.5 (n=32) | 0.7 +/- 0.2 (n=4) | 1.3 +/- 1.4 (n=3) | 0.5 +/- 0.2 (n=3) |
| 2 | $ANP_{XY}$-L4-$Fc1_{AB}$ | 41 +/- 20 (n=6) | 55 +/- 33 (n=3) | 31 +/- 16 (n=3) | 27 +/- 18 (n=3) |
| 3 | $ANP_{XY}$-L6-$Fc1_{AB}$ | 10 +/- 10 (n=12) | 25 +/- 12 (n=9) | 11 +/- 11 (n=9) | 12 +/- 8 (n=3) |
| 4 | $ANP_{XY}$-L4-$Fc2_{AB}$ | 27 +/- 17 (n=5) | 57 +/- 29 (n=5) | 46 +/- 23 (n=3) | 18 +/- 10 (n=3) |
| 5 | $ANP_{XY}$-L6-$Fc2_{AB}$ | 6 +/- 3 (n=3) | 12 +/- 9 (n=3) | 6 +/- 2 (n=3) | 5 +/- 3 (n=3) |

[1] See, *e.g.*, formulas 3 and 4

[2] Note that the Fc domains of two different chains will dimerize upon recombinant expression, thus forming a dimer of the ANP.

[00185] The recombinantly produced ANP-Fc fusion constructs were tested in NPRA cGMP induction assays. The recombinant ANP-Fc fusion proteins tested to date show good cross-reactivity in the rat, canine and monkey assays. The 11 aa linker (L4) recombinantly produced ANP-Fc fusion proteins were similar in potency to the semi-synthetic construct Construct 11 that they mimic. This indicates that the potency data generated with the semi-synthetic ANP-Fc fusions is representative of what would be seen from a recombinantly generated protein. As expected, the variation in Fc isotype (Fc1 vs. Fc2) does not have significant impact on potency. The most dramatic potency effects were seen when comparing the various linker lengths. The 20 aa linker (L6) recombinantly produced ANP-Fc fusion proteins are clearly ~2-fold more potent than the corresponding 11 aa linker (L4) fusion proteins. The semi-synthetic 16 aa linker (L5) ANP-Fc fusion protein Construct 14 has a human NPRA cGMP EC50 of 14 ± 1 nM (n=2). Taking into account the semi-synthetic and recombinant ANP-Fc fusion potency data there is a direct correlation of *in vitro* potency to the linker length with 20 aa > 16 aa > 11 aa > 2 aa.

[00186] The selectivity of the recombinant ANP-Fc fusion proteins was assessed by analyzing their efficacy in NPRB expressing cell lines. The species cross-reactivity of the ANP-Fc fusion proteins was tested in rat, canine, and monkey NPRA expressing HEK293 cell lines. The ANP-Fc fusions tested have good cross-reactivity in the rat, canine, and monkey NPRA mediated cGMP assays (Table 4). The ANP-Fc fusion proteins have nearly equivalent dose response values when tested in human, dog and monkey expressing NPRA cells. These results are not surprising given the fact that human, dog, and monkey ANP28 are 100% homologous. The dose response values for rat NPRA, on the other hand are ~2-fold weaker, likely due to the fact that rat ANP differs from human ANP by one amino acid. Wild-type rat ANP has an $EC_{50}$ slightly higher than human ANP when assayed in the human NPRA cGMP assay.

**Example 4: *In vivo* pharmacokinetic studies**

[00187] I*n vivo* pharmacokinetic studies were performed to further characterize the fusion proteins of the present invention.

[00188] Pharmacokinetic properties of the semi-synthetic 11 (L4) and 16 (L5) amino acid linked ANP-Fc fusion monomers (Construct 10 and Construct 14, respectively) and dimers (Construct 11 and Construct 15, respectively) was obtained using intravenous dosing into Wistar rats. Two different ELISAs were used to detect the protein. The ANP/Fc ELISA was used to detect intact ANP-Fc. The Fc/Fc ELISA was used to detect the Fc protein and therefore measure both intact ANP-Fc and Fc to study the possibility of ANP peptide degradation.

[00189] Both monomeric constructs (Construct 10 and Construct 14) possessed an increased $C_{max}$ over that of the dimers (Construct 11 and Construct 15), although the half-lives for the two types of constructs was similar (Table 5). This resulted in AUC's for the monomers that were >5-fold greater than those of the corresponding dimers (Table 5). No significant difference was detected between the two linker lengths on their pharmacokinetics. In addition, it was found that the ANP/Fc and Fc/Fc detection methods provided significantly different results with the Fc/Fc ELISA providing elevated serum concentrations and prolonged half-lives. This data suggests that the ANP-Fc fusion may have partially degraded with time *in vivo*.

**Table 5:** *Pharmacokinetic Parameters of Monomer and Dimer in Wistar Rats*

| Construct Number | Route | Rat | Cmax (ng/ml) | t1/2 (hr) | AUC (hr*ng /ml) | Cl (ml/hr/kg) | Vz (ml/kg) | MRT (hr) |
|---|---|---|---|---|---|---|---|---|
| 10 | i.v. | adult | 4250 | 3.9 | 13947 | 36 | 202 | 3.8 |
| 11 | i.v. | adult | 1280 | 2.8 | 2645 | 189 | 775 | 3.1 |
| 14 | i.v. | adult | 3834 | 4.5 | 14590 | 34 | 220 | 4.4 |
| 15 | i.v. | adult | 429* | 5.5 | 2525 | 198 | 1583 | 5.5 |
| 15 | p.o. | neonatal | 17.2 | 6.5 | 253 | 1799 | 18553 | 9.1 |

[00190] The pharamacokinetics of the recombinant ANP-Fc fusion candidates were assessed by running a single-dose rat PK study. Single bolus injections of ANP-Fc fusions at 1 mg/kg dose were given to Sprague Dawley rats via i.v. or s.c. injection. Vehicle control PBS injections were given to a control group of animals. Plasma samples were collected at

various timepoints (0, 0.083, 0.5, 1, 4, 8, 24, 48, 72, 96, 120, and 168 hr). Animals were sampled for 1 week with a contingency group going up to 3 weeks. After sampling the animals were quarantined and the plasma samples with EDTA and Aprotinin are assayed in a sandwich ELISA where the ANP is captured with a monoclonal antibody and detected with an anti-human Fc antibody. The ELISA has a sensitivity of 1 ng/mL and may be used to detect ANP-Fc fusion proteins in rat plasma. This rat PK data permits the comparison of the *in vivo* half-life of the ANP-Fc fusion proteins to that of the native ligand, hANP28.

[00191] The PK data obtained using the intravenous bolus dosing of the 4 recombinant ANP-Fc fusions into the rats is shown in Table 6. The data obtained demonstrates that the terminal half-life (T1/2) values for the fusion proteins (~11-17 hours) were significantly longer than the native human ANP. Native ANP is reported to have a 0.3 minute T1/2 in rat and 2-3 minute T1/2 in humans. The intravenous dosing PK data also showed that the ANP-Fc fusions showed low clearance and a moderate volume or distribution. From the intravenous dosing PK data none of the four recombinant ANP-Fc proteins could be differentiated.

**Table 6:** *Listing the clearance (CLp), volume of distribution (Vss), half-life (T1/2), and mean resonance time (MRT) data.*

| Construct Number | CLp (mL/min/kg) | Vss (L/kg) | T1/2 (hr) | MRT (hr) |
|---|---|---|---|---|
| 2 | 2.77 | 0.49 | 12.3 | 2.94 |
| 3 | 2.29 | 0.27 | 11.5 | 1.94 |
| 4 | 2.58 | 0.61 | 16.8 | 3.91 |
| 5 | 4.05 | 0.76 | 11.7 | 3.08 |

[00192] The PK data obtained from the subcutaneous dosing of the 4 recombinant ANP-Fc fusions into rats is shown in Table 7. The data obtained suggests that the fusions had a slow absorption phase that results in them having longer half-lives (~18-23 hours). The subcutaneous dosing did however result in low plasma concentration levels. From the subcutaneous dosing PK data obtained no clear differentiation could be made between the four recombinant ANP-Fc fusion proteins.

**Table 7:** *Listing of the maximum serum concentration obtained (Cmax), the area under the curve (AUC), the half-life (T1/2), and the percent bioavailability (%F) observed.*

| Construct Number | Cmax (ng/ml) | AUC (ng.hr/mL) | T1/2 (hr) | %F |
|---|---|---|---|---|
| 2 | 70.8 | 2680 | 18.2 | 22.2 |
| 3 | 71.1 | 2521 | 20.3 | 17.3 |
| 4 | 53.5 | 2671 | 23.1 | 20.6 |
| 5 | 44 | 2202 | 20.6 | 26.3 |

[00193] The presence of an Fc attached to therapeutically relevant ANP peptide may enable non-invasive delivery of the ANP-Fc protein via the FcRn transport pathway. Given that neonatal rats possess high expression levels of FcRn in the gut in the first 15 days of life they are provide a useful model to study FcRn transport. Therefore, to assess the efficiency of FcRn transport, neonatal rats are given a single oral dose of 0.5 mg/kg ANP-Fc dimer (Construct 14). The data suggests that the ANP-Fc protein is not taken up efficiently, and that there is significant breakdown of the protein in the gut as illustrated by the large signal given by the Fc-only ELISA.

**Example 5: Fusion proteins demonstrate increased resistance to proteolytic degradation by NEP**

[00194] Neutral Endopeptidase (NEP, aka Neprilysisn, CALLA neutral endopeptidse 24.11, EC 3.4.24.11) is a type II integral membrane protein. NEP is a zinc metallopeptidase involved in the degradation of several types of proteins, including natriuretic peptides, enkephalins, and substance P and is thought to be responsible for the removal of from 30 to 50 % of ANP from the circulation. See, *e.g.*, J. Kenny et al., Biochem. J. (1993) 291, 8348 (1993). An *in vitro* NEP stability assay has been developed for use as an *in vitro* tool to study this clearance mechanism.

[00195] 100 ng of recombinant human NEP (R&D Systems) was dissolved in cGMP assay buffer and added to the well of polypropylene microtiter plate containing 0.01 μM ANP (Sigma) or fusion protein. The plate was incubated for 60 minutes at room temperature. EDTA (Sigma) in calcium magnesium free PBS (Gibco) to 10 mM was added to each well to stop the reaction. The natriuretic peptide fusion protein induced cGMP assay was performed as described above. 15 μl of the NEP treated reaction mixture was added to the cell suspension and assayed for cGMP production. Data (mean of triplicate wells) indicated that

none of the constructs tested show any significant loss in potency in NPRA mediated cGMP production (see, *e.g.*, Figure 3).

**Example 6: Whole Cell Binding Demonstrated**

[00196] The binding affinity ($K_d$) of the native ligand hANP for human NPRA expressed in whole cells was determined. Human NPRA transfected HEK293 cells were incubated with known concentrations of $^{125}$I-labeled hANP28 at 4°C for 2 hours in PEI coated 96-well glass fiber filter plates. Plates were washed with ice cold buffer by vacuum filtration and allowed to dry. Scintillant was added and the plates were counted on a TopCount Scintillation counter. Equilibrium model 350 (Michaelis-Menten equation [((BLmax*x)/($K_d$+x))] is used to calculate the $K_d$ value of 0.42 nM for hANP binding to NPRA.

[00197] The radioactive ligand whole cell receptor binding assay was used to compare the relative binding affinity of Construct 1 (hANP28) to that of the fusion proteins by performing a heterologous binding competition assay. NPRA transfectants were incubated with a either a fixed amount of $^{125}$I-labeled ANP +/- excess unlabeled ANP or various concentrations of fusion protein at 4°C for 2 hours in PEI coated 96-well glass fiber filter plates. Plates were washed using vacuum filtration and allowed to dry. Scintillant was added and the plates were counted on a TopCount Scintillation counter. Curves were fit using XLfit4.2 and $IC_{50}$ values (Table 8) were determined using the Dose Response One Site Model 205, [4 parameter fit: y = A + ((B-A)/(1+((C/x)^D)))]. The $K_i$ values (see *e.g.*, Table 8) were calculated using the following formula, $K_i = IC_{50}/[1+([L]/K_d)]$ where $K_i$ is the equilibrium dissociation constant for unlabelled ligand, $IC_{50}$ is the concentration causing 50% inhibition of binding, [L] is the concentration of radioligand, and $K_d$ is the equilibrium dissociation constant for the radioligand. The candidates have 40 to 200-fold less affinity for NPRA than the native ligand. The rank affinity for each of the ligands tested is: Construct 1 > Construct 5> Construct 3 > Construct 4 > Construct 2.

**Table 8:** *$IC_{50}$, $K_i$ and relative affinity values for hANP28 and each of the recombinant ANP-Fc fusion proteins.*

| Rank Affinity Determination | | | |
|---|---|---|---|
| **Ligand** | **$IC_{50}$ (nM)** | **$K_i$ (nM)** | **Relative Affinity** |
| **Construct 1** | 5.5 ± 1 | 3.5 ±2.6 | 1 |
| **Construct 2** | 53.7 ± 1.6 | 24.1± 22.5 | 7 |
| **Construct 3** | 11.9 ± 8 | 5.5± 3.1 | 2 |
| **Construct 4** | 29.3 ± 37.2 | 12.3± 14.6 | 4 |
| **Construct 5** | 4.3 ± 2.4 | 2 ± 0.9 | 1 |

[00198] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of skill in the art in light of the teachings of this invention that certain changes and modifications maybe made thereto without departing from the spirit or scope of the disclosure herein, including the appended embodiments.

CLAIMS

1. A fusion protein comprising a natriuretic peptide and an antibody Fc domain, wherein said natriuretic peptide is conjugated to the Fc domain directly or through a linker.

2. The fusion protein of claim 1, wherein the fusion protein comprises the following formula:

$X\text{-}L_a\text{-}F\text{:}F\text{-}L_a\text{-}X$ or $X\text{-}L_a\text{-}F\text{:}F$,

wherein,

X is a natriuretic peptide;

L is a linker comprising a amino acid residues;

a is an integer of at least 0;

: is a chemical association or crosslink; and

F is at least a portion of an immunoglobulin Fc domain comprising an FcRn binding site.

3. The fusion protein of any one of claims 1 or 2, wherein the natriuretic peptide is selected from the group consisting of ANP, BNP, Urodilatin, DNP or a biologically active sequence variant thereof.

4. The fusion protein of any one of claims 1 or 2, wherein the natriuretic peptide is ANP or BNP.

5. The fusion protein of any one of claims 1 or 2, wherein the fusion protein comprises at least two natriuretic peptides.

6. The fusion protein of any one of claims 1, 2 or 5, wherein both natriuretic peptides are ANP.

7. The fusion protein of any one of claims 1, 2 or 5, wherein both natriuretic peptides are BNP.

8. The fusion protein of any one of claims 1 or 2, wherein the fusion protein

comprises at least two Fc domains.

9. The fusion protein of any one of claims 1 or 2, wherein the linker is 6 amino acids in length, 11 amino acids in length, 16 amino acids in length or 20 amino acids in length.

10. The fusion protein of any one of claims 1 or 2, wherein the linker is 6 to 11 amino acids in length, 11 to 16 amino acids in length, 16 to 20 amino acids in length, 16 to 25 amino acids in length or 20 to 30 amino acids in length.

11. The fusion protein of any one of claims 1 or 2, wherein the linker is a glycine succinate linker, an amino acid linker or combination thereof.

12. The fusion protein of any one of claims 1 or 2, wherein the amino acid linker is GlyGly (L2), $Gly(SerGlyGly)_2SerGly$ (L3) (SEQ ID NO. 2), $(GlyGlySer)_3GlyGly$ (L4) (SEQ ID NO. 3), $(GlyGlySer)_4GlyGly$ (SEQ ID NO. 4), $(GlySerGly)_5Gly$ (L5a) (SEQ ID NO. 5), $(GlyGlySer)_5Gly$ (L5) (SEQ ID NO. 6), or $(GlyGlySer)_6GlyGly$ (L6) (SEQ ID NO. 7).

13. A fusion protein comprising at least one or more natriuretic peptides separated from each other by an antibody Fc domain, wherein said natriuretic peptides are conjugated to the Fc domain directly or through a linker.

14. The fusion protein of claim 13, wherein the fusion protein comprises the following formula:

$X\text{-}L_a\text{-}F\text{:}F\text{-}La\text{-}X$,

wherein

X is one or more natriuretic peptides;

L is a linker comprising amino acid residues;

a is an integer of at least 0;

: is a chemical association or crosslink; and

F is at least a portion of an immunoglobulin Fc domain comprising an FcRn binding site.

15. The fusion protein of claim 14, wherein X is more than one natriuretic peptide.

16. The fusion protein of any one of claims 13 or 14, wherein the natriuretic peptide is selected from the group consisting of ANP, BNP, Urodilatin, DNP or a biologically active sequence variant thereof.

17. The fusion protein of any one of claims 13 or 14, wherein the natriuretic peptide is ANP or BNP.

18. The fusion protein of any one of claims 13 or 14, wherein both natriuretic peptides are ANP.

19. The fusion protein of any one of claims 13 or 14, wherein both natriuretic peptides are BNP.

20. The fusion protein of any one of claims 13 or 14, wherein one natriuretic peptide is ANP and the other is BNP.

21. The fusion protein of any one of claims 13 or 14, wherein the linker is 6 amino acids in length, 11 amino acids in length, 16 amino acids in length or 20 amino acids in length.

22. The fusion protein of any one of claims 13 or 14, wherein the linker is 6 to 11 amino acids in length, 11 to 16 amino acids in length, 16 to 20 amino acids in length, 16 to 25 amino acids in length or 20 to 30 amino acids in length.

23. The fusion protein of any one of claims 13 or 14, wherein the linker is a glycine succinate linker, an amino acid linker or combination thereof.

24. The fusion protein of any one of claims 13 or 14, wherein the amino acid linker is GlyGly (L2), $Gly(SerGlyGly)_2SerGly$ (L3) (SEQ ID NO. 2), $(GlyGlySer)_3GlyGly$ (L4) (SEQ ID NO. 3), $(GlyGlySer)_4GlyGly$ (SEQ ID NO. 4), $(GlySerGly)_5Gly$ (L5a) (SEQ ID NO. 5), $(GlyGlySer)_5Gly$ (L5) (SEQ ID NO. 6), or $(GlyGlySer)_6GlyGly$ (L6) (SEQ ID

NO. 7).

25. The fusion protein of any one of claims 13 or 14, wherein the linker is a 6 amino acids in length, 11 amino acids in length, 16 amino acids in length or 20 amino acids in length.

26. The fusion protein of any one of claims 1, 2, 13 or 14, wherein said fusion protein is more resistant to proteolytic degradation than a corresponding wild type natriuretic protein.

27. The fusion protein of any one of claims 1, 2, 13 or 14, wherein said fusion protein displays a longer half-life than a corresponding wild type natriuretic protein.

28. The fusion protein of any one of claims 1, 2, 13 or 14, wherein said fusion protein is made by recombinant techniques, synthetic chemistry or semi-synthetic chemistry.

29. A natriuretic fusion protein comprising any one of SEQ ID NOS. 8-11.

30. A natriuretic fusion protein comprising any one of SEQ ID NOS. 12-13.

31. An isolated polypeptide that exhibits at least 90% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

32. An isolated polypeptide that exhibits at least 90% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

33. An isolated polypeptide that exhibits at least 95% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

34. An isolated polypeptide that exhibits at least 95% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and

SEQ ID NO. 13.

35. An isolated polypeptide that exhibits at least 99% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

36. An isolated polypeptide that exhibits at least 99% sequence identity with a polypeptide having a sequence selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

37. An isolated nucleic acid molecule encoding a polypeptide comprising amino acid sequences selected from the group consisting of SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 and SEQ ID NO. 11.

38. An isolated nucleic acid molecule encoding a polypeptide comprising amino acid sequences selected from the group consisting of SEQ ID NO. 12 and SEQ ID NO. 13.

39. A pharmaceutical composition comprising a fusion protein of any one of claims 1, 2, 13 or 14.

40. The pharmaceutical composition of claim 39, wherein the fusion protein is adapted for intravenous, subcutaneous or oral administration.

41. The pharmaceutical composition of claim 39, wherein the fusion protein is adapted for intravenous administration.

42. The fusion protein of any one of claims 1, 2, 13 or 14, wherein said fusion protein is recombinantly produced by employing mammalian, prokaryotic, yeast, plant, or transgenic expression systems.

43. A method of treating or ameliorating a condition characterized by an excessive level of extracellular fluid, the method comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

44. A method of treating or ameliorating a pathological condition in which activation of the NPRA receptor confers a therapeutic benefit comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

45. A method of treating or ameliorating a disease associated with abnormal diruretic, natriuretic and vasodilatory activity comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

46. A method of treating or ameliorating a disease in which it is desirable to induce naturesis, diuresis, vasodilation or to modulate the renin-angiotensin II and aldosterone systems comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

47. A method to treat or ameliorate a pathological condition of the cardiovascular system selected from the group consisting of chronic heart failure (non-ischemic), reperfusion injury, left ventricular dysfunction (LVD), cardiac fibrosis, diastolic heart failure, and hypertrophic cardiomyopathy comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

48. A method to treat or ameliorate a hypertensive disorder selected from the group consisting of hypertension, pulmonary hypertension, systolic hypertension and resistant hypertension comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

49. A method to treat or ameliorate diabetic nephropathy comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of claim 39.

# FIG. 1A

$ANP_{XY}$-L4-$Fc1_{AB}$:

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtagcct
gcggagatccagctgcttcgggggcaggatggacaggattggagcccagagcggactgggctgta
acagcttccggtacggcggatccggcgggtccggcggatccggcggatgcccaccgtgcccagct
ccggaactgctgggcggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgat
ctcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagt
tcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtac
aacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaagga
gtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagcca
aagggcagccccgagaaccacaggtgtacaccctgcccccatcccgcgatgagctgaccaagaac
caggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagag
caatgggcagccggagaacaactacaagaccacgcctcccgtgttggactccgacggctccttct
tcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctcc
gtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaa
(SEQ ID NO. 24)

**metdtlllwvlllwvpgstg**slrrsscfggrmdrigaqsglgcnsfry*ggsggsggsggg*cppcpa
pellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhnaktkpreeqy
nstyrvvsvltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltkn
qvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscs
vmhealhnhytqkslslspgk (SEQ ID NO. 25)

$ANP_{XY}$-L6-$Fc1_{AB}$:

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtagcct
gcggagatccagctgcttcgggggcaggatggacaggattggagcccagagcggactgggctgta
acagcttccggtacggcggatccggcgggtccggcggctccggcggctccggcgggtccggcgga
tccggcggatgcccaccgtgcccagctccggaactgctgggcggaccgtcagtcttcctcttccc
cccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacg
tgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcataatgcc
aagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcct
gcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagccc
ccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgccc
ccatcccgcgatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcc
cagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctc
ccgtgttggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtgg
cagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaa
gagcctctccctgtctccgggtaaa (SEQ ID NO. 26)

**metdtlllwvlllwvpgstg**slrrsscfggrmdrigaqsglgcnsfry*ggsggsggsggsggsggsgg*
*sgg*cppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhna
ktkpreeqynstyrvvsvltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlp
psrdeltknqvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrw
qqgnvfscsvmhealhnhytqkslslspgk (SEQ ID NO. 27)

# FIG. 1B

$ANP_{XY}$-L4-$Fc2_{AB}$:

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtagcct
gcggagatccagctgcttcgggggcaggatggacaggattggagcccagagcggactgggctgta
acagcttccggtacggcggatccggcgggtccggcggatccggcggatgcccaccttgcccagca
ccacctgtggcaggaccttcagtcttcctcttcccccaaaacccaaggacaccctgatgatctc
cagaacccctgaggtcacgtgcgtggtggtggacgtgagccacgaagaccccgaggtccagttca
actggtacgtggacggcgtggaggtgcataatgccaagacaaagccacgggaggagcagttcaac
agcacgttccgtgtggtcagcgtcctcaccgtcgtgcaccaggactggctgaacggcaaggagta
caagtgcaaggtctccaacaaaggcctcccagcccccatcgagaaaaccatctccaaaaccaaag
ggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccag
gtcagcctgacctgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgggagagcaa
tgggcagccggagaacaactacaagaccacacctcccatgctggactccgacggctccttcttcc
tctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtg
atgcatgaggctctgcacaaccactacacacagaagagcctctccctgtctccgggtaaa (SEQ
ID NO. 28)

**metdtlllwvlllwvpgstg**slrrsscfggrmdrigaqsglgcnsfry*ggsggsggsggc*cppcpa
ppvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnaktkpreeqfn
stfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlppsreemtknq
vsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwqqgnvfscsv
mhealhnhytqkslslspgk (SEQ ID NO. 29)

$ANP_{XY}$-L6-$Fc2_{AB}$:

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtagcct
gcggagatccagctgcttcgggggcaggatggacaggattggagcccagagcggactgggctgta
acagcttccggtacggcggatccggcgggtccggcggctccggcggctccggcgggtccggcgga
tccggcggatgcccaccttgcccagcaccacctgtggcaggaccttcagtcttcctcttcccccc
aaaacccaaggacaccctgatgatctccagaacccctgaggtcacgtgcgtggtggtggacgtga
gccacgaagaccccgaggtccagttcaactggtacgtggacggcgtggaggtgcataatgccaag
acaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcgtcctcaccgtcgtgca
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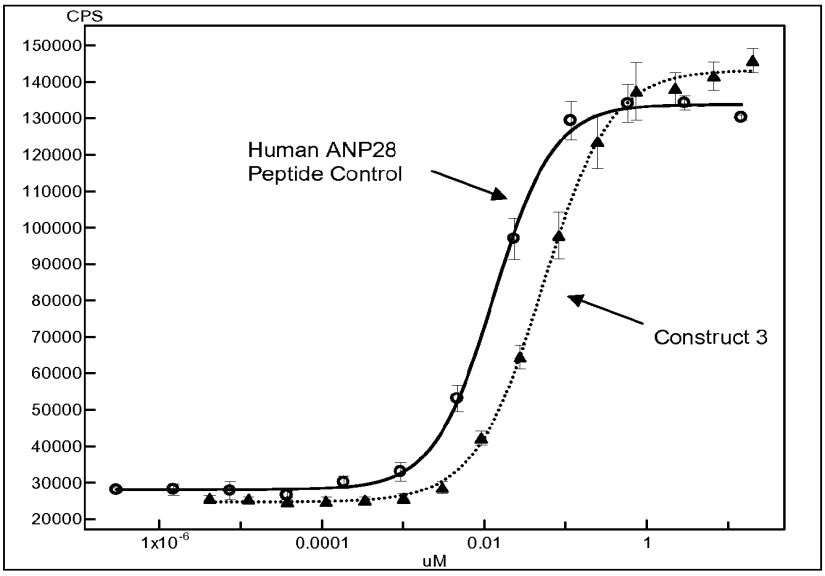
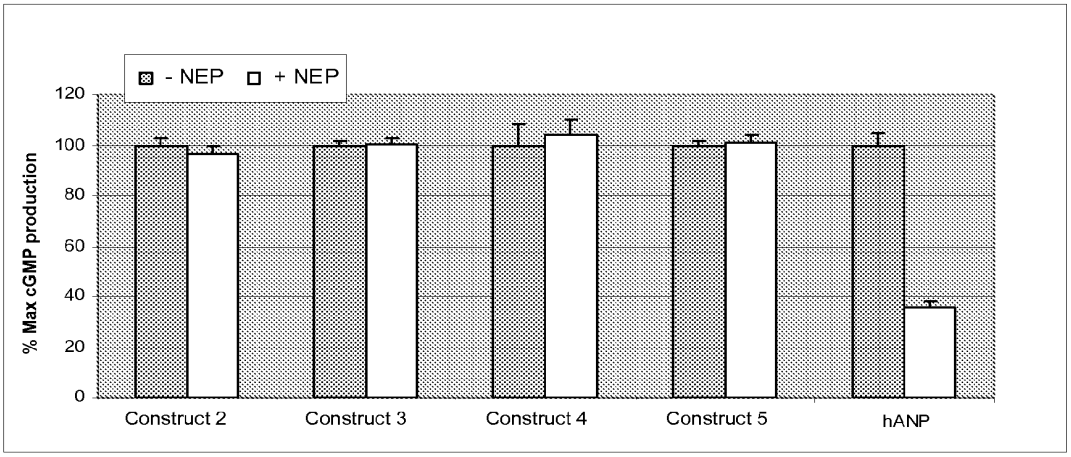
tcgagaaaaccatctccaaaaccaaagggcagccccgagaaccacaggtgtacaccctgccccca
tcccgggaggagatgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctaccccag
cgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacacctccca
tgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcag
caggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacacagaagag
cctctccctgtctccgggtaaa (SEQ ID NO. 30)

**metdtlllwvlllwvpgstg**slrrsscfggrmdrigaqsglgcnsfry*ggsggsggsggsggsggg*
*sggc*ppcpappvagpsvflfppkpkdtlmisrtpevtcvvvdvshedpevqfnwyvdgvevhnak
tkpreeqfnstfrvvsvltvvhqdwlngkeykckvsnkglpapiektisktkgqprepqvytlpp
sreemtknqvsltclvkgfypsdiavewesngqpennykttppmldsdgsfflyskltvdksrwq
qgnvfscsvmhealhnhytqkslslspgk (SEQ ID NO. 31)

# FIG. 2


CPS
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
Human ANP28
Peptide Control
Construct 3
1x10-6
0.0001
0.01
1
uM

# FIG. 3


- NEP
+ NEP
% Max cGMP production
120
100
80
60
40
20
0
Construct 2
Construct 3
Construct 4
Construct 5
hANP

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/065659

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/58 C12N15/62 A61K38/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, Sequence Search, BIOSIS, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 September 2008 | 22/09/2008 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Piret, Bernard |

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PCT/US2008/065659

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| 10/845,895 | 14 May 2004 (14.05.2004) | US |
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| 11/054,071 | 8 February 2005 (08.02.2005) | US |


(71) Applicant *(for all designated States except US)*: **MICROBIA, INC.** [US/US]; 320 Bent Street, Cambridge, MA 02141 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CURRIE, Mark, G.** [US/US]; 18 Hall Avenue, Sterling, MA 01564 (US). **MAHAJAN-MIKLOS, Shalina** [IN/US]; Stanford West, 909 Varian Way, Palo Alto, CA 94304 (US). **FRETZEN, Angelika** [DE/US]; 155R Summer Street, #1, Somerville, MA 02143 (US). **SUN, Li Jing** [CN/US]; 88 Greenwich Street, Unit 623, New York, NY 10006 (US).

(74) Agent: **MEIKLEJOHN, Anita, L.**; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF GASTROINTESTINAL DISORDERS

(57) Abstract: The present invention features compositions and related methods for treating IBS and other gastrointestinal disorders and conditions (e.g., gastrointestinal motility disorders, functional gastrointestinal disorders, gastroesophageal reflux disease (GERD), duodenogastric reflux, Crohn's disease, ulcerative colitis, Inflammatory bowel disease, functional heartburn, dyspepsia (including functional dyspepsia or nonulcer dyspepsia), gastroparesis, chronic intestinal pseudo-obstruction (or colonic pseudo-obstruction), and disorders and conditions associated with constipation, e.g., constipation associated with use of opiate pain killers, post-surgical constipation (post-operative ileus), and constipation associated with neuropathic disorders as well as other conditions and disorders using peptides and other agents that activate the guanylate cyclase C (GC-C) receptor.

WO 2005/087797 A1

# Methods and Compositions for the Treatment of Gastrointestinal Disorders

## CLAIM OF PRIORITY

This application is a continuation in part of U.S. Utility Patent Application Serial No.11/054,071, filed February 8, 2005, which is a continuation in part U.S. Utility Patent Application Serial No.10/899,806, filed July 27, 2004, which is a continuation in part of U.S. Utility Patent Application Serial No.10/845,895, filed May 14, 2004, which is a continuation in part of U.S. Utility Patent Application Serial No. 10/796,719, filed March 9, 2004, which is a continuation in part of U.S. Utility Patent Application Serial No. 10/766,735, filed January 28, 2004, which claims priority under 35 USC §119(e) to U.S. Provisional Patent Application Serial No. 60/443,098, filed on January 28, 2003; U.S. Provisional Patent Application Serial No. 60/471,288, filed on May 15, 2003 and U.S. Provisional Patent Application Serial No. 60/519,460, filed on November 12, 2003, the entire contents of which are hereby incorporated by reference.

## TECHNICAL FIELD

This invention relates to methods and compositions for treating various disorders, including gastrointestinal disorders, obesity, congestive heart failure and benign prostatic hyperplasia.

## BACKGROUND

Irritable bowel syndrome (IBS) is a common chronic disorder of the intestine that affects 20 to 60 million individuals in the US alone (Lehman Brothers, Global Healthcare-Irritable bowel syndrome industry update, September 1999). IBS is the most common disorder diagnosed by gastroenterologists (28% of patients examined) and accounts for 12% of visits to primary care physicians (Camilleri 2001, Gastroenterology 120:652-668). In the US, the economic impact of IBS is estimated at $25 billion annually, through direct costs of health care use and indirect costs of absenteeism from work (Talley 1995, Gastroenterology 109:1736-1741). Patients with IBS have three times more absenteeism from work and report a reduced quality of life.

Sufferers may be unable or unwilling to attend social events, maintain employment, or travel even short distances (Drossman 1993, Dig Dis Sci 38:1569-1580). There is a tremendous unmet medical need in this population since few prescription options exist to treat IBS.

Patients with IBS suffer from abdominal pain and a disturbed bowel pattern. Three subgroups of IBS patients have been defined based on the predominant bowel habit: constipation-predominant (c-IBS), diarrhea-predominant (d-IBS) or alternating between the two (a-IBS). Estimates of individuals who suffer from c-IBS range from 20-50% of the IBS patients with 30% frequently cited. In contrast to the other two subgroups that have a similar gender ratio, c-IBS is more common in women (ratio of 3:1) (Talley et al. 1995, Am J Epidemiol 142:76-83).

The definition and diagnostic criteria for IBS have been formalized in the "Rome Criteria" (Drossman et al. 1999, Gut 45:Suppl II: 1-81), which are well accepted in clinical practice. However, the complexity of symptoms has not been explained by anatomical abnormalities or metabolic changes. This has led to the classification of IBS as a functional GI disorder, which is diagnosed on the basis of the Rome criteria and limited evaluation to exclude organic disease (Ringel et al. 2001, Annu Rev Med 52: 319-338). IBS is considered to be a "biopsychosocial" disorder resulting from a combination of three interacting mechanisms: altered bowel motility, an increased sensitivity of the intestine or colon to pain stimuli (visceral sensitivity) and psychosocial factors (Camilleri 2001, Gastroenterology 120:652-668). Recently, there has been increasing evidence for a role of inflammation in etiology of IBS. Reports indicate that subsets of IBS patients have small but significant increases in colonic inflammatory and mast cells, increased inducible nitric oxide (NO) and synthase (iNOS) and altered expression of inflammatory cytokines (reviewed by Talley 2000, Medscape Coverage of DDW week).

## SUMMARY

The present invention features compositions and related methods for treating IBS and other gastrointestinal disorders and conditions (e.g., gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric

reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein. The compositions feature peptides that activate the guanylate cyclase C (GC-C) receptor.

The present invention also features compositions and related methods for treating obesity, congestive heart failure and benign prostatic hyperplasia (BPH).

Without being bound by any particular theory, in the case of IBS and other gastrointestinal disorders the peptides are useful because they may increase gastrointestinal motility.

Without being bound by any particular theory, in the case of IBS and other gastrointestinal disorders the peptides are useful, in part, because they may decrease inflammation.

Without being bound by any particular theory, in the case of IBS and other gastrointestinal disorders the peptides are also useful because they may decrease gastrointestinal pain or visceral pain.

The invention features pharmaceutical compositions comprising certain peptides that are capable of activating the guanylate-cyclase C (GC-C) receptor. Also within the invention are pharmaceutical compositions comprising a peptide or GC-C agonist of the invention and one or more additional therapeutic agents including, without limitation, the agents described herein. The other agents can be administered with the peptides of the invention (simultaneously or sequentially). They can also be linked to a peptide of the invention to create therapeutic conjugates.

The invention includes methods for treating various gastrointestinal disorders by administering a peptide that acts as a partial or complete agonist of the GC-C receptor. The peptide includes at least six cysteines that can form three disulfide bonds. In certain embodiments the disulfide bonds are replaced by other covalent cross-links and in some cases the cysteines are substituted by other residues to provide for alternative covalent cross-links. The peptides may also include at least one trypsin or chymotrypsin cleavage site and/or an amino or carboxy-terminal analgesic peptide or small molecule, e.g., AspPhe or some other analgesic peptide. When present within the peptide, the analgesic peptide or small molecule may be preceded by a chymotrypsin or trypsin cleavage site that allows release of the analgesic peptide or small molecule. The peptides and methods of the invention are also useful for treating pain and inflammation associated with various disorders, including gastrointestinal disorders. Certain peptides include a functional chymotrypsin or trypsin cleavage site located so as to allow inactivation of the peptide upon cleavage. Certain peptides having a functional cleavage site undergo cleavage and gradual inactivation in the digestive tract, and this is desirable in some circumstances. In certain peptides, a functional chymotrypsin site is altered, increasing the stability of the peptide *in vivo*.

The invention includes methods for treating other disorders such as congestive heart failure and benign prostatic hyperplasia by administering a peptide or small molecule (parenterally or orally) that acts as an agonist of the GC-C receptor. Such agents can be used in combination with natriuretic peptides (e.g., atrial natriuretic peptide, brain natriuretic peptide or C-type natriuretic peptide), a diuretic, or an inhibitor of angiotensin converting enzyme.

The invention features methods and compositions for increasing intestinal motility. Intestinal motility involves spontaneous coordinated dissentions and contractions of the stomach, intestines, colon and rectum to move food through the gastrointestinal tract during the digestive process.

In certain embodiments the peptides include either one or two or more contiguous negatively charged amino acids (e.g., Asp or Glu) or one or two or more contiguous positively charged

residues (e.g., Lys or Arg) or one or two or more contiguous positively or negatively charged amino acids at the carboxy terminus. In these embodiments all of the flanking amino acids at the carboxy terminus are either positively or negatively charged. In other embodiments the carboxy terminal charged amino acids are preceded by a Leu. For example, any of the following amino acid sequences can be added to the carboxy terminus of the peptide: Asp; Asp Lys; Lys Lys Lys Lys Lys Lys; Asp Lys Lys Lys Lys Lys Lys; Leu Lys Lys; and Leu Asp. It is also possible to simply add Leu at the carboxy terminus.

In a first aspect, the invention features a peptide comprising, consisting of, or consisting essentially of the amino acid sequence (I):

$Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}$
$Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (SEQ ID NO: 1)

In some embodiments $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ is Asn Ser Ser Asn Tyr or is missing or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4$ is missing.

In certain embodiments $Xaa_8$, $Xaa_9$, $Xaa_{12}$, $Xaa_{14}$, $Xaa_{16}$, $Xaa_{17}$, and $Xaa_{19}$ can be any amino acid. In certain embodiments $Xaa_8$, $Xaa_9$, $Xaa_{12}$, $Xaa_{14}$, $Xaa_{16}$, $Xaa_{17}$, and $Xaa_{19}$ can be any natural or non-natural amino acid or amino acid analog.

In certain embodiments, the peptide does not include the sequence of E. coli ST peptide. In other embodiment, the peptide does not include the sequence of any of the peptides in Table 1, below.

In certain embodiments $Xaa_5$ is Asn, Trp, Tyr, Asp, or Phe. In other embodiments, $Xaa_5$ can also be Thr or Ile. In other embodiments $Xaa_5$ is Tyr, Asp or Trp. In certain embodiments $Xaa_5$ is Asn, Trp, Tyr, Asp, Ile, Thr or Phe. In certain embodiments $Xaa_5$ is Asn.

In some embodiments $Xaa_8$ is Glu, Asp, Gln, Gly or Pro. In other embodiments $Xaa_8$ is Glu. In other embodiments $Xaa_8$ is Glu or Asp. In others it is Asn, Glu, or Asp. In others it is Glu, His, Lys, Gln, Asn, or Asp. In others it is Glu, His, Gln, Asn, or Asp. In others it is Glu, Asn, His, Gln, Lys, Asp or Ser. In still others it is Pro. In certain embodiments it is any natural or non-natural amino acid or amino acid analog.

In some embodiments $Xaa_9$ is Leu, Ile, Val, Ala, Lys, Arg, Trp, Tyr or Phe. In some embodiments $Xaa_9$ is Leu, Ile, Val, Lys, Arg, Trp, Tyr or Phe. In others it is Leu, Ile, Val, Trp, Tyr or Phe. In others it is Leu, Ile or Val. In others it is Trp, Tyr or Phe. In others it is Leu, Ile, Lys, Arg, Trp, Tyr, or Phe. In others it is Leu, Val, Ile, or Met. In others it is Leu or Phe. In others it is Leu, Phe, or Tyr. In others it is Tyr, Phe or His. In others it is Phe, His, Trp, or Tyr. In certain embodiments, $Xaa_9$ is not Leu. In others it is Tyr. In other embodiments it is any natural or non-natural aromatic amino acid or amino acid analog. In certain embodiments it is any natural or non-natural amino acid or amino acid analog.

In certain embodiments, $Xaa_{12}$ is Asn, Tyr, Asp or Ala. In others it is Asn. In others it is Asn, Met, Arg, Lys, His, or Gln. In others it is Asn, Lys, His, or Gln. In others it is Asn, Asp, Glu or Gln. In others it is Asn, Thr, Ser, Arg, Lys, Gln, or His. In others it is Asn, Ser, or His. In certain embodiments it is any natural or non-natural amino acid or amino acid analog.

In certain embodiments, $Xaa_{13}$ is is Ala, Pro or Gly. In others it is Pro or Gly. In others it is Pro and in still others it is Gly.

In certain embodiments, $Xaa_{14}$ is Ala, Leu, Ser, Gly, Val, Glu, Gln, Ile, Leu, Thr, Lys, Arg, or Asp. In others it is Ala or Gly. In others it is Val or Ala. In others it is Ala or Thr. In others it is Ala. In others it is Val, Gln, Asn, Glu, Asp, Thr, or Ala. In others it is Gly, Cys or Ser. In still others it is Thr. In certain embodiments it is any natural or non-natural amino acid or amino acid analog.

In certain embodiments $Xaa_{16}$ is Thr, Ala, Asn, Lys, Arg, Trp, Gly or Val. In others it is Thr, Ala, Asn, Lys, Arg or Trp. In others it is Thr, Ala, Lys, Arg or Trp. In certain embodiments it is Thr, Ala or Trp. In others it is Thr. In certain embodiments it is Trp, Tyr or Phe. In certain embodiments it is Thr or Ala. In certain embodiments it is Val. In certain embodiments it is Gly. In others it is Thr, Ser, Met or Val. In others it is Val, Ala, or Thr. In others it is Ile, Val, Lys, Asn, Glu, Asp, or Thr. In certain embodiments it is any natural or non-natural amino acid or amino acid analog. In certain embodiments it is any natural or non-natural non-aromatic amino acid or amino acid analog.

In certain embodiments $Xaa_{17}$ is Gly, Pro or Ala. In certain embodiments it is Gly. In certain embodiments it is Ala. In others it is Gly or Ala. In others it is Gly, Asn, Ser or Ala. In others it is Asn, Glu, Asp, Thr, Ala, Ser, or Gly. In others it is Asp, Ala, Ser, or Gly. In certain embodiments it is any natural or non-natural amino acid or amino acid analog.

In certain embodiments $Xaa_{19}$ is Trp, Tyr, Phe, Asn, Ile, Val, His, Leu, or Arg. In certain embodiments it is Trp, Tyr, Asn or Leu. In certain embodiments it is Trp, Tyr or Phe. In others it is Tyr, Phe or His. In others it is Tyr or Trp. In others it is Tyr. In certain embodiments it is Leu, Ile or Val. In certain embodiments it is His. In certain embodiments it is Trp, Tyr, Phe, Asn, Ile, Val, His or Leu. In certain embodiments it is Trp, Tyr, Phe or Leu. In certain embodiments it is Tyr or Leu. In certain embodiments it is Lys or Arg. In certain embodiments it is any amino acid other than Pro, Arg, Lys, Asp or Glu. In certain embodiments it is any amino acid other than Pro. In certain embodiments it is any natural or non-natural amino acid or amino acid analog. In certain embodiments it is missing.

In certain embodiments $Xaa_{20}$ is Asp or Asn. In certain embodiments $Xaa_{20}Xaa_{21}$ is AspPhe or is missing or $Xaa_{20}$ is Asn or Glu and $Xaa_{21}$ is missing or $Xaa_{19}Xaa_{20}Xaa_{21}$ is missing.

In certain embodiments, the invention features, a purified polypeptide comprising the amino acid sequence (II):

$Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}$
$Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$

wherein $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ is Asn Ser Ser Asn Tyr or is missing or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4$ is missing and $Xaa_5$ is Asn;

$Xaa_8$ is Glu or Asp;

$Xaa_9$ is Leu, Ile, Val, Trp, Tyr or Phe;

$Xaa_{16}$ is Thr, Ala, Trp;

$Xaa_{19}$ is Trp, Tyr, Phe or Leu or is missing; and $Xaa_{20}\ Xaa_{21}$ is AspPhe.

In various embodiments the invention features a purified polypeptide comprising the amino acid sequence (II): $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ wherein, $Xaa_9$ is Leu, Ile or Val and $Xaa_{16}$ is Trp, Tyr or Phe; $Xaa_9$ is Trp, Tyr or Phe, and $Xaa_{16}$ is Thr or Ala; $Xaa_{19}$ is Trp, Tyr, Phe and $Xaa_{20}\ Xaa_{21}$ is AspPhe; and $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4$ is missing and $Xaa_5$ is Asn; the peptide comprises fewer than 50, 40, 30 or 25 amino acids; or fewer than five amino acids precede $Cys_6$.

In certain embodiments the peptide includes a peptide comprising or consisting of the amino acid sequence $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ Cys Cys Glu $Xaa_9$ Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr $Xaa_{20}\ Xaa_{21}$ (II) (SEQ ID NO:2) wherein $Xaa_9$ is any amino acid: wherein $Xaa_9$ is any amino acid other than Leu; wherein $Xaa_9$ is selected from Phe, Trp and Tyr; wherein $Xaa_9$ is selected from any other natural or non-natural aromatic amino acid; wherein $Xaa_9$ is Tyr; wherein $Xaa_9$ is Phe; wherein $Xaa_9$ is Trp; wherein $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ is Asn Ser Ser Asn Tyr; wherein $Xaa_1$, $Xaa_2$, $Xaa_3$, $Xaa_4$, and $Xaa_5$ are missing; wherein $Xaa_1$, $Xaa_2$, $Xaa_3$ and $Xaa_4$ are missing; wherein $Xaa_1$, $Xaa_2$ and $Xaa_3$ are missing; wherein $Xaa_1$ and $Xaa_2$ are missing; wherein $Xaa_1$ is missing; wherein $Xaa_{20}\ Xaa_{21}$ is AspPhe or is missing or $Xaa_{20}$ is Asn or Glu and $Xaa_{21}$ is missing or $Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ is missing; wherein $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ and Tyr $Xaa_{20}\ Xaa_{21}$ are missing. In the case of a peptide comprising the sequence (I): $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ wherein: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ is missing and/or the sequence

$Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ is missing, the peptide can still contain additional carboxyterminal or amino terminal amino acids or both. In the case of peptides missing one or more terminal amino acids such as $Xaa_1$ or $Xaa_{21}$, the peptide can still contain additional carboxyterminal or amino terminal amino acids or both.

In certain embodiments, the peptide includes disulfide bonds between $Cys_6$ and $Cys_{11}$, between $Cys_7$ and $Cys_{15}$ and between $Cys_{10}$ and $Cys_{16}$. In other embodiments, the peptide is a reduced peptide having no disulfide bonds. In still other embodiments the peptide has one or two disulfide bonds chosen from: a disulfide bond between $Cys_6$ and $Cys_{11}$, a disulfide bond between $Cys_7$ and $Cys_{15}$ and a disulfide bond between $Cys_{10}$ and $Cys_{16}$.

In certain embodiments, one or more amino acids can be replaced by a non-naturally occurring amino acid or a naturally or non-naturally occurring amino acid analog. There are many amino acids beyond the standard 20. Some are naturally-occurring others are not (see, for example, Hunt, The Non-Protein Amino Acids: In Chemistry and Biochemistry of the Amino Acids, Barrett, Chapman and Hall, 1985). For example, an aromatic amino acid can be replaced by 3,4-dihydroxy-L-phenylalanine, 3-iodo-L-tyrosine, triiodothyronine, L-thyroxine, phenylglycine (Phg) or nor-tyrosine (norTyr). Phg and norTyr and other amino acids including Phe and Tyr can be substituted by, e.g., a halogen, -CH3, -OH, $-CH_2NH_3$, -C(O)H, $-CH_2CH_3$, -CN, $-CH_2CH_2CH_3$, -SH, or another group. Any amino acid can be substituted by the D-form of the amino acid.

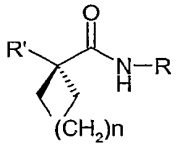
With regard to non-naturally occurring amino acids or a naturally and non-naturally occurring amino acid analogs, a number of substitutions in the peptide of formula I or the peptide of formula II are possible alone or in combination.

$Xaa_8$ can be replaced by *gamma*-Hydroxy-Glu or *gamma*-Carboxy-Glu.

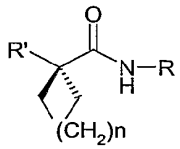
$Xaa_9$ can be replaced by an alpha substituted amino acid such as L-*alpha*-methylphenylalanine or by analogues such as: 3-Amino-Tyr; $Tyr(CH_3)$; $Tyr(PO_3(CH_3)_2)$; $Tyr(SO_3H)$; *beta*-Cyclohexyl-Ala;

*beta*-(1-Cyclopentenyl)-Ala; *beta*-Cyclopentyl-Ala; *beta*-Cyclopropyl-Ala; *beta*-Quinolyl-Ala; *beta*-(2-Thiazolyl)-Ala; *beta*-(Triazole-1-yl)-Ala; *beta*-(2-Pyridyl)-Ala; *beta*-(3-Pyridyl)-Ala; Amino-Phe; Fluoro-Phe; Cyclohexyl-Gly; *t*Bu-Gly; *beta*-(3-benzothienyl)-Ala; *beta*-(2-thienyl)-Ala; *5*-Methyl-Trp; and *4*-Methyl-Trp.

$Xaa_{13}$ can be an N(alpha)-C(*alpha*) cyclized amino acid analogues with the structure:




n = 0, 1, 2, 3 . $Xaa_{13}$ can also be homopro (L-pipecolic acid); hydroxy-Pro; 3,4-Dehydro-Pro; 4-fluoro-Pro; or *alpha*-methyl-Pro.




When $Xaa_{13}$ is Gly, Ala, Leu or Val, $Xaa_{14}$ can be: n = 0, 1, 2, 3

$Xaa_{14}$ can also be an alpha-substitued or N-methylated amino acid such as *alpha*-amino isobutyric acid (*aib*), L/D-*alpha*-ethylalanine (L/D-isovaline), L/D-methylvaline, or L/D-*alpha*-methylleucine or a non-natural amino acid such as *beta*-fluoro-Ala.

$Xaa_{17}$ can be *alpha*-amino isobutyric acid (*aib*) or L/D-*alpha*-ethylalanine (L/D-isovaline).

Further examples of unnatural amino acids include: an unnatural analogue of tyrosine; an unnatural analogue of glutamine; an unnatural analogue of phenylalanine; an unnatural analogue of serine; an unnatural analogue of threonine; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or any combination thereof; an amino

acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; an amino acid with a novel functional group; an amino acid that covalently or noncovalently interacts with another molecule; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a glycosylated or carbohydrate modified amino acid; a keto containing amino acid; amino acids comprising polyethylene glycol or polyether; a heavy atom substituted amino acid (e.g., an amino acid containing deuterium, tritium, $^{13}C$, $^{15}N$, or $^{18}O$); a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid, e.g., a sugar substituted serine or the like; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α.-hydroxy containing acid; an amino thio acid containing amino acid; an α, α disubstituted amino acid; a β-amino acid; a cyclic amino acid other than proline; an O-methyl-L-tyrosine; an L-3-(2-naphthyl)alanine; a 3-methyl-phenylalanine; a *p*-acetyl-L-phenylalanine; an 0-4-allyl-L-tyrosine; a 4-propyl-L-tyrosine; a tri-O-acetyl-GlcNAcβ-serine; an L-Dopa; a fluorinated phenylalanine; an isopropyl-L-phenylalanine; a p-azido-L-phenylalanine; a p-acyl-L-phenylalanine; a p-benzoyl-L-phenylalanine; an L-phosphoserine; a phosphonoserine; a phosphonotyrosine; a p-iodo-phenylalanine; a 4-fluorophenylglycine; a p-bromophenylalanine; a p-amino-L-phenylalanine; an isopropyl-L-phenylalanine; L-3-(2-naphthyl)alanine; an amino-, isopropyl-, or O-allyl-containing phenylalanine analogue; a dopa, O-methyl-L-tyrosine; a glycosylated amino acid; a p-(propargyloxy)phenylalanine; dimethyl-Lysine; hydroxy-proline; mercaptopropionic acid; methyl-lysine; 3-nitro-tyrosine; norleucine; pyro-glutamic acid; Z (Carbobenzoxyl); ε-Acetyl-Lysine; β-alanine; aminobenzoyl derivative; aminobutyric acid (Abu); citrulline; aminohexanoic acid; aminoisobutyric acid; cyclohexylalanine; d-cyclohexylalanine; hydroxyproline; nitro-arginine; nitro-phenylalanine; nitro-tyrosine; norvaline; octahydroindole carboxylate; ornithine; penicillamine; tetrahydroisoquinoline; acetamidomethyl protected amino acids and pegylated amino acids. Further examples of unnatural amino acids and amino acid analogs can be found in U.S. 20030108885, U.S. 20030082575, and the references cited therein.

In some embodiments, an amino acid can be replaced by a naturally-occurring, non-essential amino acid, e.g., taurine.

Methods to manfacture peptides containing unnatural amino acids can be found in, for example, U.S. 20030108885, U.S. 20030082575, Deiters et al., J Am Chem Soc. (2003) 125:11782-3, Chin et al., Science (2003) 301:964-7, and the references cited therein.

Peptides that include non-natural amino acids can also be prepared using the methods described in WO02086075

The peptides of the invention can have one or more conventional peptide bonds replaced by an alternative bond. Such replacements can increase the stability of the peptide. For example, replacement of the peptide bond between $Cys_{18}$ and $Xaa_{19}$ with an alternative bond can reduce cleavage by carboxy peptidases and may increase half-life in the digestive tract. Bonds that can replace peptide bonds include: a retro-inverso bonds (C(O)-NH instead of NH-C(O); a reduced amide bond (NH-$CH_2$); a thiomethylene bond (S-$CH_2$ or $CH_2$-S); an oxomethylene bond (O-$CH_2$ or $CH_2$-O); an ethylene bond ($CH_2$-$CH_2$); a thioamide bond (C(S)-NH); a trans-olefine bond (CH=CH); an fluoro substituted trans-olefine bond (CF=CH); a ketomethylene bond (C(O)-CHR or CHR-C(O) wherein R is H or $CH_3$; and a fluoro-ketomethylene bond (C(O)-CFR or CFR-C(O) wherein R is H or F or $CH_3$.

The peptides of the invention can be modified using standard modifications. Modifications may occur at the amino (N-), carboxy (C-) terminus, internally or a combination of any of the preceeding. In one aspect of the invention, there may be more than one type of modification of the peptide. Modifications include but are not limited to: acetylation, amidation, biotinylation, cinnamoylation, farnesylation, formylation, myristoylation, palmitoylation, phosphorylation (Ser, Tyr or Thr), stearoylation, succinylation, sulfurylation and cyclisation (via disulfide bridges or amide cyclisation), and modification by Cy3 or Cy5. The peptides of the invention may also be modified by 2, 4-dinitrophenyl (DNP), DNP-lysin, modification by 7-Amino-4-methyl-coumarin (AMC), flourescein, NBD (7-Nitrobenz-2-Oxa-1,3-Diazole), p-nitro-anilide, rhodamine B, EDANS (5-((2-aminoethyl)amino)naphthalene-1- sulfonic acid), dabcyl, dabsyl,

dansyl, texas red, FMOC, and Tamra (Tetramethylrhodamine). The peptides of the invention may also be conjugated to, for example, polyethylene glycol (PEG); alkyl groups (e.g., C1-C20 straight or branched alkyl groups); fatty acid radicals; combinations of PEG, alkyl groups and fatty acid radicals (see U.S. Patent 6,309,633; Soltero et al., 2001 Innovations in Pharmaceutical Technology 106-110); BSA and KLH (Keyhole Limpet Hemocyanin).

The peptides and agonists of the invention can be chemically modified to increase therapeutic activity by synthetically adding sugar moieties (WO 88/02756; WO 89/09786; DE 3910667 A1, EP 0 374 089 A2; and U.S. 4,861,755), adding cationic anchors (EP0363589), lipid moieties (WO91/09837; U.S. 4,837,303) or the substituents described as compounds I, II, and III in US5552520.

When $Xaa_9$ is Trp, Tyr or Phe or when $Xaa_{16}$ is Trp the peptide has a potentially functional chymotrypsin cleavage site that is located at a position where cleavage may alter GC-C receptor binding by the peptide. When $Xaa_9$ is Lys or Arg or when $Xaa_{16}$ is Lys or Arg, the peptide has a potentially functional trypsin cleavage site that is located at a position where cleavage may alter GC-C receptor binding by the peptide.

When $Xaa_{19}$ is Trp, Tyr or Phe, the peptide has a chymotrypsin cleavage site that is located at a position where cleavage will liberate the portion of the peptide carboxy-terminal to $Xaa_{19}$. When $Xaa_{19}$ is Leu, Ile or Val, the peptide can have a chymotrypsin cleavage site that is located at a position where cleavage will liberate the portion of the peptide amino-terminal to $Xaa_{19}$. At relatively high pH the same effect is seen when $Xaa_{19}$ is His. When $Xaa_{19}$ is Lys or Arg, the peptide has a trypsin cleavage site that is located at a position where cleavage will liberate portion of the peptide carboxy-terminal to $Xaa_{19}$. Thus, if the peptide includes an analgesic peptide carboxy-terminal to $Xaa_{19}$, the peptide will be liberated in the digestive tract upon exposure to the appropriate protease. Among the analgesic peptides which can be included in the peptide and/or coadministered with the peptide are: AspPhe (as $Xaa_{20}Xaa_{21}$), endomorphin-1, endomorphin-2, nocistatin, dalargin, lupron, ziconotide, and substance P and other analgesic peptides described herein. These peptides can, for example, be used to replace $Xaa_{20}Xaa_{21}$.

When $Xaa_1$ or the amino-terminal amino acid of the peptide of the invention (e.g., $Xaa_2$ or $Xaa_3$) is Trp, Tyr or Phe, the peptide has a chymotrypsin cleavage site that is located at a position where cleavage will liberate the portion of the peptide amino-terminal to $Xaa_1$ (or $Xaa_2$ or $Xaa_3$) along with $Xaa_1$, $Xaa_2$ or $Xaa_3$. When $Xaa_1$ or the amino-terminal amino acid of the peptide of the invention (e.g., $Xaa_2$ or $Xaa_3$) is Lys or Arg, the peptide has a trypsin cleavage site that is located at a position where cleavage will liberate portion of the peptide amino-terminal to $Xaa_1$ along with $Xaa_1$, $Xaa_2$ or $Xaa_3$). When $Xaa_1$ or the amino-terminal amino acid of the peptide of the invention is Leu, Ile or Val, the peptide can have a chymotrypsin cleavage site that is located at a position where cleavage will liberate the portion of the peptide amino-terminal to $Xaa_1$. At relatively high pH the same effect is seen when $Xaa_1$ is His. Thus, for example, if the peptide includes an analgesic peptide amino-terminal to $Xaa_1$, the peptide will be liberated in the digestive tract upon exposure to the appropriate protease. Among the analgesic peptides which can be included in the peptide are: AspPhe, endomorphin-1, endomorphin-2, nocistatin, dalargin, lupron, and substance p and other analgesic peptides described herein.

When fully folded, disulfide bonds may be present between: $Cys_6$ and $Cys_{11}$; $Cys_7$ and $Cys_{15}$; and $Cys_{10}$ and $Cys_{18}$. The peptides of the invention bear some sequence similarity to ST peptides. However, they include amino acid changes and/or additions that improve functionality. These changes can, for example, increase or decrease activity (e.g., increase or decrease the ability of the peptide to stimulate intestinal motility), alter the ability of the peptide to fold correctly, alter the stability of the peptide, alter the ability of the peptide to bind the GC-C receptor and/or decrease toxicity. In some cases the peptides may function more desirably than wild-type ST peptide. For example, they may limit undesirable side effects such as diarrhea and dehydration.

In some embodiments one or both members of one or more pairs of Cys residues which normally form a disulfide bond can be replaced by homocysteine, penicillamine, 3-mercaptoproline (Kolodziej et al. 1996 Int J Pept Protein Res 48:274); β, β dimethylcysteine

(Hunt et al. 1993 Int J Pept Protein Res 42:249) or diaminopropionic acid (Smith et al. 1978 J Med Chem 21:117) to form alternative internal cross-links at the positions of the normal disulfide bonds.

In addition, one or more disulfide bonds can be replaced by alternative covalent cross-links, e.g., an amide linkage ($-CH_2CH(O)NHCH_2-$ or $-CH_2NHCH(O)CH_2-$), an ester linkage, a thioester linkage, a lactam bridge, a carbamoyl linkage, a urea linkage, a thiourea linkage, a phosphonate ester linkage, an alkyl linkage ($-CH_2CH_2CH_2CH_2-$), an alkenyl linkage($-CH_2CH=CHCH_2-$), an ether linkage ($-CH_2CH_2OCH_2-$ or $-CH_2OCH_2CH_2-$), a thioether linkage ($-CH_2CH_2SCH_2-$ or $-CH_2SCH_2CH_2-$), an amine linkage ($-CH_2CH_2NHCH_2-$ or $-CH_2NHCH_2CH_2-$) or a thioamide linkage ($-CH_2CH(S)HNHCH_2-$ or $-CH_2NHCH(S)CH_2-$). For example, Ledu et al. (Proc Nat'l Acad. Sci. 100:11263-78, 2003) describe methods for preparing lactam and amide cross-links. Schafmeister et al. (J. Am. Chem. Soc. 122:5891, 2000) describes stable, hydrocarbon cross-links. Hydrocarbon cross links can be produced via metathesis (or methathesis followed by hydrogenation in the case of saturated hydrocarbons cross-links) using one or another of the Grubbs catalysts (available from Materia, Inc. and Sigma-Aldrich and described, for example, in U.S. Patent No. 5,831,108 and 6,111,121). In some cases, the generation of such alternative cross-links requires replacing the Cys residues with other residues such as Lys or Glu or non-naturally occurring amino acids. In addition the lactam, amide and hydrocarbon cross-links can be used to stabilize the peptide even if they link amino acids at postions other than those occupied by Cys. Such cross-links can occur between two amino acids that are separated by two amino acids or between two amino acids that are separated by six amino acids (see, e.g., Schafmeister et al. (J. Am. Chem. Soc. 122:5891, 2000)).

In the case of a peptide comprising the sequence (I): $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ Cys Cys Glu $Xaa_9$ Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr $Xaa_{20}\ Xaa_{21}$ (II) wherein: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5$ is missing and/or the sequence $Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ is missing, the peptide can still contain additional carboxyterminal or amino terminal amino acids

or both. For example, the peptide can include an amino terminal sequence that facilitates recombinant production of the peptide and is cleaved prior to administration of the peptide to a patient. The peptide can also include other amino terminal or carboxyterminal amino acids. In some cases the additional amino acids protect the peptide, stabilize the peptide or alter the activity of the peptide. In some cases some or all of these additional amino acids are removed prior to administration of the peptide to a patient. The peptide can include 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 80, 90, 100 or more amino acids at its amino terminus or carboxy terminus or both. The number of flanking amino acids need not be the same. For example, there can be 10 additional amino acids at the amino terminus of the peptide and none at the carboxy terminus.

In one embodiment the peptide comprises the amino acid sequence (I): $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ wherein: $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ is missing; $Xaa_8$ is Glu; $Xaa_9$ is Leu, Ile, Lys, Arg, Trp, Tyr or Phe; $Xaa_{12}$ is Asn; $Xaa_{13}$ is Pro; $Xaa_{14}$ is Ala; $Xaa_{16}$ is Thr, Ala, Lys, Arg, Trp; $Xaa_{17}$ is Gly; $Xaa_{19}$ is Tyr or Leu; and $Xaa_{20}$ $Xaa_{21}$ is AspPhe or is missing. Where $Xaa_{20}$ $Xaa_{21}$ and/or $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ are missing, there may be additional flanking amino acids in some embodiments. In certain embodiments of a composition comprising a peptide having the sequence (I): $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$, the peptide does not comprise or consist of any of the peptides of Table I.

In a second aspect, the invention also features a therapeutic or prophylactic method comprising administering to a patient a pharmaceutical composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ (I) or $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Asn_{12}$ $Pro_{13}$ $Ala_{14}$ $Cys_{15}$ $Xaa_{16}$ $Gly_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ (II) as described herein.

The peptides can be co-administered with or linked, e.g., covalently linked to any of a variety of other peptides or compounds including analgesic peptides or analgesic compounds including, without limitation, the agents described herein.

Amino acid, non-amino acid, peptide and non-peptide spacers can be interposed between a peptide that is a GC-C receptor agonsit and a peptide that has some other biological function, e.g., an analgesic peptide or a peptide used to treat obesity. The linker can be one that is cleaved from the flanking peptides *in vivo* or one that remains linked to the flanking peptides *in vivo*. For example, glycine, beta-alanine, glycyl-glycine, glycyl-beta-alanine, gamma-aminobutyric acid, 6-aminocaproic acid, L-phenylalanine, L-tryptophan and glycil-L-valil-L-phenylalanine can be used as spacers (Chaltin et al. 2003 Helvetica Chimica Acta 86:533-547; Caliceti et al. 1993 FARMCO 48:919-32) as can polyethylene glycols (Butterworth et al. 1987 J. Med. Chem 30:1295-302) and maleimide derivatives (King et al. 2002 Tetrahedron Lett. 43:1987-1990). Various other linkers are described in the literature (Nestler 1996 Molecular Diversity 2:35-42; Finn et al. 1984 Biochemistry 23:2554-8; Cook et al. 1994 Tetrahedron Lett. 35:6777-80; Brokx et al. 2002 Journal of Controlled Release 78:115-123; Griffin et al. 2003 J. Am. Chem. Soc. 125:6517-6531; Robinson et al. 1998 Proc. Natl. Acad. Sci. USA 95:5929-5934).

The peptides of the invention can be attached to one, two or more different moieties each providing the same or different functions. For example, the peptide can be linked to a molecule that is an analgesic and to a peptide that is used to treat obesity. The peptide and various moieties can be ordered in various ways. For example, a peptide of the invention can have an analgesic peptide linked to its amino terminus and an anti-obesity peptide linked to its carboxy terminus. The additional moieties can be directly covalently bonded to the peptide or can be bonded via linkers.

The peptides of the invention can be a cyclic peptide or a linear peptide. In addition, multiple copies of the same peptide can be incorporated into a single cyclic or linear peptide.

The peptides can include the amino acid sequence of a peptide that occurs naturally in a vertebrate (e.g., mammalian) species or in a bacterial species. In addition, the peptides can be partially or completely non-naturally occurring peptides. Also within the invention are peptidomimetics corresponding to the peptides of the invention.

In various embodiments, the patient is suffering from a gastrointestinal disorder; the patient is suffering from a disorder selected from the group consisting of: gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein); the patient is suffering from a gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, inflammatory bowel disease, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein); the composition is administered orally; the peptide comprises 30 or fewer amino acids, the peptide comprises 20 or fewer amino acids, and the peptide comprises no more than 5 amino acids prior to $Cys_6$; the peptide comprises 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, or 30 or fewer amino acids. In other embodiments, the peptide comprises 20 or fewer amino acids. In other embodiments the peptide comprises no more than 20, 15, 10, or 5 peptides subsequent to $Cys_{18}$. In certain embodiments $Xaa_{19}$ is a chymotrypsin or trypsin cleavage site and an analgesic peptide is present immediately following $Xaa_{19}$.

In a third aspect, the invention features a method for treating a patient suffering from constipation. Clinically accepted criteria that define constipation range from the frequency of bowel movements, the consistency of feces and the ease of bowel movement. One common definition of constipation is less than three bowel movements per week. Other definitions include abnormally hard stools or defecation that requires excessive straining (Schiller 2001, Aliment Pharmacol Ther 15:749-763). Constipation may be idiopathic (functional constipation or slow transit constipation) or secondary to other causes including neurologic, metabolic or endocrine disorders. These disorders include diabetes mellitus, hypothyroidism, hyperthyroidism, hypocalcaemia, Multiple Sclerosis, Parkinson's disease, spinal cord lesions, Neurofibromatosis, autonomic neuropathy, Chagas disease, Hirschsprung's disease and Cystic fibrosis. Constipation may also be the result of surgery (postoperative ileus) or due to the use of drugs such as analgesics (like opioids), antihypertensives, anticonvulsants, antidepressants, antispasmodics and antipsychotics. The method of treating constipation comprises administering a pharamaceutical composition comprising or consisting essentially of a peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In various embodiments, the constipation is associated with use of a therapeutic agent; the constipation is associated with a neuropathic disorder; the constipation is post-surgical constipation (postoperative ileus); and the constipation associated with a gastrointestinal disorder; the constipation is idiopathic (functional constipation or slow transit constipation); the constipation is associated with neuropathic, metabolic or endocrine disorder (e.g., diabetes mellitus, hypothyroidism, hyperthyroidism, hypocalcaemia, Multiple Sclerosis, Parkinson's disease, spinal cord lesions, neurofibromatosis, autonomic neuropathy, Chagas disease, Hirschsprung's disease or cystic fibrosis). Constipation may also be the result of surgery (postoperative ileus) or due the use of drugs such as analgesics (e.g., opioids), antihypertensives, anticonvulsants, antidepressants, antispasmodics and antipsychotics.

In a fourth aspect, the invention features a method for treating a patient suffering a gastrointestinal disorder, the method comprising administering to the patient a pharmaceutical composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In various embodiments, the patient is suffering from a gastrointestinal disorder; the patient is suffering from a disorder selected from the group consisting of: gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein), obesity, congestive heart failure, or benign prostatic hyperplasia.

In a fifth aspect, the invention features a method for increasing gastrointestinal motility in a patient, the method comprising administering to a patient a pharmaceutical composition comprising a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In a sixth aspect, the invention features a method for increasing the activity of (activating) an intestinal guanylate cyclase (GC-C) receptor in a patient, the method comprising administering to a patient a pharmaceutical composition comprising a purified peptide

comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In a seventh aspect, the invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In an eighth aspect the invention features a method for treating constipation, the method comprising administering an agonist of the intestinal guanylate cyclase (GC-C) receptor. In various embodiments: the agonist is a peptide, the peptide includes two Cys that form one disulfide bond, the peptide includes four Cys that form two disulfide bonds, and the peptide includes six Cys that form three disulfide bonds.

In a ninth aspect, the invention features a method for treating a gastrointestinal disorder, gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein), obesity, congestive heart failure, or benign prostatic hyperplasia, the method comprising administering an agonist of the intestinal guanylate cyclase (GC-C) receptor either orally, by rectal suppository, or parenterally. In various embodiments: the agonist is a peptide, the peptide includes two Cys that form one disulfide bond, the peptide includes four Cys that form two disulfide bonds, and the peptide includes six Cys that form three disulfide bonds.

In a tenth aspect, the invention features a method for treating a gastrointestinal disorder selected from the group consisting of: gastrointestinal motility disorders, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, duodenogastric reflux, dyspepsia, functional dyspepsia, nonulcer dyspepsia, a functional gastrointestinal disorder, functional heartburn, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome, post-operative ileus, ulcerative colitis, chronic constipation, and disorders and conditions associated with constipation (e.g. constipation associated with use of opiate pain killers, post-surgical constipation, and constipation associated with neuropathic disorders as well as other conditions and disorders are described herein), the method comprising administering an agonist of the intestinal guanylate cyclase (GC-C) receptor. In various embodiments the composition is administered orally; the peptide comprises 30 or fewer amino acids, the peptide comprises 20 or fewer amino acids, and the peptide comprises no more than 5 amino acids prior to $Cys_5$.

In various embodiments: the agonist is a peptide, the peptide includes two Cys that form one disulfide bond, the peptide includes four Cys that form two disulfide bonds, and the peptide includes six Cys that form three disulfide bonds.

In an eleventh aspect, the invention features a method for treating obesity, the method comprising administering a complete or partial agonist of the intestinal guanylate cyclase (GC-C) receptor. In various embodiments: the agonist is a peptide, the peptide includes two Cys that form one disulfide bond, the peptide includes four Cys that form two disulfide bonds, and the peptide includes six Cys that form three disulfide bonds. The agonist can be administered alone or in combination with one or more agents for treatment of obesity, including but not limited to the anti-obesity agents described herein. Thus, for example, $PYY_{3-36}$ can be fused to the carboxy or amino terminus of a peptide of the invention. Such a fusion protein can include a chymostrypsin or trypsin cleavage site that can permit cleavage to separate the two peptides.

In a twelfth aspect, the invention features a method for treating obesity, the method comprising administering to a patient a pharmaceutical composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In a thirteenth aspect, the invention features a composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein. In one embodiment, the composition is a pharmaceutical composition.

In a fourteenth aspect, the invention features a method for treating congestive heart failure, the method comprising administering to a patient a pharmaceutical composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

The peptide can be administered in combination with one or more agents for treatment of congestive heart failure, for example, a natriuretic peptide such as atrial natriuretic peptide, brain natriuretic peptide or C-type natriuretic peptide), a diuretic, or an inhibitor of angiotensin converting enzyme.

In a fifteenth aspect, the invention features a method for treating benign prostatic hyperplasia, the method comprising administering to a patient a pharmaceutical composition comprising a

purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein. The peptide can be administered alone or in combination with another agent for treatment of BPH, for example, a 5-alpha reductase inhibitor (e.g., finasteride) or an alpha adrenergic inhibitor (e.g., doxazosine).

In a sixteenth aspect, the invention features a method for treating or reducing pain, including visceral pain, pain associated with a gastrointestinal disorder or pain associated with some other disorder, the method comprising administering to a patient a pharmaceutical composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In a seventeenth aspect, the invention features a method for treating inflammation, including inflammation of the gastrointestinal tract, e.g., inflammation associated with a gastrointestinal disorder or infection or some other disorder, the method comprising administering to a patient a pharmaceutical composition comprising a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Xaa_{12}\ Xaa_{13}\ Xaa_{14}\ Cys_{15}\ Xaa_{16}\ Xaa_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (I) or $Xaa_1\ Xaa_2\ Xaa_3\ Xaa_4\ Xaa_5\ Cys_6\ Cys_7\ Xaa_8\ Xaa_9\ Cys_{10}\ Cys_{11}\ Asn_{12}\ Pro_{13}\ Ala_{14}\ Cys_{15}\ Xaa_{16}\ Gly_{17}\ Cys_{18}\ Xaa_{19}\ Xaa_{20}\ Xaa_{21}$ (II) as described herein.

In an eighteenth aspect, the invention features a method for treating congestive heart failure, the method comprising administering a complete or partial agonist of the intestinal guanylate cyclase (GC-C) receptor. The agonist can be administered alone or in combination with another agent for treatment of congestive heart failure, for example, a natriuretic peptide such as atrial

natriuretic peptide, brain natriuretic peptide or C-type natriuretic peptide, a diuretic, or an inhibitor of angiotensin converting enzyme.

In a nineteenth aspect, the invention features a method for treating BPH, the method comprising administering a complete or partial agonist of the intestinal guanylate cyclase (GC-C) receptor. The agonist can be administered alone or in combination with another agent for treatment of BPH, for example, a 5-alpha reductase inhibitor (e.g., finasteride) or an alpha adrenergic inhibitor (e.g., doxazosine).

In a twentieth aspect, the invention features isolated nucleic acid molecules comprising a sequence encoding a peptide of the invention. Also within the invention are vectors, e.g., expression vectors that include such nucleic acid molecules and can be used to express a peptide of the invention in a cultured cell (e.g., a eukaryotice cell or a prokaryotic cell). The vector can further include one or more regulatory elements, e.g., a heterologous promoter or elements required for translation operably linked to the sequence encoding the peptide. In some cases the nucleic acid molecule will encode an amino acid sequence that includes the amino acid sequence of a peptide of the invention. For example, the nucleic acid molecule can encode a preprotein or a preproprotein that can be processed to produce a peptide of the invention.

A vector that includes a nucleotide sequence encoding a peptide of the invention or a peptide or polypeptide comprising a peptide of the invention may be either RNA or DNA, single- or double-stranded, prokaryotic, eukaryotic, or viral. Vectors can include transposons, viral vectors, episomes, (e.g., plasmids), chromosomes inserts, and artificial chromosomes (e.g. BACs or YACs). Suitable bacterial hosts for expression of the encode peptide or polypeptide include, but are not limited to, *E. coli*. Suitable eukaryotic hosts include yeast such as *S. cerevisiae*, other fungi, vertebrate cells, invertebrate cells (e.g., insect cells), plant cells, human cells, human tissue cells, and whole eukaryotic organisms. (e.g., a transgenic plant or a transgenic animal). Further, the vector nucleic acid can be used to transfect a virus such as

vaccinia or baculovirus (for example using the Bac-to-Bac® Baculovirus expression system (Invitrogen Life Technologies, Carlsbad, CA)).

As noted above the invention includes vectors and genetic constructs suitable for production of a peptide of the invention or a peptide or polypeptide comprising such a peptide. Generally, the genetic construct also includes, in addition to the encoding nucleic acid molecule, elements that allow expression, such as a promoter and regulatory sequences. The expression vectors may contain transcriptional control sequences that control transcriptional initiation, such as promoter, enhancer, operator, and repressor sequences. A variety of transcriptional control sequences are well known to those in the art and may be functional in, but are not limited to, a bacterium, yeast, plant, or animal cell. The expression vector can also include a translation regulatory sequence (e.g., an untranslated 5' sequence, an untranslated 3' sequence, a poly A addition site, or an internal ribosome entry site), a splicing sequence or splicing regulatory sequence, and a transcription termination sequence. The vector can be capable of autonomous replication or it can integrate into host DNA.

The invention also includes isolated host cells harboring one of the forgoing nucleic acid molecules and methods for producing a peptide by culturing such a cell and recovering the peptide or a precursor of the peptide. Recovery of the peptide or precursor may refer to collecting the growth solution and need not involve additional steps of purification. Proteins of the present invention, however, can be purified using standard purification techniques, such as, but not limited to, affinity chromatography, thermaprecipitation, immunoaffinity chromatography, ammonium sulfate precipitation, ion exchange chromatography, filtration, electrophoresis and hydrophobic interaction chromatography.

The peptides can be purified. Purified peptides are peptides separated from other proteins, lipids, and nucleic acids or from the compounds from which is it synthesized. The polypeptide can constitute at least 10, 20, 50 70, 80 or 95% by dry weight of the purified preparation.

In a twenty-first aspect, the invention features a method of increasing the level of cyclic guanosine 3'-monophosphate (cGMP) in an organ, tissue (e.g, the intestinal mucosa), or cell (e.g., a cell bearing GC-A receptor) by administering to a patient a composition comprising or consisting essentially of a purified peptide comprising, consisting of or consisting essentially of the amino acid sequence: $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ (I) or $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Asn_{12}$ $Pro_{13}$ $Ala_{14}$ $Cys_{15}$ $Xaa_{16}$ $Gly_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ (II) as described herein.

In a twenty-second aspect, the invention features polypeptides comprising, consisting or consisting essentially of the amino acid sequence $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ wherein: a) $Xaa_8$ or $Xaa_9$ is not present; b) neither $Xaa_8$ or $Xaa_9$ is present; c) one of $Xaa_{12}$, $Xaa_{13}$ and $Xaa_{14}$ is not present; d) two of $Xaa_{12}$, $Xaa_{13}$ and $Xaa_{14}$ are not present; e) three of $Xaa_{12}$, $Xaa_{13}$ and $Xaa_{14}$ are not present; f) one of $Xaa_{16}$ and $Xaa_{17}$ is not present; g) neither $Xaa_{16}$ or $Xaa_{17}$ is present and combinations thereof. In various embodiments, one, two, three, four or five of $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ and $Xaa_5$ are not present. In other embodiments, one, two or three or $Xaa_{19}$ $Xaa_{20}$ and $Xaa_{21}$ are missing.

In twenty third aspect, the invention features a method for treating a disorder ameliorated by increasing cGMP levels, the method comprising administering a pharmaceutical composition comprising, consisting essentially of or consisting of a peptide or agonist of the invention and a pharmaceutically acceptable carrier.

Among the useful peptides are peptides comprising, consisting of or consisting essentially of the amino acid sequence $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ Cys Cys Glu $Xaa_9$ Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr $Xaa_{20}$ $Xaa_{21}$ (II) (SEQ ID NO:---) are the following peptides:
Gln Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Asn Thr Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Asn Leu Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Ile Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Asn Ser Ser Gln Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Gln Ser Ser Gln Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)
Ser Ser Gln Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---).
Asn Ser Ser Asn Tyr Cys Cys Glu Ala Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Asn Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Asp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Cys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Gln Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Glu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Gly Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu His Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Ile Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Met Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Pro Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Ser Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Thr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Asn Ser Ser Asn Tyr Cys Cys Glu Val Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Ala Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Asn Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Asp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Cys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Gln Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Glu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

Cys Cys Glu Gly Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu His Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Ile Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Met Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Pro Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Ser Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Thr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Val Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Ala Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Asn Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Asp Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Cys Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Gln Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Glu Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Gly Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu His Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Ile Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Met Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Pro Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Ser Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Thr Cys Cys Asn Pro Ala Cys Thr Gly Cys; (SEQ ID NO: )
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: )
Cys Cys Glu Val Cys Cys Asn Pro Ala Cys Thr Gly Cys (SEQ ID NO: ).

Also useful are peptides comprising, consisting of or consisting essentially of any of the following sequences:

Cys Cys Glu Leu Cys Cys Ala Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Val Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Leu Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Ile Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Pro Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Met Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Phe Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Trp Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Gly Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Ser Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Thr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Cys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Gln Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Asp Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Glu Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Lys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Arg Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys His Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Ala Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Val Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Leu Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Ile Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Pro Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Met Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Phe Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Trp Pro Ala Cys Thr Gly Cys Tyr

Cys Cys Glu Tyr Cys Cys Gly Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Ser Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Thr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Cys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Gln Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Tyr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Asp Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Glu Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Lys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Arg Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys His Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Ala Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Val Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Leu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Ile Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Pro Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Met Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Phe Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Trp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Gly Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Ser Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Thr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Cys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Gln Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Asp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Glu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Lys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Arg Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys His Pro Ala Cys Thr Gly Cys

Cys Cys Glu Tyr Cys Cys Ala Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Val Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Leu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Ile Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Pro Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Met Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Phe Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Trp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Gly Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Ser Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Thr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Cys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Gln Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Tyr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Asp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Glu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Lys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Arg Pro Ala Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys His Pro Ala Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Asn Pro Thr Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Asn Pro Thr Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Asn Pro Thr Cys Thr Gly Cys
Cys Cys Glu Tyr Cys Cys Asn Pro Thr Cys Thr Gly Cys
Cys Cys Glu Phe Cys Cys Asn Pro Thr Cys Thr Gly Cys Tyr
Cys Cys Glu Phe Cys Cys Asn Pro Thr Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Asn Pro Thr Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Asn Pro Thr Cys Thr Gly Cys
Cys Cys Glu Leu Cys Cys Asn Gly Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Tyr Cys Cys Asn Gly Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Leu Cys Cys Asn Gly Ala Cys Thr Gly Cys

Cys Cys Glu Tyr Cys Cys Asn Gly Ala Cys Thr Gly Cys

Cys Cys Glu Phe Cys Cys Asn Gly Ala Cys Thr Gly Cys Tyr

Cys Cys Glu Phe Cys Cys Asn Gly Ala Cys Thr Gly Cys

Cys Cys Glu Trp Cys Cys Asn Gly Ala Cys Thr Gly Cys Tyr

Cys Cys Glu Trp Cys Cys Asn Gly Ala Cys Thr Gly Cys

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Val Gly Cys Tyr

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Val Gly Cys Tyr

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Val Gly Cys

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Val Gly Cys

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Val Gly Cys Tyr

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Val Gly Cys

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Val Gly Cys Tyr

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Val Gly Cys

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Gly Gly Cys Tyr

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Gly Gly Cys Tyr

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Gly Gly Cys

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Gly Gly Cys

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Gly Gly Cys Tyr

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Gly Gly Cys

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Gly Gly Cys Tyr

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Gly Gly Cys

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Ala Cys Tyr

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Ala Cys Tyr

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Ala Cys

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Ala Cys

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Ala Cys Tyr

Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Ala Cys

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Ala Cys Tyr

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Ala Cys

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Ala

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Val
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Leu
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Ile
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Pro
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Met
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Phe
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Trp
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Gly
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Ser
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Thr
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Cys
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Asn
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Gln
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Leu
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Ile
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Pro
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Met
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Phe
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Trp
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Gly
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Ser
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Cys

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Asn
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Lys
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Cys Cys Glu Phe Cys Cys Leu Pro Ala Cys Thr Gly Cys
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Cys Cys Glu Phe Cys Cys Phe Pro Ala Cys Thr Gly Cys
Cys Cys Glu Phe Cys Cys Trp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Phe Cys Cys Gly Pro Ala Cys Thr Gly Cys
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Cys Cys Glu Phe Cys Cys Tyr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Phe Cys Cys Asp Pro Ala Cys Thr Gly Cys
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Cys Cys Glu Phe Cys Cys Arg Pro Ala Cys Thr Gly Cys
Cys Cys Glu Phe Cys Cys His Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Ala Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Val Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Leu Pro Ala Cys Thr Gly Cys Tyr

Cys Cys Glu Trp Cys Cys Ile Pro Ala Cys Thr Gly Cys Tyr
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Cys Cys Glu Trp Cys Cys Met Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Phe Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Trp Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Gly Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Ser Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Thr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Cys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Gln Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Tyr Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Asp Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Glu Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Lys Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Arg Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys His Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Glu Trp Cys Cys Ala Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Val Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Leu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Ile Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Pro Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Met Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Phe Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Trp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Gly Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Ser Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Thr Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Cys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Gln Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Tyr Pro Ala Cys Thr Gly Cys

Cys Cys Glu Trp Cys Cys Asp Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Glu Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Lys Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys Arg Pro Ala Cys Thr Gly Cys
Cys Cys Glu Trp Cys Cys His Pro Ala Cys Thr Gly Cys
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Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Leu
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Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Met
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Phe
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Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Gly
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Ser
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Thr
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Cys
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Asn
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Gln
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Asp
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Glu
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Lys
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Arg
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys His
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Ala
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Val
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Leu
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Ile
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Pro
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Met

Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Phe
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Trp
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Gly
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Ser
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Thr
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Cys
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Asn
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Gln
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Asp
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Glu
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Lys
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Arg
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys His
Cys Cys Ala Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Val Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Leu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ile Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Met Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Phe Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Trp Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Gly Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ser Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Thr Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Cys Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Asn Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Gln Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Tyr Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Asp Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Lys Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Arg Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr

Cys Cys His Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ala Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Val Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Leu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Ile Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Met Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Phe Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Trp Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Gly Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Ser Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Thr Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Cys Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Asn Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Gln Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Tyr Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Asp Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Lys Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Arg Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys His Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Ala Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Val Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Leu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ile Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Met Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Phe Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Trp Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Gly Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ser Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Thr Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Cys Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr

Cys Cys Asn Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Gln Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Tyr Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Asp Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Lys Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Arg Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys His Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
Cys Cys Ala Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Val Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Leu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Ile Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Met Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Phe Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Trp Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Gly Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Ser Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Thr Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Cys Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Asn Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Gln Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Tyr Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Asp Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Lys Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys Arg Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys
Cys Cys His Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys

The invention also features deletion variants of any of the peptides described herein in which one, two, three or four amino acids (or non-natural amino acids or natural or non-natural amino acid analogs), other than a Cys (or an amino acid substituted for Cys, e.g, an amino acid capable of forming a covalent bond to another amino acid), are deleted. Where two (or more)

amino acids are deleted and the peptide comprises the sequence: $Cys_a$ $Cys_b$ Xaa Xaa $Cys_c$ $Cys_d$ Xaa Xaa Xaa $Cys_e$ Xaa Xaa $Cys_f$, in some embodiments two or more deletions can be located between $Cys_b$ and $Cys_c$ and/or between $Cys_d$ and $Cys_e$ and/or between $Cys_e$ and $Cys_f$. However, in other embodiments there is at most one deletion between each of $Cys_b$ and $Cys_c$ or between $Cys_d$ and $Cys_e$ or between $Cys_e$ and $Cys_f$. Thus, the invention includes any of the peptides described herein comprising the sequence $Cys_a$ $Cys_b$ Xaa Xaa $Cys_c$ $Cys_d$ Xaa Xaa Xaa $Cys_e$ Xaa Xaa $Cys_f$ wherein: a) one amino acid between $Cys_b$ and $Cys_c$ is deleted; b) one amino acid between $Cys_d$ and $Cys_e$ is deleted; c) one amino acid between $Cys_e$ and $Cys_f$ is deleted; d) one amino acid between $Cys_b$ and $Cys_c$ is deleted and one amino acid between $Cys_d$ and $Cys_e$ is deleted; e) one amino acid between $Cys_d$ and $Cys_e$ is deleted and one amino acid between $Cys_e$ and $Cys_f$ is deleted; f) one amino acid between $Cys_b$ and $Cys_c$ is deleted and one amino acid between $Cys_e$ and $Cys_f$ is deleted or g) one amino acid between $Cys_b$ and $Cys_c$ is deleted, one amino acid between $Cys_d$ and $Cys_e$ is deleted and one amino acid between $Cys_e$ and $Cys_f$ is deleted. In certain embodiments, the various deletion variants are peptides that bind to and/or activate the GC-C receptor. In various embodiments, the various deletion variants are peptides that increase cGMP levels.

Deletion variants of Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3) include the peptides listed in FIG. 11. In these deletion variants, any of the amino acids can be deleted and there can be one, two, three or four amino acids deleted other than Cys.

The invention also features insertion variants of any of the peptides described herein in which one, two, three or four amino acids (e.g., Gly or Ala) are inserted before or after any amino acid in the peptide. In some embodiments no more than one amino acid is inserted between two Cys. For example, where two or more amino acids are inserted and the peptide comprises the sequence $Cys_a$ $Cys_b$ Xaa Xaa $Cys_c$ $Cys_d$ Xaa Xaa Xaa $Cys_e$ Xaa Xaa $Cys_f$, in some embodiments two or more insertions can be located between $Cys_b$ and $Cys_c$ or between $Cys_d$ and $Cys_e$ or between $Cys_e$ and $Cys_f$. However, in other embodiments no more than one insertion is located between $Cys_b$ and $Cys_c$ or between $Cys_d$ and $Cys_e$ or between $Cys_e$ and $Cys_f$. Thus, the invention features any of the peptides described herein comprising the sequence $Cys_a$

$Cys_b$ Xaa Xaa $Cys_c$ $Cys_d$ Xaa Xaa Xaa $Cys_e$ Xaa Xaa $Cys_f$ wherein: a) one amino acid is inserted between $Cys_b$ and $Cys_c$; b) one amino acid is inserted between $Cys_d$ and $Cys_e$; c) one amino acid is inserted between $Cys_e$ and $Cys_f$; d) one amino acid is inserted between $Cys_b$ and $Cys_c$ and one amino acid is inserted between $Cys_d$ and $Cys_e$; e) one amino acid is inserted between $Cys_d$ and $Cys_e$ and one amino acid is inserted between $Cys_e$ and $Cys_f$; f) one amino acid is inserted between $Cys_b$ and $Cys_c$ and one amino acid is inserted between $Cys_e$ and $Cys_f$; or g) one amino acid is inserted between $Cys_b$ and $Cys_c$, one amino acid is inserted between $Cys_d$ and $Cys_e$ and one amino acid is inserted between $Cys_e$ and $Cys_f$. In addition, one or more amino acids can be inserted preceding $Cys_a$ and/or one or more amino acids can be inserted following $Cys_f$.

In various embodiments, the various insertion variants are peptides that bind to and/or activate the GC-C receptor. In various embodiments, the various insertion variants are peptides that increase cGMP levels.

Insertion variants of Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3) include those in which up to four amino acids (i.e., 0, 1, 2, 3 or 4) can be inserted after each amino acid. Thus, the invention includes peptides having the sequence: Cys $Xaa_{(0-4)}$ Cys $Xaa_{(0-4)}$ Glu $Xaa_{(0-4)}$ Tyr $Xaa_{(0-4)}$ Cys $Xaa_{(0-4)}$ Cys $Xaa_{(0-4)}$ Asn $Xaa_{(0-4)}$ Pro $Xaa_{(0-4)}$ Ala $Xaa_{(0-4)}$ Cys $Xaa_{(0-4)}$ Thr $Xaa_{(0-4)}$ Gly $Xaa_{(0-4)}$ Cys $Xaa_{(0-4)}$ Tyr $Xaa_{(0-4))}$ (SEQ ID NO: ). The inserted amino acids can be any amino acid or amino acid analog (natural or non-natural) and can be the same or different. In certain embodiments the inserted amino acids are all Gly or all Ala or a combination of Gly and Ala.

FIG. 12 depicts insertion variants of the peptide having the sequence: Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3).

The invention also features variants of peptides having the sequence $Xaa_1$ $Xaa_2$ $Xaa_3$ $Xaa_4$ $Xaa_5$ $Cys_6$ $Cys_7$ $Xaa_8$ $Xaa_9$ $Cys_{10}$ $Cys_{11}$ $Xaa_{12}$ $Xaa_{13}$ $Xaa_{14}$ $Cys_{15}$ $Xaa_{16}$ $Xaa_{17}$ $Cys_{18}$ $Xaa_{19}$ $Xaa_{20}$ $Xaa_{21}$ (SEQ ID NO:1), e.g., variants of Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys

Tyr (SEQ ID NO:3), in which up to four amino acids are deleted and/or up to four amino acids are inserted. The insertions and deletions can be between $Cys_6$ and $Cys_{18}$ in SEQ ID NO:1 or they can be amino terminal to $Cys_6$ and/or carboxy terminal to $Cys_{18}$ in SEQ ID NO:1.

The invention also features peptides which may include one or more of the peptide modifications, one or more non-natural amino acid or amino acid analogs, one or more of the disulfide bond alternatives or one more of the alternative peptide bonds described herein.

The peptides of the invention can be present with a counterion. Useful counterions include salts of: acetate, benzenesulfonate, benzoate, calcium edetate, camsylate, carbonate, citrate, edetate (EDTA), edisylate, embonate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, iodide, bromide, chloride, hydroxynaphthoate, isethionate, lactate, lactobionate, estolate, maleate, malate, mandelate, mesylate, mucate, napsylate, nitrate, pantothenate, phosphate, salicylate, stearate, succinate, sulfate, tartarate, theoclate, acetamidobenzoate, adipate, alginate, aminosalicylate, anhydromethylenecitrate, ascorbate, aspartate, camphorate, caprate, caproate, caprylate, cinnamate, cyclamate, dichloroacetate, formate, gentisate, glucuronate, glycerophosphate, glycolate, hippurate, fluoride, malonate, napadisylate, nicotinate, oleate, orotate, oxalate, oxoglutarate, palmitate, pectinate, pectinate polymer, phenylethylbarbiturate, picrate, propionate, pidolate, sebacate, rhodanide, tosylate, and tannate.

The peptides and agonist of the intestinal guanylate cyclase (GC-C) receptor can be used to treat constipation or decreased intestinal motility, slow digestion or slow stomach emptying. The peptides can be used to relieve one or more symptoms of IBS (bloating, pain, constipation), GERD (acid reflux into the esophagus), duodenogastric reflux, functional dyspepsia, or gastroparesis (nausea, vomiting, bloating, delayed gastric emptying) and other disorders described herein.

The details of one or more embodiments of the invention are set forth in the accompanying description. All of the publications, patents and patent applications are hereby incorporated by reference.

## FIGURES

Figure 1 depicts the results of LCMS analysis of recombinant SEQ ID NO:4 peptide and SEQ ID NO:5 peptide.

Figures 1b and 1c depict the results of LCMS analysis of synthetic SEQ ID NO:3 peptide and the blank.

Figure 2 depicts the results of the intestinal GC-C receptor activity assay of synthetic SEQ ID NO:4 peptide, SEQ ID NO:5 peptide and two different SEQ ID NO:3 peptides.

Figure 3a depicts the effect of recombinant SEQ ID NO:4 peptide and Zelnorm® in an acute murine gastrointestinal transit model.

Figure 3b depicts the effect of synthetic SEQ ID NO:3 peptide and Zelnorm® in an acute murine gastrointestinal transit model.

Figures 4a and 4b depict the effect of peptidesSEQ ID NO:5, SEQ ID NO:3, and SEQ ID NO:4 in an acute murine gastrointestinal transit model.

Figure 4c depicts the effect of SEQ ID NO:3 peptide in a chronic murine gastrointestinal transit model.

Figure 5a depicts the effect of SEQ ID NO:4 peptide and Zelnorm® in a suckling mouse intestinal secretion model.

Figure 5b depicts the effects of SEQ ID NO:3 and Zelnorm® in a mouse intestinal secretion model.

Figures 6a and 6b depict the effects of SEQ ID NO:4, SEQ ID NO:3 and SEQ ID NO:5 peptides in a mouse intestinal secretion model.

Figure 7 shows the results of experiment in which SEQ ID NO:3 activity was analyzed in the TNBS colonic distention model.

Figures 8a and 8b show the effects of differing doses of SEQ ID NO:5 and SEQ ID NO:3 in the PBQ writhing assay.

Figure 9 shows the results of Kd determination analysis using SEQ ID NO:3 in a competitive radioligand binding assay.

Figures 10a and 10b show bioavailability data for IV and orally administered SEQ ID NO:3 as detected by an ELISA assay and LCMS.

Figure 11 depicts deletion variants of a peptide having the sequence of SEQ ID NO:3.

Figure 12 depicts insertion variants of a peptide having the sequence of SEQ ID NO:3.

## DETAILED DESCRIPTION

The peptides of the invention bind to the intestinal guanylate cyclase (GC-C) receptor, a key regulator of fluid and electrolyte balance in the intestine. When stimulated, this receptor, which is located on the apical membrane of the intestinal epithelial surface, causes an increase in intestinal epithelial cyclic GMP (cGMP). This increase in cGMP is believed to cause a decrease in water and sodium absorption and an increase in chloride and potassium ion secretion, leading to changes in intestinal fluid and electrolyte transport and increased intestinal

motility. The intestinal GC-C receptor possesses an extracellular ligand binding region, a transmembrane region, an intracellular protein kinase-like region and a cyclase catalytic domain. Proposed functions for the GC-C receptor are fluid and electrolyte homeostasis, the regulation of epithelial cell proliferation and the induction of apoptosis (Shalubhai 2002 Curr Opin Drug Dis Devel 5:261-268).

In addition to being expressed in the intestine by gastrointestinal epithelial cells, GC-C is expressed in extra-intestinal tissues including kidney, lung, pancreas, pituitary, adrenal, developing liver and gall bladder (reviewed in Vaandrager 2002 Mol Cell Biochem 230:73-83, Kulaksiz et al. 2004, Gastroenterology 126:732-740) and male and female reproductive tissues (reviewed in Vaandrager 2002 Mol Cell Biochem 230:73-83). This suggests that the GC-C receptor agonists can be used in the treatment of disorders outside the GI tract, for example, congestive heart failure and benign prostatic hyperplasia.

Ghrelin, a peptide hormone secreted by the stomach, is a key regulator of appetite in humans. Ghrelin expression levels are regulated by fasting and by gastric emptying (Kim et al. 2003 Neuroreprt 14:1317-20; Gualillo et al. 2003 FEBS Letts 552: 105-9). Thus, by increasing gastrointestinal motility, GC-C receptor agonists may also be used to regulate obesity.

In humans, the GC-C receptor is activated by guanylin (Gn) (U.S. 5,96,097), uroguanylin (Ugn) (U.S. 5,140,102) and lymphoguanylin (Forte et al. 1999 *Endocrinology* 140:1800-1806). Interestingly, these agents are 10-100 fold less potent than a class of bacterially derived peptides, termed ST (reviewed in Gianella 1995 J Lab Clin Med 125:173-181). ST peptides are considered super agonists of GC-C and are very resistant to proteolytic degradation.

ST peptide is capable of stimulating the enteric nervous system (Rolfe et al., 1994, J Physiolo 475: 531-537; Rolfe et al. 1999 Gut 44: 615-619; Nzegwu et al. 1996 Exp Physiol 81: 313-315). Also, cGMP has been reported to have antinociceptive effects in multiple animal models of pain (Lazaro Ibanez et al. 2001 Eur J Pharmacol 426: 39-44; Soares et al. 2001 British J Pharmacol 134: 127-131; Jain et al. 2001 Brain Res 909:170-178; Amarante et al. 2002 Eur J

Pharmacol 454:19-23). Thus, GC-C agonists may have both an analgesic as well an anti-inflammatory effect.

In bacteria, ST peptides are derived from a preproprotein that generally has at least 70 amino acids. The pre and pro regions are cleaved as part of the secretion process, and the resulting mature protein, which generally includes fewer than 20 amino acids, is biologically active.

Among the known bacterial ST peptides are: *E. coli* ST Ib (Moseley et al. 1983 Infect. Immun. 39:1167) having the mature amino acid sequence Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:__); *E. coli* ST Ia (So and McCarthy 1980 Proc. Natl. Acad. Sci. USA 77:4011) having the mature amino acid sequence Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr (SEQ ID NO:__); *E. coli* ST I* (Chan and Giannella 1981 J. Biol. Chem. 256:7744) having the mature amino acid sequence Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys Asn (SEQ ID NO:___); *C.freundii* ST peptide (Guarino et al. 1989b *Infect. Immun.* 57:649) having the mature amino acid sequence Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr (SEQ ID NO:__); *Y. enterocolitica* ST peptides, Y-ST(Y-STa), Y-STb, and Y-STc (reviewed in Huang et al. 1997 Microb. Pathog. 22:89) having the following pro-form amino acid sequences: Gln Ala Cys Asp Pro Pro Ser Pro Pro Ala Glu Val Ser Ser Asp Trp Asp Cys Cys Asp Val Cys Cys Asn Pro Ala Cys Ala Gly Cys (SEQ ID NO:___) (as well as a Ser-7 to Leu-7 variant of Y-STa (SEQ ID NO:___), (Takao et al. 1985 Eur. J. Biochem. 152:199)); Lys Ala Cys Asp Thr Gln Thr Pro Ser Pro Ser Glu Glu Asn Asp Asp Trp Cys Cys Glu Val Cys Cys Asn Pro Ala Cys Ala Gly Cys (SEQ ID NO:___); Gln Glu Thr Ala Ser Gly Gln Val Gly Asp Val Ser Ser Ser Thr Ile Ala Thr Glu Val Ser Glu Ala Glu Cys Gly Thr Gln Ser Ala Thr Thr Gln Gly Glu Asn Asp Trp Asp Trp Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Phe Gly Cys (SEQ ID NO:___), respectively; *Y. kristensenii* ST peptide having the mature amino acid sequence Ser Asp Trp Cys Cys Glu Val Cys Cys Asn Pro Ala Cys Ala Gly Cys (SEQ ID NO:___); V. *cholerae* non-01 ST peptide (Takao et al. (1985) *FEBS lett.* 193:250) having the mature amino acid sequence Ile Asp Cys Cys Glu Ile Cys Cys Asn Pro Ala Cys Phe Gly Cys Leu Asn (SEQ ID NO:__); and *V. mimicus* ST peptide (Arita et al. 1991 FEMS Microbiol. Lett. 79:105)

having the mature amino acid sequence Ile Asp Cys Cys Glu Ile Cys Cys Asn Pro Ala Cys Phe Gly Cys Leu Asn (SEQ ID NO:__). Table I below provides sequences of all or a portion of a number of mature ST peptides. Such peptides are useful GCC agonists.

Table I

| **GenBank® Accession No.** | **GenBank® GI No.** | **Sequence** |
|---|---|---|
| QHECIB | 69638 | NSSNYCCELCCNPACTGCY(SEQ ID NO:__) |
| P01559 | 123711 | NTFYCCELCCNPACAGCY(SEQ ID NO:__) |
| AAA24653 | 147878 | NTFYCCELCCNPACAPCY(SEQ ID NO:__) |
| P01560 | 123707 | NTFYCCELCCYPACAGCN(SEQ ID NO:__) |
| AAA27561 | 295439 | IDCCEICCNPACFGCLN(SEQ ID NO:__) |
| P04429 | 123712 | IDCCEICCNPACFGCLN(SEQ ID NO:__) |
| S34671 | 421286 | IDCCEICCNPACF(SEQ ID NO:__) |
| CAA52209 | 395161 | IDCCEICCNPACFG(SEQ ID NO:__) |
| A54534 | 628844 | IDCCEICCNPACFGCLN(SEQ ID NO:__) |
| AAL02159 | 15592919 | IDRCEICCNPACFGCLN(SEQ ID NO:__) |
| AAA18472 | 487395 | DWDCCDVCCNPACAGC(SEQ ID NO:__) |
| S25659 | 282047 | DWDCCDVCCNPACAGC(SEQ ID NO:__) |
| P74977 | 3913874 | NDDWCCEVCCNPACAGC(SEQ ID NO:__) |
| BAA23656 | 2662339 | WDWCCELCCNPACFGC(SEQ ID NO:__) |
| P31518 | 399947 | SDWCCEVCCNPACAGC(SEQ ID NO:__) |
| | | QACDPPSPPAEVSSDWDCCDVCCDPAC AGC |
| | | QACDPPSPPAEVSSDWDCCDVCCNPACAG C |
| | | KACDTQTPSPSEENDDTCCEVCCNPACAG C |
| | | QETASGQVGDVSSSTIATEVSEAECGTQSATTQGE NDWDWCCELCCNPACFGC |
| | | MKKLMLAIFISVLSFPSFSQSTESLDS SKEKITLETKKCDVVKNNSEKKSEN MNNTFYCCELCCNPACAGCY |

| | | |
|---|---|---|
| | | MKKSILFIFLSVLSFSPFAQDAKPVES SKEKITLESKKCNIAKKSNKSGPESM NSSNYCCELCCNPACTGCY |
| | | MKKIVFVLVLMLSSFGAFGQETVSG QFSDALSTPITAEVYKQACDPPLPPA EVSSDWDCCDVCCNPACAGC |
| | | GNLIDCCEICCNPACFGCLN |
| | | GNLIDRCEICCNPACFGCLN |
| | | PPAEVSSDWDCCDVCCNPACAGC |

The immature (including pre and pro regions) form of *E. coli* ST-1A (ST-P) protein has the sequence: mkklmlaifisvlsfpsfsqstesldsskekitletkkcdvvknnsekksenmnntfyccelccnpacagcy (SEQ ID NO:___; see GenBank® Accession No. P01559 (gi:123711). The pre sequence extends from aa 1-19. The pro sequence extends from aa 20-54. The mature protein extends from 55-72. The immature (including pre and pro regions) form of *E. coli* ST-1B (ST-H) protein has the sequence: mkksilfiflsvlsfspfaqdakpvesskekitleskkcniakksnksgpesmnssnyccelccnpactgcy (SEQ ID NO:___; see GenBank® Accession No. P07965 (gi:3915589)). The immature (including pre and pro regions) form of *Y. enterocolitica* ST protein has the sequence: mkkivfvlvlmlssfgafgqetvsgqfsdalstpitaevykqacdpplppaevssdwdccdvccnpacagc (SEQ ID NO:___; see GenBank® Accession No. S25659 (gi:282047)).

The peptides of the invention, like the bacterial ST peptides, have six Cys residues. These six Cys residues form three disulfide bonds in the mature and active form of the peptide. If the six Cys residues are identified, from the amino to carboxy terminus of the peptide, as A, B, C, D, E, and F, then the disulfide bonds form as follows: A-D, B-E, and C-F. The formation of these bonds is thought to be important for GC-C receptor binding. Certain of the peptides of the invention include a potentially functional chymotrypsin cleavage site, e.g., a Trp, Tyr or Phe located between either Cys B and Cys D or between Cys E and Cys F. Cleavage at either chymotrypsin cleavage site may reduce or eliminates the ability of the peptide to bind to the GC-C receptor.

In the human body an inactive form of chymotrypsin, chymotrypsinogen is produced in the pancreas. When this inactive enzyme reaches the small intestine it is converted to active chymotrypsin by the excision of two di-peptides. Active chymotrypsin can potentially cleave peptides at the peptide bond on the carboxy-terminal side of Trp, Tyr or Phe. The presence of active chymotrypsin in the intestinal tract can potentially lead to cleavage of certain of the peptides of the invention having an appropriately positioned functional chymotrypsin cleavage site. It is expected that chymotrypsin cleavage will moderate the action of a peptide of the invention having an appropriately positioned chymotrypsin cleavage site as the peptide passes through the intestinal tract.

Trypsinogen, like chymotrypsin, is a serine protease that is produced in the pancreas and is present in the digestive tract. The active form, trypsin, will cleave peptides having a Lys or Arg. The presence of active trypsin in the intestinal tract can lead to cleavage of certain of the peptides of the invention having an appropriately positioned functional trypsin cleavage site. It is expected that chymotrypsin cleavage will moderate the action of a peptide of the invention having an appropriately positioned trypsin cleavage site as the peptide passes through the intestinal tract.

Many gastrointestinal disorders, including IBS, are associated with abdominal or visceral pain. Certain of the peptides of the invention include analgesic or antinociceptive tags such as the carboxy-terminal sequence AspPhe immediately following a Trp, Tyr or Phe that creates a functional chymotrypsin cleavage site or following Lys or Arg that creates a functional trypsin cleavage site. Chymotrypsin in the intestinal tract can potentially cleave such peptides immediately carboxy terminal to the Trp, Phe or Tyr residue, releasing the dipeptide, AspPhe. This dipeptide has been shown to have analgesic activity in animal models (Abdikkahi et al. 2001 Fundam Clin Pharmacol 15:117-23; Nikfar et al 1997, 29:583-6; Edmundson et al 1998 Clin Pharmacol Ther 63:580-93). In this manner such peptides can treat both pain and inflammation. Other analgesic peptides can be present at the amino or carboxy terminus of the peptide (e.g., following a functional cleavage site) including: endomorphin-1, endomorphin-2, nocistatin, dalargin, lupron, and substance P.

A number of the useful peptides are based on the core sequence: Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr. To create a variant having a potentially functional chymotrypsin cleavage site capable of inactivating the peptide, either the Leu (underlined) or the Thr (underlined) can be replaced by Trp, Phe or Tyr or both the Leu and the Thr can be replaced by (independently) Trp, Phe or Tyr. To create a variant having an analgesic di-peptide, the core sequence is followed by Asp Phe. The carboxy terminal Tyr in the core sequence can allow the Asp Phe dipeptide to be released by chymotrypsin in the digestive tract. The core sequence can be optionally be preceded by Asn Ser Ser Asn Tyr or Asn.

Thus, useful variants based on the core sequence include:

Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:4)

Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr (SEQ ID NO:---)

Asn Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:5)

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:6)

Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr (SEQ ID NO:---)

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3)

Asn Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:---)

Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)

Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr Asp Phe

(SEQ ID NO:---)
Asn Ser Ser Asn Tyr Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Ser Ser Asn Tyr Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Ser Ser Asn Tyr Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Ser Ser Asn Tyr Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Trp Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Phe Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Trp Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Arg Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)
Asn Cys Cys Glu Lys Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr Asp Phe (SEQ ID NO:---)

In some cases, the peptides of the invention are produced as a prepro protein that includes the amino terminal leader sequence: mkksilfiflsvlsfspfaqdakpvesskekitleskkcniakksnksgpesmn. Where the peptide is produced by a bacterial cell, e.g., *E. coli*, the forgoing leader sequence will be cleaved and the mature peptide will be efficiently secreted from the bacterial cell. U.S.

Patent No. 5,395,490 describes vectors, expression systems and methods for the efficient production of ST peptides in bacterial cells and methods for achieving efficient secretion of mature ST peptides. The vectors, expression systems and methods described in U.S. Patent No. 5,395,490 can be used to produce the ST peptides and variant ST peptides of the present invention

Variant Peptides

The invention includes variant peptides which can include one, two, three, four, five, six, seven, eight, nine, or ten (in some embodiments fewer than 5 or fewer than 3 or 2 or fewer) amino acid substitutions and/or deletions compared to SEQ ID NOs:____ to ___. The substitution(s) can be conservative or non-conservative. The naturally-occurring amino acids can be substituted by D-isomers of any amino acid, non-natural amino acids, natural and natural amino acid analogs and other groups. A conservative amino acid substitution results in the alteration of an amino acid for a similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity. At some positions, even conservative amino acid substitutions can alter the activity of the peptide. A conservative substitution can substitute a naturally-occurring amino acid for a non-naturally-occurring amino acid. The amino acid substitutions among naturally-occurring amino acids are listed in Table II.

Table II

| For Amino Acid | Code | Replace with any of |
|---|---|---|
| Alanine | Ala | Gly, Cys, Ser |
| Arginine | Arg | Lys, His |
| Asparagine | Asn | Asp, Glu, Gln, |
| Aspartic Acid | Asp | Asn, Glu, Gln |
| Cysteine | Cys | Met, Thr, Ser |
| Glutamine | Gln | Asn, Glu, Asp |
| Glutamic Acid | Glu | Asp, Asn, Gln |
| Glycine | Gly | Ala |
| Histidine | His | Lys, Arg |
| Isoleucine | Ile | Val, Leu, Met |
| Leucine | Leu | Val, Ile, Met |
| Lysine | Lys | Arg, His |
| Methionine | Met | Ile, Leu, Val |
| Phenylalanine | Phe | Tyr, His, Trp |
| Proline | Pro | |
| Serine | Ser | Thr, Cys, Ala |
| Threonine | Thr | Ser, Met, Val |
| Tryptophan | Trp | Phe, Tyr |
| Tyrosine | Tyr | Phe, His |
| Valine | Val | Leu, Ile, Met |

In some circumstances it can be desirable to treat patients with a variant peptide that binds to and activates intestinal GC-C receptor, but is less active than the non-variant form the peptide. This reduced activity can arise from reduced affinity for the receptor or a reduced ability to activate the receptor once bound or reduced stability of the peptide.

Production of peptides

Useful peptides can be produced either in bacteria including, without limitation, *E. coli*, or in other existing systems for peptide or protein production (e.g., *Bacillus subtilis,* baculovirus expression systems using Drosophila Sf9 cells, yeast or filamentous fungal expression systems, mammalian cell expression systems), or they can be chemically synthesized.

If the peptide or variant peptide is to be produced in bacteria, e.g., *E. coli*, the nucleic acid molecule encoding the peptide will preferably also encode a leader sequence that permits the secretion of the mature peptide from the cell. Thus, the sequence encoding the peptide can include the pre sequence and the pro sequence of, for example, a naturally-occurring bacterial ST peptide. The secreted, mature peptide can be purified from the culture medium.

The sequence encoding a peptide of the invention is preferably inserted into a vector capable of delivering and maintaining the nucleic acid molecule in a bacterial cell. The DNA molecule may be inserted into an autonomously replicating vector (suitable vectors include, for example, pGEM3Z and pcDNA3, and derivatives thereof). The vector nucleic acid may be a bacterial or bacteriophage DNA such as bacteriophage lambda or M13 and derivatives thereof. Construction of a vector containing a nucleic acid described herein can be followed by transformation of a host cell such as a bacterium. Suitable bacterial hosts include but are not limited to, *E. coli, B. subtilis*, *Pseudomonas*, *Salmonella*. The genetic construct also includes, in addition to the encoding nucleic acid molecule, elements that allow expression, such as a promoter and regulatory sequences. The expression vectors may contain transcriptional control sequences that control transcriptional initiation, such as promoter, enhancer, operator, and repressor sequences. A variety of transcriptional control sequences are well known to those in the art. The expression vector can also include a translation regulatory sequence (e.g., an untranslated 5' sequence, an untranslated 3' sequence, or an internal ribosome entry site). The vector can be capable of autonomous replication or it can integrate into host DNA to ensure stability during peptide production.

The protein coding sequence that includes a peptide of the invention can also be fused to a nucleic acid encoding a polypeptide affinity tag, e.g., glutathione S-transferase (GST), maltose E binding protein, protein A, FLAG tag, hexa-histidine, myc tag or the influenza HA tag, in order to facilitate purification. The affinity tag or reporter fusion joins the reading frame of the peptide of interest to the reading frame of the gene encoding the affinity tag such that a translational fusion is generated. Expression of the fusion gene results in translation of a single polypeptide that includes both the peptide of interest and the affinity tag. In some instances

where affinity tags are utilized, DNA sequence encoding a protease recognition site will be fused between the reading frames for the affinity tag and the peptide of interest.

Genetic constructs and methods suitable for production of immature and mature forms of the peptides and variants of the invention in protein expression systems other than bacteria, and well known to those skilled in the art, can also be used to produce peptides in a biological system.

Mature peptides and variants thereof can be synthesized by the solid-phase chemical synthesis. For example, the peptide can be synthesized on Cyc(4-$CH_2$ Bxl)-$OCH_2$-4-(oxymethyl)-phenylacetamidomethyl resin using a double coupling program. Protecting groups must be used appropriately to create the correct disulfide bond pattern. For example, the following protecting groups can be used: t-butyloxycarbonyl (alpha-amino groups); acetamidomethyl (thiol groups of Cys residues B and E); 4-methylbenyl (thiol groups of Cys residues C and F); benzyl (y-carboxyl of glutamic acid and the hydroxyl group of threonine, if present); and bromobenzyl (phenolic group of tyrosine, if present). Coupling is effected with symmetrical anhydride of t-butoxylcarbonylamino acids or hydroxybenzotriazole ester (for asparagine or glutamine residues), and the peptide is deprotected and cleaved from the solid support in hydrogen fluoride, dimethyl sulfide, anisole, and p-thiocresol using 8/1/1/0.5 ratio (v/v/v/w) at 0°C for 60 min. After removal of hydrogen fluoride and dimethyl sulfide by reduced pressure and anisole and p-thiocresol by extraction with ethyl ether and ethyl acetate sequentially, crude peptides are extracted with a mixture of 0.5M sodium phosphate buffer, pH 8.0 and N, N-dimethylformamide using 1/1 ratio, v/v. The disulfide bond for Cys residues B and E is the formed using dimethyl sulfoxide (Tam et al. (1991) *J. Am. Chem. Soc.* 113:6657-62). The resulting peptide is the purified by reverse-phase chromatography. The disulfide bond between Cys residues C and F is formed by first dissolving the peptide in 50% acetic acid in water. Saturated iodine solution in glacial acetic acid is added (1 ml iodine solution per 100 ml solution). After incubation at room temperature for 2 days in an enclosed glass container, the solution is diluted five-fold with deionized water and extracted with ethyl ether four times for removal of unreacted iodine. After removal of the residual amount of ethyl ether by rotary

evaporation the solution of crude product is lyophilized and purified by successive reverse-phase chromatography.

Peptides can also be synthesized by many other methods including solid phase synthesis using traditional FMOC protection (i.e., coupling with DCC-HOBt and deprotection with piperdine in DMF). Cys thiol groups can be trityl protected. Treatment with TFA can be used for final deprotection of the peptide and release of the peptide from the solid-state resin. In many cases air oxidation is sufficient to achieve proper disulfide bond formation.

Intestinal GC-C receptor binding assay

The ability of peptides and other agents to bind to the intestinal GC-C receptor can be tested as follows. Cells of the T84 human colon carcinoma cell line (American Type Culture Collection (Bethesda, Md.)) are grown to confluence in 24-well culture plates with a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum. Cells used in the assay are typically between passages 54-60. Briefly, T84 cell monolayers in 24-well plates are washed twice with 1 ml of binding buffer (DMEM containing 0.05% bovine serum albumin and 25 mM HEPES, pH 7.2), then incubated for 30 min at 37°C in the presence of mature radioactively labeled *E. coli* ST peptide and the test material at various concentrations. The cells are then washed four times with 1 ml of DMEM and solubilized with 0.5 ml/well 1N NaOH. The level of radioactivity in the solubilized material is then determined using standard methods.

**Example 1: Preparation of variant ST peptides and wild-type ST peptide**

**1a: Preparation of recombinant variant ST peptides and wild-type ST peptide**

A variant ST peptide having the sequence Asn Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:5) was produced recombinantly and tested in an

animal model. A peptide having the sequence of the wild-type ST peptide was also created (SEQ ID NO:4).

SEQ ID NO:5 and SEQ ID NO:4 peptides were produced as preproproteins using vectors produced as follows. A sequence encoding a heat-stable enterotoxin pre-pro sequence was amplified from pGK51/pGSK51 (ATCC 67728) using oligonucleotide MO3514 (5' CACACCATATGAAGAAATCAATATTATTTATTTTCTTTCTG 3' (SEG ID NO: )) and oligonucelotide MO3515 (5' CACACCTCGAGTTAGGTCTCCATGCTTTCAGGACCACTTTTATTAC 3' (SEQ ID NO: __)). The amplification product fragment was digested with NdeI/XhoI and ligated to the T7 expression vector, pET26b(+) (Novagen) digested with NdeI/XhoI thereby creating plasmid MB3976. The region encoding the pre-pro protein was sequenced and found to encode the amino acid sequence: mkksilfiflsvlsfspfaqdakpagsskekitleskkcnivkk**s**n**k**s**g**pesm (SEQ ID NO: __) which differs from the amino acid sequence of heat-stable enterotoxin a2 precursor (sta2; mkksilfiflsvlsfspfaqdakpagsskekitleskkcnivkknnesspesm (SEQ ID NO:___); GenBank® Accession No. Q47185, GI: 3913876) at three positions (indicated by underlining and bold text) near the C-terminus. To create expression vectors with the pre-pro sequence, complementary oligos encoding each ST peptide variant or wild-type ST peptide were annealed and cloned into the MB3976 expression vector. To create MB3984 (encoding SEQ ID NO:4 peptide (wild-type ST peptide) as a prepro protein), containing the amino acid sequence, NSSNYCCELCCNPACTGCY (SEQ ID NO:___) fused downstream of the pre-pro sequence, MB 3976 was digested with BsaI/XhoI and ligated to annealed oligos MO3621 (5' GCATGAATAGTAGCAATTACTGCTGTGAATTGTGTTGTAATCCTGCTTGTACCGGGTG CTATTAATAAC 3' (SEQ ID NO:__)) and MO3622 (5' TCGAGTTATTAATAGCACCCGGTACAAGCAGGATTACAACACAATTCACAGCAGTAA TTGCTACTATTC 3'(SEQ ID NO:__) ). To create MB3985 (encoding SEQ ID NO:5 as a prepro protein) containing the following amino acid sequence, NSSNYCCEYCCNPACTGCY fused downstream of the pre-pro sequence, MB 3976 was digested with BsaI/XhoI and ligated to annealed oligos MO3529 (5' GCATGAATAGTAGCAATTACTGCTGTGAATATTGTTGTAATCCTGCTTGTACCGGGTG

CTATTAATAAC 3' (SEQ ID NO:__)) and MO3530 (5' TCGAGTTATTAATAGCACCCGGTACAAGCAGGATTACAACAATATTCACAGCAGTAA TTGCTACTATTC 3'(SEQ ID NO:__)).

The SEQ ID NO:5 peptide and the SEQ ID NO:4 peptide were produced as follows. The expression vectors were transformed into *E. coli* bacterial host BL21 λ DE3 (Invitrogen). A single colony was innoculated and grown shaking overnight at 30°C in L broth + 25 mg/l kanamycin. The overnight culture was added to 3.2 L of batch medium (Glucose 25 g/l, Caseamino Acids 5 g/l, Yeast Extract 5 g/l, $KH_2PO_4$ 13.3 g/l, $(NH_4)_2HPO_4$ 4 g/l, $MgSO_4$-$7H_20$ 1.2 g/l, Citric Acid 1.7 g/l, EDTA 8.4 mg/l, $CoCl_2$-$6H_2O$ 2.5 mg/l, $MnCl_2$-$4H_2O$ 15 mg/l, $CuCl_2$-$4H_2O$ 1.5 mg/l, $H_3BO_3$ 3 mg/l, $Na_2MoO_4$-$2H_20$ 2.5 mg/l, Zn Acetate-$2H_20$ 13 mg/l, Ferric Citrate 100 mg/l, Kanamycin 25 mg/l, Antifoam $DF_20_4$ 1 ml/l) and fermented using the following process parameters : pH 6.7 - control with base only (28% $NH_4OH$), 30°C, aeration : 5 liters per minute. After the initial consumption of batch glucose (based on monitoring dissolved oxygen (DO) levels), 1.5 L of feed medium (Glucose 700 g/l, Caseamino Acids 10 g/l, Yeast Extract 10 g/l, $MgSO_4$-$7H_20$ 4 g/l, EDTA 13 mg/l, $CoCl_2$-$6H_2O$ 4 mg/l, $MnCl_2$-$4H_2O$ 23.5 mg/l, $CuCl_2$-$4H_20$ 2.5 mg/l, $H_3BO_3$ 5 mg/l, $Na_2MoO_4$-$2H_20$ 4 mg/l, Zn Acetate-$2H_20$ 16 mg/l, Ferric Citrate 40 mg/l, Antifoam $DF_20_4$ 1 ml/l) was added at a feed rate controlled to maintain 20% DO. IPTG was added to 0.2 mM 2 hours post feed start. The total run time was approximately 40-45 hours (until feed exhaustion).

Cells were collected by centrifugation at 5,000 g for 10 minutes. The cell pellet was discarded and the supernatant was passed through a 50 Kd ultrafiltration unit. The 50 Kd filtrate (0.6 liters) was loaded onto a 110 ml Q-Sepharose fast Flow column (Amersham Pharmacia, equilibrated with 20 mM Tris-HCl pH 7.5) at a flow rate of 400 ml/hour. The column was washed with six volumes of 20 mM Tris-HCl pH 7.5 and proteins were eluted with 50 mM acetic acid collecting 50 ml fractions. Fractions containing ST peptide variant or wild-type ST peptide were pooled and the solvent was removed by rotary evaporation. The dried proteins were resuspended in 10 ml of 8% acetic acid, 0.1% trifluoroacetic acid (TFA) and loaded onto a Varian Polaris C18-A column (250 X 21.2 mm 10 μm, equilibrated in the same buffer) at a

flow rate of 20 ml/min. The column was washed with 100 ml of 8% methanol, 0.1% TFA and developed with a gradient (300 ml) of 24 to 48% methanol, 0.1% TFA, collecting 5-ml fractions. Fractions containing peptide were pooled and the solvent was removed by rotary evaporation. The peptides were dissolved in 0.1%TFA and lyophilized.

The SEQ ID NO:5 peptide and SEQ ID NO:4 peptide fractions were analyzed by standard LCMS and HPLC. LCMS analysis revealed that SEQ ID NO:5 peptide is more homogeneous than SEQ ID NO: 4 peptide (see Figure 1a; note that SEQ ID NO:5 peptide exhibits fewer peaks (Panel B) than SEQ ID NO:4 peptide (Panel A)).

**1b: Preparation of synthetic variant ST peptides and wild-type ST peptide**

Peptides were chemically synthesized by a commercial peptide synthesis company. Varying yields of peptides were obtained depending on the efficiency of chemical synthesis. Thus, the four peptides, in decreasing order of yield were: Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3), 10-20% yield; Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:6); Asn Ser Ser Asn Tyr Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:5); Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:SEQ ID NO:4), <5% yield. Thus the specific amino acid changes introduced into the peptides can create improved manufacturing properties.

Figure 1b shows the total ion chromatograph profile of synthetically manufacturedSEQ ID NO:3 peptide. Figure 1c shows the total ion chromatograph profile of the control blank sample. There is one major peak present in the SEQ ID NO:3 peptide sample that is not also present in the control sample. Quantitative analysis suggests the SEQ ID NO:3 peptide is >98% pure.

**Example 2: Activation of the intestinal GC-C receptor by a variant ST peptide and ST peptide**

The ability of SEQ ID NO:5 , SEQ ID NO:4, and SEQ ID NO:3 to activate the intestinal GC-C receptor was assessed in an assay employing the T84 human colon carcinoma cell line (American Type Culture Collection (Bethesda, Md)). For the assays cells were grown to confluency in 24-well culture plates with a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum and were used at between passages 54 and 60.

Briefly, monolayers of T84 cells in 24-well plates were washed twice with 1 ml/well DMEM, then incubated at 37°C for 10 min with 0.45 ml DMEM containing 1 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor. Test peptides (50µl) were then added and incubated for 30 minutes at 37°C. The media was aspirated and the reaction was then terminated by the addition of ice cold 0.5 ml of 0.1N HCl. The samples were held on ice for 20 minutes and then evaporated to dryness using a heat gun or vacuum centrifugation. The dried samples were resuspended in 0.5ml of phosphate buffer provided in the Cayman Chemical Cyclic GMP EIA kit (Cayman Chemical, Ann Arbor, MI). Cyclic GMP was measured by EIA according to procedures outlined in the Cayman Chemical Cyclic GMP EIA kit.

Figure 2 shows the activity of chemically synthesized peptide variants in this GC-C receptor activity assay. In this assay, SEQ ID NO:4 and two different SEQ ID NO:3 peptides (SEQ ID NO:3(a) and SEQ ID NO:3(b), synthesized by two different methods) had activity comparable to SEQ ID NO:4. SEQ ID NO:5 and SEQ ID NO:4 peptide were chemically synthesized in a manner identical to that of SEQ ID NO:3(b).

**Example 3: SEQ ID NO:5 and SEQ ID NO:4 increase intestinal transit in mice**

In order to determine whether the peptides increase the rate of gastrointestinal transit, the peptides and controls were tested using a murine gastrointestinal transit (GIT) assay (Moon et al. *Infection and Immunity* 25:127, 1979). In this assay, charcoal, which can be readily visualized in the gastrointestinal tract is administered to mice after the administration of a test compound. The distance traveled by the charcoal is measured and expressed as a percentage of the total length of the colon.

Mice were fasted with free access to water for 12 to 16 hours before the treatment with peptide or control buffer. The peptides were orally administered at 1μg/kg – 1mg/kg of peptide in buffer (20mM Tris pH 7.5) 7 minutes before being given an oral dose of 5% Activated Carbon (Aldrich 242276-250G). Control mice were administered buffer only before being given a dose of Activated Carbon. After 15 minutes, the mice were sacrificed and their intestines from the stomach to the cecum were dissected. The total length of the intestine as well as the distance traveled from the stomach to the charcoal front was measured for each animal and the results are expressed as the percent of the total length of the intestine traveled by the charcoal front. All results are reported as the average of 10 mice ± standard deviation. A comparison of the distance traveled by the charcoal between the mice treated with peptide versus the mice treated with vehicle alone was performed using a Student's t test and a statistically significant difference was considered for $P<0.05$. P-values are calculated using a two-sided T-Test assuming unequal variances.

As can be seen in Figure 3a and Figure 3b, wild-type ST peptide (SEQ ID NO:4, (Sigma-Aldrich, St Louis, MO); 0.1 mg/kg), synthetically manufactured SEQ ID NO:3 and Zelnorm® (0.1 mg/kg), a drug approved for IBS that is an agonist for the serotonin receptor 5HT4, increase gastrointestinal transit rate in this model. Figure 4a shows the result of a study demonstrating that intestinal transit rate increases with an increasing dosage of either recombinantly synthesized SEQ ID NO:4 or SEQ ID NO:5. Figure 4b shows the results of a

study demonstrating both chemically synthesized SEQ ID NO:4 or SEQ ID NO:3 peptide increase intestinal transit rates more than either Tris buffer alone or an equivalent dose of Zelnorm®.

The identical experiment was performed to determine if SEQ ID NO:3 is effective in a chronic dosing treatment regimen. Briefly, 8 week old CD1 female mice are dosed orally once a day for 5 days with either SEQ ID NO:3 (0.06mg/kg or 0.25mg/kg in 20mM Tris pH 7.5) or vehicle alone (20mM Tris pH 7.5). On the 5$^{th}$ day, a GIT assay is performed identical to that above except 200µl of a 10% charcoal solution is administered. Figure 4c shows the results of a study demonstrating both chemically synthesized SEQ ID NO:3 or Zelnorm® are effective in a mouse gastrointestinal motility assay upon chronic dosing (daily for 5 days). The results are shown side by side with acute dosing (1 day).

**Example 4: SEQ ID NO:5 peptide and SEQ ID NO:4 peptide increase intestinal secretion in suckling mice (SuMi assay)**

SEQ ID NO:4 peptide and SEQ ID NO:5 were tested for their ability to increase intestinal secretion using a suckling mouse model of intestinal secretion. In this model a test compound is administered to suckling mice that are between 7 and 9 days old. After the mice are sacrificed, the gastrointestinal tract from the stomach to the cecum is dissected ("guts"). The remains ("carcass") as well as the guts are weighed and the ratio of guts to carcass weight is calculated. If the ratio is above 0.09, one can conclude that the test compound increases intestinal secretion. Figure 5a shows a dose response curve for wild-type ST peptide (SEQ ID NO:4) in this model. Figure 5b shows dose response curve for the SEQ ID NO:3 peptide in this model. These data show that wild-type ST peptide (purchased from TDT, Inc. West Chester, PA) and the SEQ ID NO:3 peptide increase intestinal secretion. The effect of Zelnorm® was also studied. As can be seen from Figure 5, Zelnorm® at 0.2 mg/kg does not increase intestinal secretion in this model. Figure 6a shows a dose response curve for the recombinant SEQ ID NO:4 peptide described above and the recombinant SEQ ID NO:5 peptide described above. As can be seen from Figure 6a, both peptides increase intestinal secretion in this model.

Similarly figure 6b shows a dose response curve for chemically synthesized SEQ ID NO:5, SEQ ID NO:3 and SEQ ID NO:4 as well as wild-type ST peptide (purchased from Sigma-Aldrich, St Louis, MO).

Colonic hyperalgesia animal models

Hypersensitivity to colorectal distension is common in patients with IBS and may be responsible for the major symptom of pain. Both inflammatory and non-inflammatory animal models of visceral hyperalgesia to distension have been developed to investigate the effect of compounds on visceral pain in IBS.

I. Trinitrobenzenesulphonic acid (TNBS)-induced rectal allodynia model

Male Wistar rats (220-250 g) were premedicated with 0.5 mg/kg of acepromazine injected intraperitoneally (IP) and anesthetized by intramuscular administration of 100 mg/kg of ketamine. Pairs of nichrome wire electrodes (60 cm in length and 80 μm in diameter) were implanted in the striated muscle of the abdomen, 2 cm laterally from the white line. The free ends of electrodes were exteriorized on the back of the neck and protected by a plastic tube attached to the skin. Electromyographic (EMG) recordings were started 5 days after surgery. Electrical activity of abdominal striated muscle was recorded with an electroencephalograph machine (Mini VIII, Alvar, Paris, France) using a short time constant (0.03 sec.) to remove low-frequency signals (<3 Hz).

Ten days post surgical implantation, trinitrobenzenesulphonic acid (TNBS) was administered to induce rectal inflammation. TNBS (80 mg $kg^{-1}$ in 0.3 ml 50 % ethanol) was administered intrarectally through a silicone rubber catheter introduced at 3 cm from the anus under light diethyl-ether anesthesia, as described (Morteau et al. 1994 Dig Dis Sci 39:1239). Following TNBS administration, rats were placed in plastic tunnels where they were severely limited in mobility for several days before colorectal distension (CRD). Experimental compound was administered one hour before CRD which was performed by insertion into the rectum, at 1 cm of the anus, a 4 cm long balloon made from a latex condom (Gue et al, 1997

*Neurogastroenterol. Motil.* 9:271). The balloon was fixed on a rigid catheter taken from an embolectomy probe (Fogarty). The catheter attached balloon was fixed at the base of the tail. The balloon, connected to a barostat, was inflated progressively by step of 15 mmHg, from 0 to 60 mmHg, each step of inflation lasting 5 min. Evaluation of rectal sensitivity, as measured by EMG, was performed before (1-2 days) and 3 days following rectal instillation of TNBS.

The number of spike bursts that corresponds to abdominal contractions was determined per 5 min periods. Statistical analysis of the number of abdominal contractions and evaluation of the dose-effects relationships was performed by a one way analysis of variance (ANOVA) followed by a post-hoc (Student or Dunnett tests) and regression analysis for ED50 if appropriate.

Figure 7 shows the results of experiment in which SEQ ID NO:3 activity was analyzed in the TNBS colorectal model. Significant decreases in abdominal response are observed at 0.3 μg/kg and 3 μg/kg SEQ ID NO:3. These results demonstrate that SEQ ID NO:3 reduces pain associated with colorectal distension in this animal model.

II. Stress-induced hyperalgesia model

Male Wistar Rats (200-250 g) are surgically implanted with nichrome wire electrodes as in the TNBS model. Ten days post surgical implantation, partial restraint stress (PRS), is performed as described by Williams et al. for two hours (Williams et al. 1988 Gastroenterology 64:611). Briefly, under light anesthesia with ethyl-ether, the foreshoulders, upper forelimbs and thoracic trunk are wrapped in a confining harness of paper tape to restrict, but not prevent body movements. Control sham-stress animals are anaesthetized but not wrapped. Thirty minutes before the end of the PRS session, the animals are administered test-compound or vehicle. Thirty minutes to one hour after PRS completion, the CRD distension procedure is performed as described above for the TNBS model with barostat at pressures of 15, 30, 45 and 60mm Hg. Statistical analysis on the number of bursts is determined and analyzed as in the TNBS model above.

Phenylbenzoquinone-induced writhing model

The PBQ-induced writhing model can be used to assess pain control activity of the peptides and GC-C receptor agonists of the invention. This model is described by Siegmund et al. (1957 Proc. Soc. Exp. Bio. Med. 95:729-731). Briefly, one hour after oral dosing with a test compound, e.g., a peptide, morphine or vehicle, 0.02% phenylbenzoquinone (PBQ) solution (12.5 mL/kg) is injected by intraperitoneal route into the mouse. The number of stretches and writhings are recorded from the $5^{th}$ to the $10^{th}$ minute after PBQ injection, and can also be counted between the $35^{th}$ and $40^{th}$ minute and between the $60^{th}$ and $65^{th}$ minute to provide a kinetic assessment. The results are expressed as the number of stretches and writhings (mean ± SEM) and the percentage of variation of the nociceptive threshold calculated from the mean value of the vehicle-treated group. The statistical significance of any differences between the treated groups and the control group is determined by a Dunnett's test using the residual variance after a one-way analysis of variance ($P< 0.05$) using SigmaStat Software.

Figures 8a and 8b show the effect of different doses of SEQ ID NO:5 and SEQ ID NO:3 in the PBQ writhing assay. Indomethacin, an NSAID (nonsteroidal anti-inflammatory drug) with known pain control activity, was used as the positive control in the assay. Significant reductions in writhings were observed for SEQ ID NO:5 (1 mg/kg dose) and SEQ ID NO:3 (2.5 mg/kg dose) compared to the vehicle control. Loss of efficacy at the highest dose tested has also been observed for multiple other compounds (such as 5HT-3 antagonists) tested in similar assays. The results of this study suggest that both SEQ ID NO:5 and SEQ ID NO:3 have antinociceptive effects in this visceral pain model comparable to the intermediate doses of indomethacin.

**Example 5: SEQ ID NO:3 Kd determination**

To determine the affinity of SEQ ID NO:3 for GC-C receptors found in rat intestinal mucosa, a competition binding assay was performed using rate intestinal epithelial cells. Epithelial cells from the small intestine of rats were obtained as described by Kessler et al. (*J. Biol. Chem.*

245: 5281-5288 (1970)). Briefly, animals were sacrificed and their abdominal cavities exposed. The small intestine was rinsed with 300 ml ice cold saline or PBS. 10 cm of the small intestine measured at 10 cm from the pylorus was removed and cut into 1 inch segments. Intestinal mucosa was extruded from the intestine by gentle pressure between a piece of parafilm and a P-1000 pipette tip. Intestinal epithelial cells were placed in 2 ml PBS and pipetted up and down with a 5 ml pipette to make a suspension of cells. Protein concentration in the suspension was measured using the Bradford method (*Anal. Biochem.* 72: 248-254 (1976)).

A competition binding assay was performed based on the method of Giannella et al. (*Am. J. Physiol.* 245: G492-G498) between [$^{125}$I] labeled SEQ ID NO:4 and SEQ ID NO:3. The assay mixture contained: 0.5 ml of DME with 20 mM HEPES-KOH pH 7.0, 0.9 mg of the cell suspension listed above, 21.4 fmol [$^{125}$I]-SEQ ID NO:4 (42.8 pM), and different concentrations of competitor SEQ ID NO:3 (0.01 to 1000 nM). The mixture was incubated at room temperature for 1 hour, and the reaction stopped by applying the mixture to GF/B glass-fiber filters (Whatman). The filters were washed with 5 ml ice-cold PBS and radioactivity was measured. Figure 9 shows that the Kd for SEQ ID NO:3 in this assay is 4.5 nm. %B/Bo is the percentage of the ratio of radioactivity trapped in each sample (B) compared to the radioactivity retained in a control sample with no cold competitor (Bo). Giannella et al. (*Am. J. Physiol.*245: G492-G498) observed that the Kd for wild-type ST peptide in this same assay was ~13 nm.

**Example 6: Pharmacokinetic properties of SEQ ID NO:3**

To study the pharmacokinetics of SEQ ID NO:3, absorbability studies in mice were performed by administering SEQ ID NO:3 intravaneously via tail vein injection or orally by gavage to 8-week-old CD1 mice. Serum was collected from the animals at various time points and tested for the presence of SEQ ID NO:3 using a competitive enzyme-linked immunoabsorbent assay (Oxoid, ST EIA kit, Cat#TD0700). The assay utilized monoclonal antibodies against ST peptide (antibodies are provided in the Oxoid kit) and synthetically manufactured SEQ ID NO:3. Figure 10a shows absorption data for intravenously and orally administered SEQ ID

NO:3 as detected by the ELISA assay. SEQ ID NO:3 appears to be minimally systemically absorbed and is < 2.2% bioavailable.

A similar bioavailability study was performed in which LCMS rather than ELISA was used to detect SEQ ID NO:3. Initially, serum samples were extracted from the whole blood of exposed and control mice, then injected directly (10mL) onto an in-line solid phase extraction (SPE) column (Waters Oasis HLB 25mm column, 2.0 x 15mm direct connect) without further processing. The sample on the SPE column was washed with a 5% methanol, 95% $dH_2O$ solution (2.1 mL/min, 1.0 minute), then loaded onto an analytical column using a valve switch that places the SPE column in an inverted flow path onto the analytical column (Waters Xterra MS C8 5mm IS column, 2.1 x 20mm). The sample was eluted from the analytical column with a reverse phase gradient (Mobile Phase A: 10 mM ammonium hydroxide in $dH_2O$, Mobile Phase B: 10 mM ammonium hydroxide in 80% acetonitrile and 20% methanol; 20% B for the first 3 minutes then ramping to 95% B over 4 min. and holding for 2 min., all at a flow rate of 0.4 mL/min.). At 9.1 minutes, the gradient returns to the initial conditions of 20%B for 1 min. SEQ ID NO:3 eluted from the analytical column at 1.45 minutes, and was detected by triple-quadrapole mass spectrometry (MRM, 764 (+2 charge state)>182 (+1 charge state) Da; cone voltage = 30V; collision = 20 eV; parent resolution = 2 Da at base peak; daughter resolution = 2 Da at base peak). Instrument response was converted into concentration units by comparison with a standard curve using known amounts of chemically synthesized SEQ ID NO:3 prepared and injected in mouse serum using the same procedure.

Figure 10b shows absorption data for IV and orally administered SEQ ID NO:3 as detected by LCMS. In this assay, SEQ ID NO:3 appears similarly minimally systemically absorbed and is < 0.11 % bioavailable.

Administration of peptides and GC-C receptor agonists

For treatment of gastrointestinal disorders, the peptides and agonists of the invention are preferably administered orally, e.g., as a tablet or cachet containing a predetermined amount of

the active ingredient, pellet, gel, paste, syrup, bolus, electuary, slurry, sachet; capsule; powder; lyophilized powder; granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, via a liposomal formulation (see, e.g., EP 736299) or in some other form. Orally administered compositions can include binders, lubricants, inert diluents, lubricating, surface active or dispersing agents, flavoring agents, and humectants. Orally administered formulations such as tablets may optionally be coated or scored and may be formulated so as to provide sustained, delayed or controlled release of the active ingredient therein. The peptides and agonists can be co-administered with other agents used to treat gastrointestinal disorders including but not limited to the agents described herein. The peptides and agonists can also be administered by rectal suppository. For the treatment of disorders outside the gastrointestinal tract such as congestive heart failure and benign prostatic hypertrophy, peptides and agonists are preferably administered parenterally or orally.

The peptides described herein can be administered alone or in combination with other agents. For example, the peptides can be administered together with an analgesic peptide or compound. The analgesic peptide or compound can be covalently attached to a peptide described herein or it can be a separate agent that is administered together with or sequentially with a peptide described herein in a combination therapy.

Combination therapy can be achieved by administering two or more agents, e.g., a peptide described herein and an analgesic peptide or compound, each of which is formulated and administered separately, or by administering two or more agents in a single formulation. Other combinations are also encompassed by combination therapy. For example, two agents can be formulated together and administered in conjunction with a separate formulation containing a third agent. While the two or more agents in the combination therapy can be administered simultaneously, they need not be. For example, administration of a first agent (or combination of agents) can precede administration of a second agent (or combination of agents) by minutes, hours, days, or weeks. Thus, the two or more agents can be administered within minutes of each other or within 1, 2, 3, 6, 9, 12, 15, 18, or 24 hours of each other or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 days of each other or within 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks of each other.

In some cases even longer intervals are possible. While in many cases it is desirable that the two or more agents used in a combination therapy be present in within the patient's body at the same time, this need not be so.

Combination therapy can also include two or more administrations of one or more of the agents used in the combination. For example, if agent X and agent Y are used in a combination, one could administer them sequentially in any combination one or more times, e.g., in the order X-Y-X, X-X-Y, Y-X-Y, Y-Y-X, X-X-Y-Y, etc.

The agents, alone or in combination, can be combined with any pharmaceutically acceptable carrier or medium. Thus, they can be combined with materials that do not produce an adverse, allergic or otherwise unwanted reaction when administered to a patient. The carriers or mediums used can include solvents, dispersants, coatings, absorption promoting agents, controlled release agents, and one or more inert excipients (which include starches, polyols, granulating agents, microcrystalline cellulose, diluents, lubricants, binders, disintegrating agents, and the like), etc. If desired, tablet dosages of the disclosed compositions may be coated by standard aqueous or nonaqueous techniques.

Compositions of the present invention may also optionally include other therapeutic ingredients, anti-caking agents, preservatives, sweetening agents, colorants, flavors, desiccants, plasticizers, dyes, and the like. Any such optional ingredient must be compatible with the compound of the invention to insure the stability of the formulation.
The composition may contain other additives as needed, including for exanple lactose, glucose, fructose, galactose, trehalose, sucrose, maltose, raffinose, maltitol, melezitose, stachyose, lactitol, palatinite, starch, xylitol, mannitol, myoinositol, and the like, and hydrates thereof, and amino acids, for example alanine, glycine and betaine, and peptides and proteins, for example albumen.

Examples of excipients for use as the pharmaceutically acceptable carriers and the pharmaceutically acceptable inert carriers and the aforementioned additional ingredients include, but are not limited to binders, fillers, disintegrants, lubricants, anti-microbial agents,

and coating agents such as:

BINDERS: corn starch, potato starch, other starches, gelatin, natural and synthetic gums such as acacia, sodium alginate, alginic acid, other alginates, powdered tragacanth, guar gum, cellulose and its derivatives (*e.g.*, ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl cellulose), polyvinyl pyrrolidone, methyl cellulose, pre-gelatinized starch (*e.g.*, STARCH 1500® and STARCH 1500 LM®, sold by Colorcon, Ltd.), hydroxypropyl methyl cellulose, microcrystalline cellulose (*e.g.* AVICEL™, such as, AVICEL-PH-101™, -103™ and -105™, sold by FMC Corporation, Marcus Hook, PA, USA), or mixtures thereof,

FILLERS: talc, calcium carbonate (*e.g.*, granules or powder), dibasic calcium phosphate, tribasic calcium phosphate, calcium sulfate (*e.g.*, granules or powder), microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol, starch, pre-gelatinized starch, or mixtures thereof,

DISINTEGRANTS: agar-agar, alginic acid, calcium carbonate, microcrystalline cellulose, croscarmellose sodium, crospovidone, polacrilin potassium, sodium starch glycolate, potato or tapioca starch, other starches, pre-gelatinized starch, clays, other algins, other celluloses, gums, or mixtures thereof,

LUBRICANTS: calcium stearate, magnesium stearate, mineral oil, light mineral oil, glycerin, sorbitol, mannitol, polyethylene glycol, other glycols, stearic acid, sodium lauryl sulfate, talc, hydrogenated vegetable oil (*e.g.*, peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil and soybean oil), zinc stearate, ethyl oleate, ethyl laurate, agar, syloid silica gel (AEROSIL 200, W.R. Grace Co., Baltimore, MD USA), a coagulated aerosol of synthetic silica (Deaussa Co., Plano, TX USA), a pyrogenic silicon dioxide (CAB-O-SIL, Cabot Co., Boston, MA USA), or mixtures thereof,

ANTI-CAKING AGENTS: calcium silicate, magnesium silicate, silicon dioxide, colloidal

silicon dioxide, talc, or mixtures thereof,

ANTIMICROBIAL AGENTS: benzalkonium chloride, benzethonium chloride, benzoic acid, benzyl alcohol, butyl paraben, cetylpyridinium chloride, cresol, chlorobutanol, dehydroacetic acid, ethylparaben, methylparaben, phenol, phenylethyl alcohol, phenoxyethanol, phenylmercuric acetate, phenylmercuric nitrate, potassium sorbate, propylparaben, sodium benzoate, sodium dehydroacetate, sodium propionate, sorbic acid, thimersol, thymo, or mixtures thereof, and

COATING AGENTS: sodium carboxymethyl cellulose, cellulose acetate phthalate, ethylcellulose, gelatin, pharmaceutical glaze, hydroxypropyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl methyl cellulose phthalate, methylcellulose, polyethylene glycol, polyvinyl acetate phthalate, shellac, sucrose, titanium dioxide, carnauba wax, microcrystalline wax, or mixtures thereof.

The agents either in their free form or as a salt can be combined with a polymer such as polylactic-glycoloic acid (PLGA), poly-(I)-lactic-glycolic-tartaric acid (P(I)LGT) (WO 01/12233), polyglycolic acid (U.S. 3,773,919), polylactic acid (U.S. 4,767,628), poly(ε-caprolactone) and poly(alkylene oxide) (U.S. 20030068384) to create a sustained release formulation. Such formulations can be used to implants that release a peptide or another agent over a period of a few days, a few weeks or several months depending on the polymer, the particle size of the polymer, and the size of the implant (see, e.g., U.S. 6,620,422). Other sustained release formulations and polymers for use in are described in EP 0 467 389 A2, WO 93/24150, U.S. 5,612,052, WO 97/40085, WO 03/075887, WO 01/01964A2, U.S. 5,922,356, WO 94/155587, WO 02/074247A2, WO 98/25642, U.S. 5,968,895, U.S. 6,180,608, U.S. 20030171296, U.S. 20020176841, U.S. 5,672,659, U.S. 5,893,985, U.S. 5,134,122, U.S. 5,192,741, U.S. 5,192,741, U.S. 4,668,506, U.S. 4,713,244, U.S. 5,445,832 U.S. 4,931,279, U.S. 5,980,945, WO 02/058672, WO 9726015, WO 97/04744, and. US20020019446. In such sustained release formulations microparticles of peptide are combined with microparticles of polymer. One or more sustained release implants can be placed in the large intestine, the small

intestine or both. U.S. 6,011,011 and WO 94/06452 describe a sustained release formulation providing either polyethylene glycols (i.e. PEG 300 and PEG 400) or triacetin. WO 03/053401 describes a formulation which may both enhance bioavailability and provide controlled releaseof the agent within the GI tract. Additional controlled release formulations are described in WO 02/38129, EP 326 151, U.S. 5,236,704, WO 02/30398, WO 98/13029; U.S. 20030064105, U.S. 20030138488A1, U.S. 20030216307A1, U.S. 6,667,060, WO 01/49249, WO 01/49311, WO 01/49249, WO 01/49311, and U.S. 5,877,224.

The agents can be administered, e.g., by intravenous injection, intramuscular injection, subcutaneous injection, intraperitoneal injection, topical, sublingual, intraarticular (in the joints), intradermal, buccal, ophthalmic (including intraocular), intranasaly (including using a cannula), intraspinally, intrathecally, or by other routes. The agents can be administered orally, e.g., as a tablet or cachet containing a predetermined amount of the active ingredient, gel, pellet, paste, syrup, bolus, electuary, slurry, capsule, powder, lyophilized powder, granules, sachet, as a solution or a suspension in an aqueous liquid or a non-aqueous liquid, as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, via a micellar formulation (see, e.g. WO 97/11682) via a liposomal formulation (see, e.g., EP 736299,WO 99/59550 and WO 97/13500), via formulations described in WO 03/094886 or in some other form. Orally administered compositions can include binders, lubricants, inert diluents, lubricating, surface active or dispersing agents, flavoring agents, and humectants. Orally administered formulations such as tablets may optionally be coated or scored and may be formulated so as to provide sustained, delayed or controlled release of the active ingredient therein. The agents can also be administered transdermally (i.e. via reservoir-type or matrix-type patches, microneedles, thermal poration, hypodermic needles, iontophoresis, electroporation, ultrasound or other forms of sonophoresis, jet injection, or a combination of any of the preceding methods (Prausnitz et al. 2004, Nature Reviews Drug Discovery 3:115-124)). The agents can be administered using high-velocity transdermal particle injection techniques using the hydrogel particle formulation described in U.S. 20020061336. Additional particle formulations are described in WO 00/45792, WO 00/53160, and WO 02/19989. An example of a transdermal formulation containing plaster and the absorption promoter dimethylisosorbide can be found in

WO 89/04179. WO 96/11705 provides formulations suitable for transdermal adminisitration. The agents can be administered in the form a suppository or by other vaginal or rectal means. The agents can be administered in a transmembrane formulation as described in WO 90/07923. The agents can be administed non-invasively via the dehydrated particicles described in U.S. 6,485,706. The agent can be administered in an enteric-coated drug formulation as described in WO 02/49621. The agents can be administered intranassaly using the formulation described in U.S. 5,179,079. Formulations suitable for parenteral injection are described in WO 00/62759. The agents can be administered using the casein formulation described in U. S. 20030206939 and WO 00/06108. The agents can be administered using the particulate formulations described in U.S. 20020034536.

The agents, alone or in combination with other suitable components, can be administered by pulmonary route utilizing several techniques including but not limited to intratracheal instillation (delivery of solution into the lungs by syringe), intratracheal delivery of liposomes, insufflation (administration of powder formulation by syringe or any other similar device into the lungs) and aerosol inhalation. Aerosols (e.g., jet or ultrasonic nebulizers, metered-dose inhalers (MDIs), and dry-powder inhalers (DPIs)) can also be used in intranasal applications. Aerosol formulations are stable dispersions or suspensions of solid material and liquid droplets in a gaseous medium and can be placed into pressurized acceptable propellants, such as hydrofluroalkanes (HFAs, i.e. HFA-134a and HFA-227, or a mixture thereof), dichlorodifluoromethane (or other chlorofluocarbon propellants such as a mixture of Propellants 11, 12, and/or 114), propane, nitrogen, and the like. Pulmonary formulations may include permeation enhancers such as fatty acids, and saccharides, chelating agents, enzyme inhibitors (e.g., protease inhibitors), adjuvants (e.g., glycocholate, surfactin, span 85, and nafamostat), preservatives (e.g., benzalkonium chloride or chlorobutanol), and ethanol (normally up to 5% but possibly up to 20%, by weight). Ethanol is commonly included in aerosol compositions as it can improve the function of the metering valve and in some cases also improve the stability of the dispersion. Pulmonary formulations may also include surfactants which include but are not limited to bile salts and those described in U.S. 6,524,557 and references therein. The surfactants described in U.S. 6,524,557, e.g., a C8-C16 fatty acid

salt, a bile salt, a phospholipid, or alkyl saccaride are advantageous in that some of them also reportedly enhance absorption of the peptide in the formulation. Also suitable in the invention are dry powder formulations comprising a therapeutically effective amount of active compound blended with an appropriate carrier and adapted for use in connection with a dry-powder inhaler. Absorption enhancers which can be added to dry powder formulations of the present invention include those described in U.S. 6,632,456. WO 02/080884 describes new methods for the surface modification of powders. Aerosol formulations may include U.S. 5,230,884, U.S. 5,292,499, WO 017/8694, WO 01/78696, U.S. 2003019437, U. S. 20030165436, and WO 96/40089 (which includes vegetable oil). Sustained release formulations suitable for inhalation are described in U.S. 20010036481A1, 20030232019A1, and U.S. 20040018243A1 as well as in WO 01/13891, WO 02/067902, WO 03/072080, and WO 03/079885. Pulmonary formulations containing microparticles are described in WO 03/015750, U.S. 20030008013, and WO 00/00176. Pulmonary formulations containing stable glassy state powder are described in U.S. 20020141945 and U.S. 6,309,671. Other aerosol formulations are desribed in EP 1338272A1 WO 90/09781, U. S. 5,348,730, U.S. 6,436,367, WO 91/04011, and U.S. 6,294,153 and U.S. 6,290,987 describes a liposomal based formulation that can be administered via aerosol or other means. Powder formulations for inhalation are described in U.S. 20030053960 and WO 01/60341. The agents can be administered intranasally as described in U.S. 20010038824.

Solutions of medicament in buffered saline and similar vehicles are commonly employed to generate an aerosol in a nebulizer. Simple nebulizers operate on Bernoulli's principle and employ a stream of air or oxygen to generate the spray particles. More complex nebulizers employ ultrasound to create the spray particles. Both types are well known in the art and are described in standard textbooks of pharmacy such as Sprowls' American Pharmacy and Remington's The Science and Practice of Pharmacy. Other devices for generating aerosols employ compressed gases, usually hydrofluorocarbons and chlorofluorocarbons, which are mixed with the medicament and any necessary excipients in a pressurized container, these devices are likewise described in standard textbooks such as Sprowls and Remington.

The agents can be a free acid or base, or a pharmacologically acceptable salt thereof. Solids can be dissolved or dispersed immediately prior to administration or earlier. In some circumstances the preparations include a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injection can include sterile aqueous or organic solutions or dispersions which include, e.g., water, an alcohol, an organic solvent, an oil or other solvent or dispersant (e.g., glycerol, propylene glycol, polyethylene glycol, and vegetable oils). The formulations may contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Pharmaceutical agents can be sterilized by filter sterilization or by other suitable means. The agent can be fused to immunoglobulins or albumin, or incorporated into a lipsome to improve half-life. The agent can also be conjugated to polyethylene glycol (PEG) chains. Methods for pegylation and additional formulations containing PEG-conjugates (i.e. PEG-based hydrogels, PEG modified liposomes) can be found in Harris and Chess, Nature Reviews Drug Discovery 2: 214-221 and the references therein. Peptides can also be modified with alkyl groups (e.g., C1-C20 straight or branched alkyl groups); fatty acid radicals; and combinations of PEG, alkyl groups and fatty acid radicals (see U.S. Patent 6,309,633; Soltero et al., 2001 Innovations in Pharmaceutical Technology 106-110). The agent can be administered via a nanocochleate or cochleate delivery vehicle (BioDelivery Sciences International). The agents can be delivered transmucosally (i.e. across a mucosal surface such as the vagina, eye or nose) using formulations such as that described in U.S. 5,204,108. The agents can be formulated in microcapsules as described in WO 88/01165. The agent can be administered intra-orally using the formulations described in U.S. 20020055496, WO 00/47203, and U.S. 6,495,120. The agent can be delivered using nanoemulsion formulations described in WO 01/91728A2.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of the active compound(s) with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the

intended use. The techniques of preparation are generally well known in the art, as exemplified by Remington's Pharmaceutical Sciences (18th Edition, Mack Publishing Company, 1995).

The agents described herein and combination therapy agents can be packaged as a kit that includes single or multiple doses of two or more agents, each packaged or formulated individually, or single or multiple doses of two or more agents packaged or formulated in combination. Thus, one or more agents can be present in first container, and the kit can optionally include one or more agents in a second container. The container or containers are placed within a package, and the package can optionally include administration or dosage instructions. A kit can include additional components such as syringes or other means for administering the agents as well as diluents or other means for formulation.

Methods to increase chemical and/or physical stability of the agents the described herein are found in U.S. 6,541,606, U.S. 6,068,850, U.S. 6,124,261, U.S. 5,904,935, and WO 00/15224, U.S. 20030069182 (via the additon of nicotinamide), U.S. 20030175230A1, U.S. 20030175230A1, U.S. 20030175239A1, U.S. 20020045582, U.S. 20010031726, WO 02/26248, WO 03/014304, WO 98/00152A1, WO 98/00157A1, WO 90/12029, WO 00/04880, and WO 91/04743, WO 97/04796 and the references cited therein.

Methods to increase bioavailability of the agents described herein are found in U.S. 6,008,187, U.S. 5,424,289, U.S. 20030198619, WO 90/01329, WO 01/49268, WO 00/32172, and WO 02/064166. Glycyrrhizinate can also be used as an absorption enhancer (see, e.g., EP397447). WO 03/004062 discusses Ulex europaeus I (UEAl) and UEAI mimetics which may be used to target the agents of the invention to the GI tract.

The agents described herein can be fused to a modified version of the blood serum protein transferrin. U.S. 20030221201, U.S. 20040023334, U.S. 20030226155, WO 04/020454, and WO 04/019872 discuss the manufacture and use of transferrin fusion proteins. Transferrin fusion proteins may improve circulatory half life and efficacy, decrease undesirable side effects and allow reduced dosage.

The peptides and agonists of the invention can be recombinantly expressed in bacteria. Bacteria expressing the peptide or agonists can be administered orally, rectally, mucosally or in via some other mode of administration including but not limited to those described herein.

Analgesic Agents in combitherapy

The peptides and agonists described herein can be used in combination therapy with an analgesic agent, e.g., an analgesic compound or an analgesic peptide. These peptides and compounds can be administered with the peptides of the invention (simultaneously or sequentially). They can also be optionally covalently linked or attached to an agent described herein to create therapeutic conjugates. Among the useful analgesic agents are: Ca channel blockers, 5HT receptor antagonists (for example 5HT3, 5HT4 and 5HT1 receptor antagonists), opioid receptor agonists (loperamide, fedotozine, and fentanyl), NK1 receptor antagonists, CCK receptor agonists (e.g., loxiglumide), NK1 receptor antagonists, NK3 receptor antagonists, norepinephrine-serotonin reuptake inhibitors (NSRI), vanilloid and cannabanoid receptor agonists, and sialorphin. Analgesics agents in the various classes are described in the literature.

Among the useful analgesic peptides are sialorphin-related peptides, including those comprising the amino acid sequence QHNPR (SEQ ID NO: ), including: VQHNPR (SEQ ID NO: ); VRQHNPR (SEQ ID NO: ); VRGQHNPR (SEQ ID NO: ); VRGPQHNPR (SEQ ID NO: ); VRGPRQHNPR (SEQ ID NO: ); VRGPRRQHNPR (SEQ ID NO: ); and RQHNPR (SEQ ID NO: ). Sialorphin-related peptides bind to neprilysin and inhibit neprilysin-mediated breakdown of substance P and Met-enkephalin. Thus, compounds or peptides that are inhibitors of neprilysin are useful analgesic agents which can be administered with the peptides of the invention in a co-therapy or linked to the peptides of the invention, e.g., by a covalent bond. Sialophin and related peptides are described in U.S. Patent 6,589,750; U.S. 20030078200 A1; and WO 02/051435 A2.

Opioid receptor antagonists and agonists can be administered with the peptides of the invention in co-therapy or linked to the agent of the invention, e.g., by a covalent bond. For example, opioid receptor antagonists such as naloxone, naltrexone, methyl nalozone, nalmefene, cypridime, beta funaltrexamine, naloxonazine, naltrindole, and nor-binaltorphimine are thought to be useful in the treatment of IBS. It can be useful to formulate opioid antagonists of this type is a delayed and sustained release formulation such that initial release of the antagonist is in the mid to distal small intestine and/or ascending colon. Such antagonists are described in WO 01/32180 A2. Enkephalin pentapeptide (HOE825; Tyr-D-Lys-Gly-Phe-L-homoserine) is an agonist of the mu and delta opioid receptors and is thought to be useful for increasing intestinal motility (*Eur. J. Pharm.* 219:445, 1992), and this peptide can be used in conjunction with the peptides of the invention. Also useful is trimebutine which is thought to bind to mu/delta/kappa opioid receptors and activate release of motilin and modulate the release of gastrin, vasoactive intestinal peptide, gastrin and glucagons. Kappa opioid receptor agonists such as fedotozine, asimadoline, and ketocyclazocine, and compounds described in WO 03/097051 A2 can be used with or linked to the peptides of the invention. In addition, mu opioid receptor agonists such as morphine, diphenyloxylate, frakefamide (H-Tyr-D-Ala-Phe(F)-Phe-$NH_2$; WO 01/019849 A1) and loperamide can be used.

Tyr-Arg (kyotorphin) is a dipeptide that acts by stimulating the release of met-enkephalins to elicit an analgesic effect (*J. Biol. Chem* 262:8165, 1987). Kyotorphin can be used with or linked to the peptides of the invention.

Chromogranin-derived peptide (CgA 47–66; see, e.g., Ghia et al. 2004 Regulatory Peptides 119:199) can be used with or linked to the peptides of the invention.

CCK receptor agonists such as caerulein from amphibians and other species are useful analgesic agents that can be used with or linked to the peptides of the invention.

Conotoxin peptides represent a large class of analgesic peptides that act at voltage gated Ca channels, NMDA receptors or nicotinic receptors. These peptides can be used with or linked to the peptides of the invention.

Peptide analogs of thymulin (FR Application 2830451) can have analgesic activity and can be used with or linked to the peptides of the invention.

CCK (CCKa or CCKb) receptor antagonists, including loxiglumide and dexloxiglumide (the R-isomer of loxiglumide) (WO 88/05774) can have analgesic activity and can be used with or linked to the peptides of the invention.

Other useful analgesic agents include 5-HT4 agonists such as tegaserod (Zelnorm®), mosapride, metoclopramide, zacopride, cisapride, renzapride, benzimidazolone derivatives such as BIMU 1 and BIMU 8, and lirexapride. Such agonists are described in: EP1321142 A1, WO 03/053432A1, EP 505322 A1, EP 505322 B1, US 5,510,353, EP 507672 A1, EP 507672 B1, and US 5,273,983.

Calcium channel blockers such as ziconotide and related compounds described in, for example, EP625162B1, US 5,364,842, US 5,587,454, US 5,824,645, US 5,859,186, US 5,994,305, US 6,087,091, US 6,136,786, WO 93/13128 A1, EP 1336409 A1, EP 835126 A1, EP 835126 B1, US 5,795,864, US 5,891,849, US 6,054,429, WO 97/01351 A1, can be used with or linked to the peptides of the invention.

Various antagonists of the NK-1, NK-2, and NK-3 receptors (for a review see Giardina et al. 2003 *Drugs* 6:758) can be can be used with or linked to the peptides of the invention.

NK1 receptor antagonists such as: aprepitant (Merck & Co Inc), vofopitant, ezlopitant (Pfizer, Inc.), R-673 (Hoffmann-La Roche Ltd), SR-48968 (Sanofi Synthelabo), CP-122,721 (Pfizer, Inc.), GW679769 (Glaxo Smith Kline), TAK-637 (Takeda/Abbot), SR-14033, and related

compounds described in, for example, EP 873753 A1, US 20010006972 A1, US 20030109417 A1, WO 01/52844 A1, can be used with or linked to the peptides of the invention.

NK-2 receptor antagonists such as nepadutant (Menarini Ricerche SpA), saredutant (Sanofi-Synthelabo), GW597599 (Glaxo Smith Kline), SR-144190 (Sanofi-Synthelabo) and UK-290795 (Pfizer Inc) can be used with or linked to the peptides of the invention.

NK3 receptor antagonists such as osanetant (SR-142801; Sanofi-Synthelabo), SSR-241586, talnetant and related compounds described in, for example, WO 02/094187 A2, EP 876347 A1, WO 97/21680 A1, US 6,277,862, WO 98/11090, WO 95/28418, WO 97/19927, and Boden et al. (*J Med Chem.* 39:1664-75, 1996) can be used with or linked to the peptides of the invention.

Norepinephrine-serotonin reuptake inhibitors (NSRI) such as milnacipran and related compounds described in WO 03/077897 A1 can be used with or linked to the peptides of the invention.

Vanilloid receptor antagonists such as arvanil and related compouds described in WO 01/64212 A1 can be used with or linked to the peptides of the invention.

The analgesic peptides and compounds can be administered with the peptides and agonists of the invention (simultaneously or sequentially). The analgesic agents can also be covalently linked to the peptides and agonists of the invention to create therapeutic conjugates. Where the analgesic is a peptide and is covalently linked to an agent described herein the resulting peptide may also include at least one trypsin cleavage site. When present within the peptide, the analgesic peptide may be preceded by (if it is at the carboxy terminus) or followed by (if it is at the amino terminus) a trypsin cleavage site that allows release of the analgesic peptide.

In addition to sialorphin-related peptides, analgesic peptides include: AspPhe, endomorphin-1, endomorphin-2, nocistatin, dalargin, lupron, ziconotide, and substance P.

Other Agents for Use in Combitherapy

Also within the invention are pharmaceutical compositions comprising a peptide or agonists of the invention and a second therapeutic agent. The second therapeutic agent can be administered to treat any condition for which it is useful, including conditions that are not considered to be the primary indication for treatment with the second therapeutic agent. The second therapeutic agent can be administered simultaneously or sequentially. The second therapeutic agent can be covalently linked to the peptides and agonists of the invention to create a therapeutic conjugate. When the second therapeutic agent is another peptide, a linker including those described herein may be used between the peptide of the invention and the second therapeutic peptide.

Examples of additional therapeutic agents to treat gastrointestinal and other disorders include:

(1) agents to treat constipation (e.g., a chloride channel activator such as the bicylic fatty acid, Lubiprostone (formerly known as SPI-0211; Sucampo Pharmaceuticals, Inc.; Bethesda, MD), a laxative such as MiraLax; Braintree Laboratories, Braintree MA);

(2) acid reducing agents such as proton pump inhibitors (e.g., omeprazole (Prilosec®), esomeprazole (Nexium®), lansoprazole (Prevacid®), pantoprazole (Protonix®) and rabeprazole (Aciphex®)) and Histamine H2-receptor antagonist (also known as H2 receptor blockers including cimetidine, ranitidine, famotidine and nizatidine);

(3) prokinetic agents including metoclopramide (Reglan®), domperidone (Motilium®), erythromycin or cisapride (propulsid®)

(4) pro-motility agents such as the vasostatin-derived peptide, chromogranin A (4–16) (see, e.g., Ghia et al. 2004 Regulatory Peptides 121:31) or motilin agonists (e.g., GM-611 or mitemcinal fumarate);

(5) complete or partial 5HT (e.g. 5HT1, 5HT2, 5HT3, 5HT4) receptor agonists or antagonists (including 5HT4 receptor agonists (such as tegaserod (ZELNORM®), mosapride, metoclopramide, zacopride, cisapride, renzapride, benzimidazolone derivatives such as BIMU 1 and BIMU 8, and lirexapride. Such agonists are described in: EP1321142 A1, WO 03/053432A1, EP 505322 A1, EP 505322 B1, US 5,510,353, EP 507672 A1, EP 507672

B1, and US 5,273,983); 5HT3 receptor agonists such as MKC-733; and 5HT3 receptor antagonists such as alosetron and ATI-7000 (Aryx Therapeutics, Santa Clara CA);

(6) muscarinic receptor agonists;

(7) anti-inflammatory agents;

(8) antispasmodics;

(9) antidepressants;

(10) centrally-acting analgesic agents such as opioid receptor agonists, opioid receptor antagonists (e.g., naltrexone);

(11) agents for the treatment of Inflammatory bowel disease;

(12) agents for the treatment of Crohn's disease and/or ulcerative colitis (e.g., alequel (Enzo Biochem, Inc.; Farmingsale, NY), the anti-inflammatory peptide RDP58 (Genzyme, Inc.; Cambridge, MA), and TRAFICET-EN™ (ChemoCentryx, Inc.; San Carlos, CA);

(13) agents that treat gastrointestinal or visceral pain;

(14) PDE (phosphodiesterase) inhibitors including but not limited to those disclosed herein;

(15) purgatives that draw fluids to the intestine (e.g., VISICOL®, a combination of sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrate);

(16) Corticotropin Releasing Factor (CRF) receptor antagonists (including NBI-34041 (Neurocrine Biosciences, San Diego, CA), CRH9-41, astressin, R121919 (Janssen Pharmaceutica), CP154,526, NBI-27914, Antalarmin, DMP696 (Bristol-Myers Squibb) CP-316,311 (Pfizer, Inc.), GW876008 (Neurocrine/Glaxo Smith Kline), ONO-2333Ms (Ono Pharmaceuticals), TS-041 (Janssen), AAG561 (Novartis) and those disclosed in US 5,063,245, US 5,861,398, US20040224964, US20040198726, US20040176400, US20040171607, US20040110815, and US20040006066);

(17) glucagon-like peptides (glp-1) and analogues thereof (including exendin-4) and inhibitors of DPP-IV (DPP-IV mediates the inactivation of glp-1);

(18) tofisopam, enantiomerically-pure R-tofisopam, and pharmaceutically-acceptable salts thereof (US 20040229867)

(19) the tricyclic anti-depressant of the dibenzothiazepine type, tianeptine (Stablon®) and other agents described in U.S. 6,683,072;

(20) (E)-4 (1,3bis(cyclohexylmethyl)-1,2,34,-tetrahydro-2,6-diono-9H-purin-8-yl)cinnamic acid nonaethylene glycol methyl ether ester and related compounds described in WO 02/067942;

and

(21) the probiotic PROBACTRIX® (The BioBalance Corporation; New York, NY) which contains microorganisms useful in the treatment of gastrointestinal disorders.

The peptides and agonists of the invention can be used in combination therapy with insulin and related compounds including primate, rodent, or rabbit insulin including biologically active variants thereof including allelic variants, more preferably human insulin available in recombinant form. Sources of human insulin include pharmaceutically acceptable and sterile formulations such as those available from Eli Lilly (Indianapolis, Ind. 46285) as Humulin.TM. (human insulin rDNA origin). See the THE PHYSICIAN'S DESK REFERENCE, 55.sup.th Ed. (2001) Medical Economics, Thomson Healthcare (disclosing other suitable human insulins). The peptides of the invention can also be used in combination therapy with agents that can boost insulin effects or levels of a subject upon administration, e.g. glipizide and/or rosiglitazone. The peptides and agonists of the invention can be used in combitherapy with SYMLIN® (pramlintide acetate) and Exenatide® (synthetic exendin-4; a 39 aa peptide).

The peptides and agonists of the invention can also be used in combination therapy with agents (e.g., Entereg(TM) (alvimopan; formerly called adolor/ ADL 8-2698), conivaptan and related agents describe in US 6,645,959) for the treatment of postoperative ileus.

The peptides and agonists of the invention can be used in combination therapy with an anti-hypertensive agent including but not limited to:

(1) diuretics, such as thiazides, including chlorthalidone, chlorthiazide, dichlorophenamide, hydroflumethiazide, indapamide, polythiazide, and hydrochlorothiazide; loop diuretics, such as bumetanide, ethacrynic acid, furosemide, and torsemide; potassium sparing agents, such as amiloride, and triamterene; and aldosterone antagonists, such as spironolactone, epirenone, and the like;

(2) beta-adrenergic blockers such as acebutolol, atenolol, betaxolol, bevantolol, bisoprolol, bopindolol, carteolol, carvedilol, celiprolol, esmolol, indenolol, metaprolol, nadolol, nebivolol, penbutolol, pindolol, propanolol, sotalol, tertatolol, tilisolol, and timolol, and the like;

(3) calcium channel blockers such as amlodipine, aranidipine, azelnidipine, barnidipine, benidipine, bepridil, cinaldipine, clevidipine, diltiazem, efonidipine, felodipine, gallopamil, isradipine, lacidipine, lemildipine, lercanidipine, nicardipine, nifedipine, nilvadipine, nimodepine, nisoldipine, nitrendipine, manidipine, pranidipine, and verapamil, and the like;

(4) angiotensin converting enzyme (ACE) inhibitors such as benazepril; captopril; ceranapril; cilazapril; delapril; enalapril; enalopril; fosinopril; imidapril; lisinopril; losinopril; moexipril; quinapril; quinaprilat; ramipril; perindopril; perindropril; quanipril; spirapril; tenocapril; trandolapril, and zofenopril, and the like;

(5) neutral endopeptidase inhibitors such as omapatrilat, cadoxatril and ecadotril, fosidotril, sampatrilat, AVE7688, ER4030, and the like;

(6) endothelin antagonists such as tezosentan, A308165, and YM62899, and the like;

(7) vasodilators such as hydralazine, clonidine, minoxidil, and nicotinyl alcohol, and the like;

(8) angiotensin II receptor antagonists such as aprosartan, candesartan, eprosartan, irbesartan, losartan, olmesartan, pratosartan, tasosartan, telmisartan, valsartan, and EXP-3137, FI6828K, and RNH6270, and the like;

(9) $\alpha/\beta$ adrenergic blockers such as nipradilol, arotinolol and amosulalol, and the like;

(10) alpha 1 blockers, such as terazosin, urapidil, prazosin, tamsulosin, bunazosin, trimazosin, doxazosin, naftopidil, indoramin, WHP 164, and XEN010, and the like;

(11) alpha 2 agonists such as lofexidine, tiamenidine, moxonidine, rilmenidine and guanobenz, and the like;

(12) aldosterone inhibitors, and the like; and

(13) angiopoietin-2-binding agents such as those disclosed in WO03/030833.

The peptides and agonists of the invention can be used in combination therapy with one or more of the following agents useful in the treatment of respiratory and other disorders:

(1) β-agonists including but not limited to: albuterol (PROVENTIL®, SALBUTAMOl®, VENTOLIN®), bambuterol, bitoterol, clenbuterol, fenoterol, formoterol, isoetharine (BRONKOSOL®, BRONKOMETER®), metaproterenol (ALUPENT®, METAPREL®), pirbuterol (MAXAIR®), reproterol, rimiterol, salmeterol, terbutaline (BRETHAIRE®, BRETHINE®, BRICANYL®), adrenalin, isoproterenol (ISUPREL®), epinephrine bitartrate (PRIMATENE®), ephedrine, orciprenline, fenoterol and isoetharine;

(2) steroids, including but not limited to beclomethasone, beclomethasone dipropionate, betamethasone, budesonide, bunedoside, butixocort, dexamethasone, flunisolide, fluocortin, fluticasone, hydrocortisone, methyl prednisone, mometasone, predonisolone, predonisone, tipredane, tixocortal, triamcinolone, and triamcinolone acetonide;

(3) β2-agonist-corticosteroid combinations [e.g., salmeterol-fluticasone (ADVAIR®), formoterol-budesonid (SYMBICORT®)];

(4) leukotriene D4 receptor antagonists/leukotriene antagonists/LTD4 antagonists (i.e., any compound that is capable of blocking, inhibiting, reducing or otherwise interrupting the interaction between leukotrienes and the Cys LTI receptor) including but not limited to: zafirlukast, montelukast, montelukast sodium (SINGULAIR®), pranlukast, iralukast, pobilukast, SKB-106,203 and compounds described as having LTD4 antagonizing activity described in U.S. Patent No. 5,565,473;

(5) 5-lipoxygenase inhibitors and/or leukotriene biosynthesis inhibitors [e.g., zileuton and BAY1005 (CA registry 128253-31-6)];

(6) histamine H1 receptor antagonists/antihistamines (i.e., any compound that is capable of blocking, inhibiting, reducing or otherwise interrupting the interaction between histamine and its receptor) including but not limited to: astemizole, acrivastine, antazoline, azatadine, azelastine, astamizole, bromopheniramine, bromopheniramine maleate, carbinoxamine, carebastine, cetirizine, chlorpheniramine, chloropheniramine maleate, cimetidine ,clemastine, cyclizine, cyproheptadine, descarboethoxyloratadine, dexchlorpheniramine, dimethindene, diphenhydramine, diphenylpyraline, doxylamine succinate, doxylarnine, ebastine, efletirizine, epinastine, farnotidine, fexofenadine, hydroxyzine, hydroxyzine, ketotifen, levocabastine, levocetirizine, levocetirizine, loratadine, meclizine, mepyramine, mequitazine, methdilazine, mianserin, mizolastine, noberastine, norasternizole,

noraztemizole, phenindamine, pheniramine, picumast, promethazine, pynlamine, pyrilamine, ranitidine, temelastine, terfenadine, trimeprazine, tripelenamine, and triprolidine;

(7) an anticholinergic including but not limited to: atropine, benztropine, biperiden, flutropium, hyoscyamine, ilutropium, ipratropium, ipratropium bromide, methscopolamine, oxybutinin, rispenzepine, scopolamine, and tiotropium;

(8) an anti-tussive including but not limited to: dextromethorphan, codeine, and hydromorphone;

(9) a decongestant including but not limited to: pseudoephedrine and phenylpropanolamine;

(10) an expectorant including but not limited to: guafenesin, guaicolsulfate, terpin, ammonium chloride, glycerol guaicolate, and iodinated glycerol;

(11) a bronchodilator including but not limited to: theophylline and aminophylline;

(12) an anti-inflammatory including but not limited to: fluribiprofen, diclophenac, indomethacin, ketoprofen, S-ketroprophen, tenoxicam;

(13) a PDE (phosphodiesterase) inhibitor including but not limited to those disclosed herein;

(14) a recombinant humanized monoclonal antibody [e.g. xolair (also called omalizumab), rhuMab, and talizumab];

(15) a humanized lung surfactant including recombinant forms of surfactant proteins SP-B, SP-C or SP-D [e.g. SURFAXIN®, formerly known as dsc-104 (Discovery Laboratories)],

(16) agents that inhibit epithelial sodium channels (ENaC) such as amiloride and related compounds;

(17) antimicrobial agents used to treat pulmonary infections such as acyclovir, amikacin, amoxicillin, doxycycline, trimethoprin sulfamethoxazole, amphotericin B, azithromycin, clarithromycin, roxithromycin, clarithromycin, cephalosporins( ceffoxitin, cefmetazole etc), ciprofloxacin, ethambutol, gentimycin, ganciclovir, imipenem, isoniazid, itraconazole, penicillin, ribavirin, rifampin, rifabutin,amantadine, rimantidine, streptomycin, tobramycin, and vancomycin;

(18) agents that activate chloride secretion through Ca++ dependent chloride channels (such as purinergic receptor (P2Y(2) agonists);

(19) agents that decrease sputum viscosity, such as human recombinant DNase 1, (Pulmozyme®);

(20) nonsteroidal anti-inflammatory agents (acemetacin, acetaminophen, acetyl salicylic acid, alclofenac, alminoprofen, apazone, aspirin, benoxaprofen, bezpiperylon, bucloxic acid, carprofen, clidanac, diclofenac, diclofenac, diflunisal, diflusinal, etodolac, fenbufen, fenbufen, fenclofenac, fenclozic acid, fenoprofen, fentiazac, feprazone, flufenamic acid, flufenisal, flufenisal, fluprofen, flurbiprofen, flurbiprofen, furofenac, ibufenac, ibuprofen, indomethacin, indomethacin, indoprofen, isoxepac, isoxicam, ketoprofen, ketoprofen, ketorolac, meclofenamic acid, meclofenamic acid, mefenamic acid, mefenamic acid, miroprofen, mofebutazone, nabumetone oxaprozin, naproxen, naproxen, niflumic acid , oxaprozin, oxpinac, oxyphenbutazone, phenacetin, phenylbutazone, phenylbutazone, piroxicam, piroxicam, pirprofen, pranoprofen, sudoxicam,tenoxican , sulfasalazine, sulindac, sulindac, suprofen, tiaprofenic acid, tiopinac, tioxaprofen, tolfenamic acid, tolmetin, tolmetin, zidometacin, zomepirac, and zomepirac); and

(21) aerosolized antioxidant therapeutics such as S-Nitrosoglutathione.

The peptides and agonists of the invention can be used in combination therapy with an anti-obesity agent including but not limited to:

(1) 11$\beta$ HSD-1 (11-beta hydroxy steroid dehydrogenase type 1) inhibitors, such as BVT 3498, BVT 2733, 3-(1-adamanty1)-4-ethyl-5-(ethy1thio)- 4H-1,2,4-triazole, 3-(l-adamantyl)-5-(3,4,5-trimethoxypheny1)-4-methy1-4H-1,2,4-triazole, 3- adamantanyl-4,5,6,7,8,9,10,11,12,3a-decahydro-l,2,4-triazolo[4,3-a][11]annulene, and those compounds disclosed in WO01/90091, WO01/90090, WO01/90092 and WO02/072084;

(2) 5HT (serotonin) transporter inhibitors, such as paroxetine, fluoxetine (Prozac®), fenfluramine, fluvoxamine, sertraline, and imipramine, and those disclosed in WO03/00663;

(3) 5HT antagonists such as those in WO03/037871, WO03/037887, and the like;

(4) 5HT1a modulators such as those disclosed in WO03/031439, and the like;

(5) 5HT-2 agonists;

(6) 5HT2c (serotonin receptor 2c) agonists, such as BVT933, DPCA37215, IK264, PNU 22394, WAY161503, R-1065, SB 243213 (Glaxo Smith Kline) and YM 348 and those disclosed in U.S. Patent No. 3,914,250 and PCT publication Nos. WO02/36596, WO02/48124, WO02/10169, WO01/66548, WO02/44152, WO02/51844, WO02/40456, and WO02/40457;

(7) 5HT6 receptor modulators, such as those in WO03/030901, WO03/035061, WO03/039547, and the like;

(8) ACC2 (acetyl-CoA carboxylase-2) inhibitors;

(9) acyl-estrogens, such as oleoyl-estrone, disclosed in del Mar-Grasa, M. et al., Obesity Research, 9:202-9 (2001) and Japanese Patent Application No. JP 2000256190;

(10) alpha-lipoic acid (alpha-LA);

(11) anorectic bicyclic compounds such as 1426 (Aventis) and 1954 (Aventis), and the compounds disclosed in WO00/18749, WO01/32638, WO01/62746, WO01/62747, and WO03/015769;

(12) AOD9604;

(13) appetite suppressants such as those in WO03/40107;

(14) ATL-962 (Alizyme PLC);

(15) benzocaine;

(16) benzphetamine hydrochloride (Didrex);

(17) bladderwrack (focus vesiculosus);

(18) BRS3 (bombesin receptor subtype 3) agonists;

(19) bupropion;

(20) caffeine;

(21) CB 1 (cannabinoid-1 receptor) antagonist/inverse agonists, such as rimonabant (Acomplia; Sanofi Synthelabo), SR-147778 (Sanofi Synthelabo), BAY 65-2520 (Bayer), and SLV 319 (Solvay), and those disclosed in US Patent Nos. 4,973,587, 5,013,837, 5,081,122, 5,112,820, 5,292,736, 5,532,237, 5,624,941, 6,028,084, and 6,509,367 and WO96/33159, WO97/29079, WO98/31227, WO98/33765, WO98/37061, WO98/41519, WO98/43635, WO98/43636, WO99/02499, WO00/10967, WO00/10968, WO01/09120, WO01/58869, WO01/64632, WO01/64633, WO01/64634, WO01/70700, WO01/96330,

WO02/076949, WO03/006007, WO03/007887, WO03/020217, WO03/026647, WO03/026648, WO03/027069, WO03/027076, WO03/027114, WO03/037332, WO03/040107, WO03/086940, WO03/084943 and US6,509,367 and EPO Application No. EP-658546;

(22) CCK agonists;

(23) CCK-A (cholecystokinin-A) agonists, such as AR-R 15849, GI 181771, JMV-180, A-71378, A-71623 and SR146131, and those described in U.S. Pat. No. 5,739,106;

(24) chitosan;

(25) chromium;

(26) CNTF (Ciliary neurotrophic factors), such as GI-181771 (Glaxo-SmithKline), SR146131 (Sanofi Synthelabo), butabindide, PD170,292, and PD 149164 (Pfizer);

(27) CNTF derivatives, such as axokine (Regeneron), and those disclosed in PCT Application Nos. WO 94/09134, WO 98/22128, and WO 99/43813;

(28) conjugated linoleic acid;

(29) corticotropin-releasing hormone agonists;

(30) dehydroepiandrosterone;

(31) DGAT1 (diacylglycerol acyltransferase 1) inhibitors;

(32) DGAT2 (diacylglycerol acyltransferase 2) inhibitors;

(33) dicarboxylate transporter inhibitors;

(34) diethylpropion hydrochloride (Tenuate);

(35) dipeptidyl peptidase IV (DP-IV) inhibitors, such as isoleucine thiazolidide, valine pyrrolidide, NVP-DPP728, LAF237, P93/01, TSL 225, TMC-2A/2B/2C, FE 999011, P9310/K364, VIP 0177, SDZ 274-444 and the compounds disclosed in PCT publication Nos. WO02/083128, WO02/062764, WO03/000180, WO03/000181, WO03/000250, WO03/002530, WO03/002531, WO03/002553, WO03/002593, WO03/004498, WO03/004496,WO03/017936, WO03/024942, WO03/024965, WO03/033524, WO03/037327 and EP1258476;

(36) ephedra;

(37) exendin-4 (an inhibitor of glp-1)

(38) FAS (fatty acid synthase) inhibitors, such as Cerulenin and C75;

(39) fat resorption inhibitors such as those in WO03/053451, and the like;

(40) fatty acid transporter inhibitors;

(41) fiber (psyllium, plantago, guar fiber);

(42) galanin antagonists;

(43) galega (Goat's Rue, French Lilac);

(44) garcinia cambogia;

(45) germander (teucrium chamaedrys);

(46) ghrelin antagonists, such as those disclosed in PCT Application Nos. WO 01/87335, and WO 02/08250;

(47) GLP-1 (glucagon-like peptide 1) agonists (e.g. exendin-4);

(48) glp-1 (glucagon-like peptide-1);

(49) glucocorticoid antagonists;

(50) glucose transporter inhibitors;

(51) growth hormone secretagogue receptor agonists/antagonists, such as NN703, hexarelin, MK-0677, SM-130686, CP-424,391, L-692,429 and L-163,255, and such as those disclosed in U.S. Pat. No. 6,358,951, U.S. Patent Application Nos. 2002/049196 and 2002/022637, and PCT Application Nos. WO 01/56592 and WO 02/32888;

(52) growth hormone secretagogues, such as those disclosed and specifically described in U.S. Pat. No. 5,536,716;

(53) H3 (histamine H3) antagonist/inverse agonists, such as thioperamide, 3-(1H-imidazol-4-yl)propyl N-(4-pentenyl)carbamate), clobenpropit, iodophenpropit, imoproxifan, GT2394 (Gliatech), and A331440, and those disclosed in PCT publication No. WO02/15905 and O-[3-(1H-imidazol-4-yl)propanol]carbamates (Kiec-Kononowicz, K. et al., Pharmazie, 55:349-55 (2000)), piperidine-containing histamine H3-receptor antagonists (Lazewska, D. et al., Pharmazie, 56:927-32 (2001), benzophenone derivatives and related compounds (Sasse, A. et al., Arch. Pharm.(Weinheim) 334:45-52 (2001)), substituted N-phenylcarbamates (Reidemeister, S. et al., Pharmazie, 55:83-6 (2000)), and proxifan derivatives (Sasse, A. et al., J. Med. Chem.. 43:3335-43 (2000)) and histamine H3 receptor modulators such as those disclosed in WO03/024928 and WO03/024929;

(54) interleukin-6 (IL-6) and modulators thereof, as in WO03/057237, and the like;

(55) L-carnitine;

(56) leptin derivatives, such as those disclosed in U.S. Pat. Nos. 5,552,524, 5,552,523, 5,552,522, 5,521,283, and PCT International Publication Nos. WO 96/23513, WO 96/23514, WO 96/23515, WO 96/23516, WO 96/23517, WO 96/23518, WO 96/23519, and WO 96/23520;

(57) leptin, including recombinant human leptin (PEG-OB, Hoffman La Roche) and recombinant methionyl human leptin (Amgen);

(58) lipase inhibitors, such as tetrahydrolipstatin (orlistat/Xenical®), Triton WR1339, RHC80267, lipstatin, teasaponin, and diethylumbelliferyl phosphate, FL-386, WAY-121898, Bay-N-3176, valilactone, esteracin, ebelactone A, ebelactone B, and RHC 80267, and those disclosed in PCT publication No. WO01/77094, and U.S. Patent Nos. 4,598,089,4,452,813, 5,512,565, 5,391,571, 5,602,151, 4,405,644, 4,189,438, and 4,242,453;

(59) lpid metabolism modulators such as maslinic acid, erythrodiol, ursolic acid uvaol, betulinic acid, betulin, and the like and compounds disclosed in WO03/011267;

(60) Mc3r (melanocortin 3 receptor) agonists;

(61) Mc4r (melanocortin 4 receptor) agonists, such as CHIR86036 (Chiron), ME-10142, ME-10145, and HS-131 (Melacure), and those disclosed in PCT publication Nos. WO99/64002, WO00/74679, WO01/991752, WO01/25192, WO01/52880, WO01/74844, WO01/70708, WO01/70337, WO01/91752, WO02/059095, WO02/059107, WO02/059108, WO02/059117, WO02/06276, WO02/12166, WO02/11715, WO02/12178, WO02/15909, WO02/38544, WO02/068387, WO02/068388, WO02/067869, WO02/081430, WO03/06604, WO03/007949, WO03/009847, WO03/009850, WO03/013509, and WO03/031410;

(62) Mc5r (melanocortin 5 receptor) modulators, such as those disclosed in WO97/19952, WO00/15826, WO00/15790, US 20030092041;

(63) MCH2R (melanin concentrating hormone 2R) agonist/antagonists;

(64) melanin concentrating hormone antagonists;

(65) melanin-concentrating hormone 1 receptor (MCHR) antagonists, such as T-226296 (Takeda), SNP-7941 (Synaptic), and those disclosed WO01/21169, WO01/82925,

WO01/87834, WO02/051809, WO02/06245, WO02/076929, WO02/076947, WO02/04433, WO02/51809, WO02/083134, WO02/094799, WO03/004027, WO03/13574, WO03/15769, WO03/028641, WO03/035624, WO03/033476, WO03/033480 and Japanese Patent Application Nos. JP 13226269, and JP1437059;

(66) melanocortin agonists, such as Melanotan II or those described in WO 99/64002 and WO 00/74679;

(67) Metformin (GLUCOPHAGE®);

(68) mGluR5 modulators such as those disclosed in WO03/029210, WO03/047581, WO03/048137, WO03/051315, WO03/051833, WO03/053922, WO03/059904, and the like;

(69) monoamine reuptake inhibitors, such as sibutratmine (Meridia®/Reductil®) and salts thereof, and those compounds disclosed in U.S. Patent Nos. 4,746,680, 4,806,570, and 5,436,272, and U.S. Patent Publication No. 2002/0006964, and WO01/27068, and WO01/62341;

(70) NE (norepinephrine) transport inhibitors, such as GW 320659, despiramine, talsupram, and nomifensine;

(71) nomame herba;

(72) non-selective serotonin/norepinephrine transport inhibitors, such as sibutramine or fenfluramine;

(73) NPY 1 antagonists, such as BIBP3226, J-115814, BIBO 3304, LY-357897, CP-671906, GI-264879A, and those disclosed in U.S. Pat. No. 6,001,836, and PCT Patent Publication Nos. WO 96/14307, WO 01/23387, WO 99/51600, WO 01/85690, WO 01/85098, WO 01/85173, and WO 01/89528;

(74) NPY5 (neuropeptide Y Y5) antagonists, such as 152,804, GW-569180A, GW-594884A, GW-587081X, GW-548118X, FR235208, FR226928, FR240662, FR252384, 1229U91, GI-264879A, CGP71683A, LY-377897, LY-366377, PD-160170, SR- 120562A, SR-120819A, JCF-104, and H409/22 and those compounds disclosed in U.S. Patent Nos. 6,140,354, 6,191,160, 6,258,837, 6,313,298, 6,326,375, 6,329,395, 6,335,345, 6,337,332, 6,329,395, and 6,340,683, European Patent Nos. EP-01010691, and EP-01044970 and PCT Publication Nos. WO97/19682, WO97/20820, WO97/20821, WO97/20822, WO97/20823,

WO98/27063, WO00/107409, WO00/185714, WO00/185730, WO00/64880, WO00/68197, WO00/69849, WO01/09120, WO01/14376, WO01/85714, WO01/85730, WO01/07409, WO01/02379, WO01/23388, WO01/23389, WO01/44201, WO01/62737, WO01/62738, WO01/09120, WO02/20488, WO02/22592, WO02/48152, WO02/49648, WO02/051806, WO02/094789, WO03/009845, WO03/014083, WO03/022849, WO03/028726 and Norman et al., J. Med. Chem. 43:4288-4312 (2000);

(75) opioid antagonists, such as nalmefene (REVEX ®), 3-methoxynaltrexone, naloxone, and naltrexone and those disclosed in WO00/21509;

(76) orexin antagonists, such as SB-334867-A and those disclosed in PCT publication Nos. WO01/96302, WO01/68609, WO02/44172, WO02/51232, WO02/51838, WO02/089800, WO02/090355, WO03/023561, WO03/032991, and WO03/037847;

(77) PDE (phosphodiesterase) inhibitors including but not limited to those disclosed herein;

(78) peptide YY and fragments and variants thereof (e.g. YY3–36 (PYY3-36 )(N. Engl. J. Med. 349:941, 2003; IKPEAPGE DASPEELNRY YASLRHYLNL VTRQRY (SEQ ID NO:XXX)) and PYY agonists such as those disclosed in WO03/026591;

(79) phendimetrazine;

(80) phentermine,

(81) phosphate transporter inhibitors;

(82) phosphodiesterase-3B (PDE3B) inhibitors;

(83) phytopharm compound 57 (CP 644,673);

(84) pyruvate;

(85) SCD-1 (stearoyl-CoA desaturase-1) inhibitors;

(86) serotonin reuptake inhibitors, such as dexfenfluramine, fluoxetine, and those in U.S. Patent No. 6,365,633, and WO01/27060, and WO01/162341;

(87) T71 (Tularik; Inc.; Boulder CO);

(88) thyroid hormone $\beta$ agonists, such as KB-2611 (KaroBioBMS), and those disclosed in WO02/15845 and Japanese Patent Application No. JP 2000256190;

(89) Topiramate (TOPIMAX®);

(90) transcription factor modulators such as those disclosed in WO03/026576;

(91) UCP-1 (uncoupling protein-1), 2, or 3 activators, such as phytanic acid, 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propeny- 1]benzoic acid (TTNPB), retinoic acid, and those disclosed in PCT Patent Application No. WO 99/00123;

(92) β3 (beta adrenergic receptor 3) agonists, such as AD9677/TAK677 (Dainippon/Takeda), CL-316,243, SB 418790, BRL-37344, L-796568, BMS-196085, BRL-35135A, CGP12177A, BTA-243, GW 427353, Trecadrine, Zeneca D7114, N-5984 (Nisshin Kyorin), LY-377604 (Lilly), and SR 59119A, and those disclosed in US Patent Nos. 5,705,515, US 5,451,677 and PCT publication Nos. WO94/18161, WO95/29159, WO97/46556, WO98/04526 and WO98/32753, WO01/74782, WO02/32897, WO03/014113, WO03/016276, WO03/016307, WO03/024948, WO03/024953 and WO03/037881;

(93) β-hydroxy steroid dehydrogenase-1 inhibitors (β -HSD-1) and

(94) β-hydroxy-β-methylbutyrate.

Peptides and agonists of the invention useful in the treatment of obesity can be administered as a cotherapy with electrostimulation (US20040015201).

The peptides and agonists of the invention can be used in combination therapy with agents that activate soluble guanylate cyclase, for example those described in US20040192680.

The peptides and agonists of the invention can be used in combination therapy with a phosphodiesterase inhibitor. PDE inhibitors within the meaning of the present invention are those compounds which slow the degradation of cyclic AMP (cAMP) and/or cyclic GMP (cGMP) by inhibition of the phosphodiesterases, which can lead to a relative increase in the intracellular concentration of cAMP and cGMP. Possible PDE inhibitors within the meaning of the present invention are primarily those substances which are to be numbered among the class consisting of the PDE3 inhibitors, the class consisting of the PDE4 inhibitors and/or the class consisting of the PDE5 inhibitors, in particular those substances which can be designated as mixed types of PDE3/4 inhibitors or as mixed types of PDE3/4/5 inhibitors. By way of example, those PDE inhibitors may be mentioned such as are described and/or claimed in the

following patent applications and patents: DE1470341, DE2108438, DE2123328, DE2305339, DE2305575, DE2315801, DE2402908, DE2413935, DE2451417, DE2459090, DE2646469, DE2727481, DE2825048, DE2837161, DE2845220, DE2847621, DE2934747, DE3021792, DE3038166, DE3044568, EP000718, EP0008408, EP0010759, EP0059948, EP0075436, EP0096517, EP0112987, EP0116948, EP0150937, EP0158380, EP0161632, EP0161918, EP0167121, EP0199127, EP0220044, EP0247725, EP0258191, EP0272910, EP0272914, EP0294647, EP0300726, EP0335386, EP0357788, EP0389282, EP0406958, EP0426180, EP0428302, EP0435811, EP0470805, EP0482208, EP0490823, EP0506194, EP0511865, EP0527117, EP0626939, EP0664289, EP0671389, EP0685474, EP0685475, EP0685479, JP92234389, JP94329652, JP95010875, U.S. Pat. Nos. 4,963,561, 5,141,931, WO9117991, WO9200968, WO9212961, WO9307146, WO9315044, WO9315045, WO9318024, WO9319068, WO9319720, WO9319747, WO9319749, WO9319751, WO9325517, WO9402465, WO9406423, WO9412461, WO9420455, WO9422852, WO9425437, WO9427947, WO9500516, WO9501980, WO9503794, WO9504045, WO9504046, WO9505386, WO9508534, WO9509623, WO9509624, WO9509627, WO9509836, WO9514667, WO9514680, WO9514681, WO9517392, WO9517399, WO9519362, WO9522520, WO9524381, WO9527692, WO9528926, WO9535281, WO9535282, WO9600218, WO9601825, WO9602541, WO9611917, DE3142982, DE1116676, DE2162096, EP0293063, EP0463756, EP0482208, EP0579496, EP0667345 US6,331,543, US20050004222 (including those disclosed in formulas I-XIII and paragraphs 37-39, 85-0545 and 557-577) and WO9307124, EP0163965, EP0393500, EP0510562, EP0553174, WO9501338 and WO9603399. PDE5 inhibitors which may be mentioned by way of example are RX-RA-69, SCH-51866, KT-734, vesnarinone, zaprinast, SKF-96231, ER-21355, BF/GP-385, NM-702 and sildenafil (Viagra®). PDE4 inhibitors which may be mentioned by way of example are RO-20-1724, MEM 1414 (R1533/R1500; Pharmacia Roche), DENBUFYLLINE, ROLIPRAM, OXAGRELATE, NITRAQUAZONE, Y-590, DH-6471, SKF-94120, MOTAPIZONE, LIXAZINONE, INDOLIDAN, OLPRINONE, ATIZORAM, KS-506-G, DIPAMFYLLINE, BMY-43351, ATIZORAM, AROFYLLINE, FILAMINAST, PDB-093, UCB-29646, CDP-840, SKF-107806, PICLAMILAST, RS-17597, RS-25344-000, SB-207499, TIBENELAST, SB-210667, SB-211572, SB-211600, SB-212066, SB-212179, GW-3600,

CDP-840, MOPIDAMOL, ANAGRELIDE, IBUDILAST, AMRINONE, PIMOBENDAN, CILOSTAZOL, QUAZINONE and N-(3,5-dichloropyrid-4-yl)-3-cyclopropylmethoxy4-difluoromethoxybenzamide. PDE3 inhibitors which may be mentioned by way of example are SULMAZOLE, AMPIZONE, CILOSTAMIDE, CARBAZERAN, PIROXIMONE, IMAZODAN, CI-930, SIGUAZODAN, ADIBENDAN, SATERINONE, SKF-95654, SDZ-MKS-492, 349-U-85, EMORADAN, EMD-53998, EMD-57033, NSP-306, NSP-307, REVIZINONE, NM-702, WIN-62582 and WIN-63291, ENOXIMONE and MILRINONE. PDE3/4 inhibitors which may be mentioned by way of example are BENAFENTRINE, TREQUINSIN, ORG-30029, ZARDAVERINE, L-686398, SDZ-ISQ-844, ORG-20241, EMD-54622, and TOLAFENTRINE. Other PDE inhibitors include: cilomilast, pentoxifylline, roflumilast, tadalafil(Cialis®), theophylline, and vardenafil(Levitra®), zaprinast (PDE5 specific).

Methods of Treatment

A number of disorders might be treated with GC-C receptor agonists and agents that increase cGMP levels including the peptides and agonists of the invention.

The peptides and agonists of the invention can be used alone or in combination therapy for the treatment or prevention of congestive heart failure. Such agents can be used in combination with natriuretic peptides (e.g., atrial natriuretic peptide, brain natriuretic peptide or C-type natriuretic peptide), a diuretic, or an inhibitor of angiotensin converting enzyme.

The peptides and agonists of the invention can be used alone or in combination therapy for the treatment or prevention of benign prostatic hyperplasia (BPH). Such agents can be used in combination with one or more agents for treatment of BPH, for example, a 5-alpha reductase inhibitor (e.g., finasteride) or an alpha adrenergic inhibitor (e.g., doxazosine).

The peptides and agonists of the invention can be used alone or in combination therapy for the treatment, prevention or reduction of visceral pain associated with a gastrointestinal disorder or pain associated with another disorder.

The peptides and agonists of the invention can be used alone or in combination therapy for the treatment or prevention of obesity-related disorders (e.g. disorders that are associated with, caused by, or result from obesity). Examples of obesity-related disorders include overeating and bulimia, hypertension, diabetes, elevated plasma insulin concentrations and insulin resistance, dyslipidemias, hyperlipidemia, endometrial, breast, prostate and colon cancer, osteoarthritis, obstructive sleep apnea, cholelithiasis, gallstones, heart disease, abnormal heart rhythms and arrhythmias, myocardial infarction, congestive heart failure, coronary heart disease, sudden death, stroke, polycystic ovarian disease, craniopharyngioma, the Prader-Willi Syndrome, Frohlich's syndrome, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, e.g., children with acute lymphoblastic leukemia. The agents of the invention may be used to reduce or control body weight (or fat) or to prevent and/or treat obesity or other appetite related disorders related to the excess consumption of food, ethanol and other appetizing substances. The agents may be used to modulate lipid metabolism, reduce body fat (e.g. via increasing fat utilization) or reduce (or suppress) appetite (e.g. via inducing satiety). Further examples of obesity-related disorders are metabolic syndrome, also known as syndrome X, insulin resistance syndrome, sexual and reproductive dysfunction, such as infertility, hypogonadism in males and hirsutism in females, gastrointestinal motility disorders, such as obesity-related gastroesophageal reflux, respiratory disorders, such as obesity-hypoventilation syndrome (Pickwickian syndrome), cardiovascular disorders, inflammation, such as systemic inflammation of the vasculature, arteriosclerosis, hypercholesterolemia, hyperuricaemia, lower back pain, gallbladder disease, gout, and kidney cancer. The agents of the present invention are also useful for reducing the risk of secondary outcomes of obesity, such as reducing the risk of left ventricular hypertrophy.

The peptides and agonists of the invention can be used alone or in combination therapy for the treatment or prevention of gastrointestinal related disorders including: chronic intestinal pseudo-obstruction (Ogilvie's syndrome), colonic pseudoobstruction, Crohn's disease, dyspepsia (including functional dyspepsia or nonulcer dyspepsia), duodenogastric reflux, functional bowel disorder, functional gastrointestinal disorders, functional heartburn, gastroesophageal reflux disease (GERD), gastrointestinal motility disorders, gastroparesis (e.g. idopathic gastroparesis), hypertrophic pyloric stenosis, Inflammatory bowel disease, irritable bowel syndrome (IBS), post-operative ileus, and ulcerative colitis. The peptides and agonists of the invention can be used alone or in combination therapy to patient suffering from or susceptible to GI disorders relating to damage to the GI tract stemming from impact or surgical intervention. The peptides and agonists of the invention can be used alone or in combination therapy to patients at risk for or having particular diseases associated with hypomotility or stasis in the GI tract. For example, diabetic neuropathy, anorexia nervosa, and achlorhydria are frequently accompanied by gastric hypomotility. Damage to the GI tract following surgical intervention, for instance, can result in substantial gastric stasis. The peptides and agonists of the invention can be administered alone or in combination therapy to patients susceptible to or having a GI disorder associated with diabetes (e.g. diabetic gastropathy). The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat GI disorders characterized by at least one of nausea, vomiting, heartburn, postprandial discomfort, diarrhea, constipation, indigestion or related symptoms. The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat GI disorders associated with at least one of diabetes, anorexia nervosa, bulimia, achlorhydria, achalasia, anal fissure, irritable bowel syndrome, intestinal pseudoobstruction, scleroderma and gastrointestinal damage.

The peptides and agonists of the invention can be used to prevent and/or treat constipation (e.g. constipation associated with use of a therapeutic agent; constipation associated with a neuropathic, metabolic or endocrine disorder (including autonomic neuropathy, Chagas disease, cystic fibrosis, diabetes mellitus, Hirschsprung disease, hyperthyroidism, hypocalcaemia, hypothyroidism, Multiple Sclerosis, neurofibromatosis, Parkinson's disease, and spinal cord

lesions); post-surgical constipation (postoperative ileus); constipation associated with a gastrointestinal disorder; idiopathic constipation (functional constipation or slow transit constipation); constipation associated with the use of analgesic drugs (e.g. opioid induced constipation); constipation associated with the use of other agents (e.g. antihypertensives, anticonvulsants, antidepressants, antispasmodics and antipsychotics); megacolon; and chronic constipation).

The peptides and agonists of the invention can be used to treat decreased intestinal motility, slow digestion or slow stomach emptying. The peptides and agonists can be used to relieve one or more symptoms of IBS (bloating, pain, constipation), GERD (acid reflux into the esophagus), duodenogastric reflux, functional dyspepsia, or gastroparesis (nausea, vomiting, bloating, delayed gastric emptying) and other disorders described herein.

The peptides and agonists of the invention can be used to increase intestinal motility and to prevent and/or treat gastrointestinal immotility and other conditions calling for laxative or stool softener therapy. Gastrointestinal immotility can include constipation, and also includes delayed oral cecal transit time, irregular Taxation, and other related gastrointestinal motility disfunction including impaction. Impaction is a condition where a large mass of dry, hard stool develops in the rectum, often due to chronic constipation. This mass may be so hard that it cannot be excreted. The subjects affected by constipation or gastrointestinal immotility can be refractory to laxative therapy and/or stool softener therapy.

The peptides and agonists of the invention can be used for the treatment or prevention of cancer, pre-cancerous growths, or metastatic growths. For example, they can be used for the prevention or treatment of: colorectal/local metastasized colorectal cancer, intestinal polyps, gastrointestinal tract cancer, lung cancer, cancer or pre-cancerous growths or metastatic growths of epithelial cells, polyps, breast, colorectal, lung, ovarian, pancreatic, prostatic, renal, stomach, bladder, liver, esophageal and testicular carcinoma, carcinoma (e.g., basal cell, basosquamous, Brown-Pearce, ductal carcinoma, Ehrlich tumor, Krebs, Merkel cell, small or non-small cell lung, oat cell, papillary, bronchiolar, squamous cell, transitional cell, (Walker),

leukemia (e.g., B-cell, T-cell, HTLV, acute or chronic lymphocytic, mast cell, myeloid), histiocytonia, histiocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, plasmacytoma, reticuloendotheliosis, adenoma, adeno-carcinoma, adenofibroma, adenolymphoma, ameloblastoma, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, sclerosing angioma, angiomatosis, apudoma, branchionia, malignant carcinoid syndrome, carcinoid heart disease, carcinosarcoma, cementoma, cholangioma, cholesteatoma, chondrosarcoma, chondroblastoma, chondrosarcoma, chordoma, choristoma, craniopharyngioma, chrondrorna, cylindroma, cystadenocarcinoma, cystadenoma, cystosarconia phyllodes, dysgenninoma, ependymoma, Ewing sarcoma, fibroma, fibrosarcoma, giant cell tumor, ganglioneuroma, glioblastoma, glomangioma, granulosa cell tumor, gynandroblastoma, hamartoma, hemangioendothelioma, hemangioma, hemangio-pericytoma, hemangiosarcoma, hepatoma, islet cell tumor, Kaposi sarcoma, leiomyoma, leiomyosarcoma, leukosarcoma, Leydig cell tumor, lipoma, liposarcoma, lymphaugioma, lymphangiomyoma, lymphangiosarcoma, medulloblastoma, meningioma, mesenchymoma, mesonephroma, mesothelioma, myoblastoma, myoma, myosarcoma, myxoma, myxosarcoma, neurilemmoma, neuroma, neuroblastoma, neuroepithelioma, neurofibroma, neurofibromatosis, odontoma, osteoma, osteosarcoma, papilloma, paraganglioma, paraganglionia. nonchroinaffin, pinealoma, rhabdomyoma, rhabdomyosarcoma, Sertoli cell tumor, teratoma, theca cell tumor, and other diseases in which cells have become dysplastic, immortalized, or transformed.

The peptides and agonists of the invention can be used for the treatment or prevention of: Familial Adenomatous Polyposis (FAP) (autosomal dominant syndrome) that precedes colon cancer, hereditary nonpolyposis colorectal cancer (HNPCC), and inherited autosomal dominant syndrome.

For treatment or prevention of cancer, pre-cancerous growths and metastatic growths, the peptides and agonists of the invention can be used in combination therapy with radiation or chemotherapeutic agents, an inhibitor of a cGMP-dependent phosphodiesterase or a selective cyclooxygenase-2 inhibitor. A number of selective cyclooxygenase-2 inhibitors are described in US20010024664, U.S. Pat. No. 5,380,738, U.S. Pat. No. 5,344,991, U.S. Pat. No. 5,393,790,

U.S. Pat. No. 5,434,178, U.S. Pat. No. 5,474,995, U.S. Pat. No. 5,510,368, WO02/062369, WO 96/06840, WO 96/03388, WO 96/03387, WO 96/19469, WO 96/25405, WO 95/15316, WO 94/15932, WO 94/27980, WO 95/00501, WO 94/13635, WO 94/20480, and WO 94/26731, the disclosures of which are herein incorporated by reference. [Pyrazol-1-yl]benzenesulfonamides have also been described as inhibitors of cyclooxygenase-2.

The peptides and agonists of the invention can be used in the treatment or prevention of inflammation. Thus, they can be used alone or in combination with an inhibitor of cGMP-dependent phosphodiesterase or a selective cyclooxygenase-2 inhibitor for treatment of: organ inflammation, IBD (e.g, Crohn's disease, ulcerative colitis), asthma, nephritis, hepatitis, pancreatitis, bronchitis, cystic fibrosis, ischemic bowel diseases, intestinal inflammations/allergies, coeliac disease, proctitis, eosnophilic gastroenteritis, mastocytosis, and other inflammatory disorders. The peptides and agonists of the invention can be used alone or in combination therapy in the treatment or prevention of gastrointestinal tract inflammation (e.g. inflammation associated with a gastrointestinal disorder, gastrointestinal tract infection, or another disorder).

The peptides and agonists of the invention can also be used to treat or prevent insulin-related disorders, for example: II diabetes mellitus, hyperglycemia, obesity, disorders associated with disturbances in glucose or electrolyte transport and insulin secretion in cells, or endocrine disorders. They can be also used in insulin resistance treatment and post-surgical and non-post surgery decrease in insulin responsiveness.

The peptides and agonists of the invention can be used to prevent and/or treat pulmonary and respiratory related disorders, including, inhalation, ventilation and mucus secretion disorders, pulmonary hypertension, chronic obstruction of vessels and airways, and irreversible obstructions of vessels and bronchi. One may administer an agent of the invention for treating bronchospasm, for inducing bronchodilation, for treating chronic obstructive pulmonary disease (including chronic bronchitis with normal airflow), for treating asthma (including bronchial asthma, intrinsic asthma, extrinsic asthma, chronic or inveterate asthma (e.g. late asthma and airways hyper-responsiveness), dust-induced asthma, allergen-induced asthma,

viral-induced asthma, cold-induced asthma, pollution-induced asthma and exercise-induced asthma) and for treating rhinitis (including acute-, allergic, hatrophic rhinitis or chronic rhinitis (such as rhinitis caseosa, hypertrophic rhinitis, rhinitis purulenta, rhinitis sicca), rhinitis medicamentosa, membranous rhinitis (including croupous, fibrinous and pseudomembranous rhinitis), scrofulous rhinitis, perennial allergic rhinitis, seasonal rhinitis (including rhinitis nervosa (hay fever) and vasomotor rhinitis). The peptides of the invention may also be useful in the treatment of dry eye disease and chronic sinusitis. The peptides of the invention may also be used to prevent and/or treat disorders characterized by acute pulmonary vasoconstriction such as may result from pneumonia, traumatic injury, aspiration or inhalation injury, fat embolism in the lung, acidosis inflammation of the lung, adult respiratory distress syndrome, acute pulmonary edema, acute mountain sickness, post-cardiac surgery, acute pulmonary hypertension, persistent pulmonary hypertension of the newborn, perinatal aspiration syndrome, hyaline membrane disease, acute pulmonary thromboembolism, herapin-protamine reactions, sepsis, status asthmaticus or hypoxia (including iatrogenic hypoxia) and other forms of reversible pulmonary vasoconstriction. Such pulmonary disorders also are also characterized by inflammation of the lung including those associated with the migration into the lung of nonresident cell types including the various leucocyte subclasses. Also included in the respiratory disorders contemplated are: bullous disease, cough, chronic cough associated with inflammation or iatrogenic induced, airway constriction, pigeon fancier's disease, eosinophilic bronchitis, asthmatic bronchitis, chronic bronchitis with airway obstruction (chronic obstructive bronchitis), eosinophilic lung disease, emphysema, farmer's lung, allergic eye diseases (including allergic conjunctivitis, vernal conjunctivitis, vernal keratoconjunctivitis, and giant papillary conjunctivitis), idiopathic pulmonary fibrosis, cystic fibrosis, diffuse pan bronchiolitis and other diseases which are characterized by inflammation of the lung and/or excess mucosal secretion. Other physiological events which are contemplated to be prevented, treated or controlled include platelet activation in the lung, chronic inflammatory diseases of the lung which result in interstitial fibrosis, such as interstitial lung diseases (ILD) (e.g., idiopathic pulmonary fibrosis, or ILD associated with rheumatoid arthritis, or other autoimmune conditions), chronic obstructive pulmonary disease (COPD)(such as irreversible COPD), chronic sinusitis, fibroid lung, hypersensitivity lung diseases, hypersensitivity

pneumonitis, idiopathic interstitial pneumonia, nasal congestion, nasal polyposis, and otitis media.

The peptides and agonists of the invention can be used alone or in combitherapy to prevent or treat: retinopathy, nephropathy, diabetic angiopathy, and edema formation

The peptides and agonists of the invention can be used alone or in combitherapy to prevent or treat neurological disorders, for example, headache, migraines, anxiety, stress, cognitive disorders, cerebral ischemia, brain trauma, movement disorders, aggression, psychosis, seizures, panic attacks, hysteria, sleep disorders, depression, schizoaffective disorders, sleep apnea, attention deficit syndromes, memory loss, dementia, memory and learning disorders as discussed in Moncada and Higgs 1995 FASEB J. 9:1319-1330; Severina 1998 Biochemistry 63:794; Lee et al. 2000 PNAS 97: 10763-10768; Hobbs 1997 TIPS 18:484-491; Murad 1994 Adv. Pharmacol. 26:1-335; and Denninger et al. 1999 Biochim. Biophys. Acta 1411:334-350 and narcolepsy. They may also be used as a sedative.

The peptides and detectably peptides and agonists of the invention can be used as markers to identify, detect, stage, or diagnosis diseases and conditions of small intestine, including, without limitation: Crohn's disease, colitis, inflammatory bowel disease, tumors, benign tumors, such as benign stromal tumors, adenoma, angioma, adenomatous (pedunculated and sessile) polyps, malignant, carcinoid tumors, endocrine cell tumors, lymphoma, adenocarcinoma, foregut, midgut, and hindgut carcinoma, gastroinstestinal stromal tumor (GIST), such as leiomyorna, cellular leiomyoma, leiomyoblastoma, and leiomyosarcoma, gastrointestinal autonomic nerve tumor, malabsorption syndromes, celiac diseases, diverticulosis, Meckel's diverticulum, colonic diverticula, megacolon, Hirschsprung's disease, irritable bowel syndrome, mesenteric ischemia, ischemic colitis, colorectal cancer, colonic polyposis, polyp syndrome, intestinal adenocarcinoma, Liddle syndrome, Brody myopathy, infantile convulsions, and choreoathetosis

The peptides and agonists of the invention can be conjugated to another molecule (e.g., a diagnostic or therapeutic molecule) to target cells bearing the GC-C receptor, e.g., cystic fibrosis lesions and specific cells lining the intestinal tract. Thus, they can be used to target radioactive moieties or therapeutic moieties to the intestine to aid in imaging and diagnosing or treating colorectal/metastasized or local colorectal cancer and to deliver normal copies of the p53 tumor suppressor gene to the intestinal tract. The peptides and agonists of the invention can also be used to increase the number of GC-C molecules on the surface of a cell. In some embodiments the cell is a metastasized colorectal cancer cell. In one embodiment the peptide or agonist of the invention is therapeutically conjugated to a second agent. In certain embodiments, the second agent can be radioactive or radiostable. In certain embodiments the second agent can be selected from the group consisting of a compound that causes cell death, a compound that inhibits cell division, a compound that induces cell differentiation, a chemotherapeutic, a toxin and a radiosensitizing agent. In certain embodiments the second agent can be selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, etoposide, 5-4 fluorouracil, melphalan, chlorambucil, cis-platin, vindesine, mitomycin, bleomycin, purothionin, macromomycin, 1,4-benzoquinone derivatives, trenimon, ricin, ricin A chain, Pseudomonas exotoxin, diphtheria toxin, Clostridium perfringens phospholipase C, bovine pancreatic ribonuclease, pokeweed antiviral protein, abrin, abrin A chain, cobra venom factor, gelonin, saporin, modeccin, viscumin, volkensin, nitroimidazole, metronidazole and misonidazole.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat inner ear disorders, e.g., to prevent and/or treat Meniere's disease (including symptoms thereof such as vertigo, hearing loss, tinnitus, sensation of fullness in the ear), Mal de débarquement syndrome, otitis externa, otitis media, otorrhea, acute mastoiditis, otosclerosis, otic pain, otic bleeding, otic inflammation, Lermoyez's syndrome, vestibular neuronitis, benign paroxysmal positional vertigo (BPPV), herpes zoster oticus, Ramsay Hunt's syndrome, herpes, labyrinthitis, purulent labyrinthitis, perilymph fistulas, presbycusis, ototoxicity (including drug-induced ototoxicity), neuromias (including acoustic neuromas), aerotitis media, infectious myringitis, bullous myringitis, squamous cell carcinoma, basal cell

carcinoma, pre-cancerous otic conditions, nonchromaffin paragangliomas, chemodectomas, glomus jugulare tumors, glomus tympanicum tumors, perichondritis, aural eczematoid dermatitis, malignant external otitis, subperichondrial hematoma, ceruminomas, impacted cerumen, sebaceous cysts, osteomas, keloids, otalgia, tinnitus, tympanic membrane infection, tympanitis, otic furuncles, petrositis, conductive and sensorineural hearing loss, epidural abscess, lateral sinus thrombosis, subdural empyema, otitic hydrocephalus, Dandy's syndrome, bullous myringitis, diffuse external otitis, foreign bodies, keratosis obturans, otic neoplasm, otomycosis, trauma, acute barotitis media, acute eustachian tube obstruction, postsurgical otalgia, cholesteatoma, infections related to an otic surgical procedure, and complications associated with any of said disorders. The peptides and agonists of the invention can be used alone or in combination therapy to maintain fluid homeostasis in the inner ear. neuronitis (including viral neuronitis), ganglionitis, geniculate

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat disorders associated with fluid and sodium retention, e.g., diseases of the electrolyte-water/electrolyte transport system within the kidney, gut and urogenital system, congestive heart failure, hypertension, hypotension, salt dependent forms of high blood pressure, hepatic edema, and liver cirrhosis. In addition they can be used to facilitate diuresis or control intestinal fluid. The peptides and agonists of the invention can also be used to treat disorders where there is abnormal proliferation of epithelial cells within the kidney (e.g. as in the case of renal cancer).

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat kidney disease. "Kidney disease" includes renal failure (including acute renal failure), renal insufficiency, nephrotic edema, glomerulonephritis, pyelonephritis, kidney failure, chronic renal failure, nephritis, nephrosis, azotemia, uremia, immune renal disease, acute nephritic syndrome, rapidly progressive nephritic syndrome, nephrotic syndrome, Berger's Disease, chronic nephritic/proteinuric syndrome, tubulointerstital disease, nephrotoxic disorders, renal infarction, atheroembolic renal disease, renal cortical necrosis, malignant nephroangiosclerosis, renal vein thrombosis, renal tubular acidosis, renal glucosuria,

nephrogenic diabetes insipidus, Bartter's Syndrome, Liddle's Syndrome, polycystic kidney disease, medullary cystic disease, medullary sponge kidney, hereditary nephritis, and nail-patella syndrome, along with any disease or disorder that relates to the renal system and related disorders, as well as symptoms indicative of, or related to, renal or kidney disease and related disorders.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent or treat polycystic kidney disease. Polycystic kidney disease" "PKD" (also called "polycystic renal disease") refers to a group of disorders characterized by a large number of cysts distributed throughout dramatically enlarged kidneys. The resultant cyst development leads to impairment of kidney function and can eventually cause kidney failure. "PKD" specifically includes autosomal dominant polycystic kidney disease (ADPKD) and recessive autosomal recessive polycystic kidney disease (ARPKD), in all stages of development, regardless of the underlying cause.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat disorders associated with bicarbonate secretion, e.g., Cystic Fibrosis.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat disorders associated with bile secretion. In addition, they can be used to facilitate or control chloride and bile fluid secretion in the gall bladder.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat disorders associated with liver cell regeneration. This may include administration of the peptides and agonists to liver transplant recipients and to patients with drug or alcohol induced-liver damage. Furthermore, the peptides and agonists may be useful to treat liver damage as in the case of viral mediated hepatitis. The peptides and agonists of the invention may be used alone or in combination to prevent and/or treat liver abscess, liver cancer (either primary or metastatic), cirrhosis (such as cirrhosis caused by the alcohol consumption or primary biliary cirrhosis), amebic liver abscess, autoimmune hepatitis, biliary

atresia, coccidioidomycosis disseminated, δ agent (hepatitis δ), hemochromatosis, hepatitis a, hepatitis b, hepatitis c, or any other acute, subacute, fulminant or chronic hepatitis of viral, metabolic or toxic etiology, hepatocellular carcinoma, pyogenic liver abscess, Reye's syndrome, sclerosing cholangitis, Wilson's disease, drug induced hepatotoxicity, or fulminant or acute liver failure. The peptides and agonists may be used in stimulating hepatic regeneration after surgical hepatectomy.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat myocardial infraction, diastolic dysfunction, angina pectoris, stable, unstable and variant (Prinzmetal) angina, atherosclerosis, thrombosis, endothelial dysfunction, cardiac edema, stroke, conditions of reduced blood vessel patency, e.g., postpercutaneous transluminal coronary angioplasty (post-PTCA) and peripheral vascular disease.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat glaucoma.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat immunodeficiency.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat bladder outlet obstruction and incontinence.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat male (e.g. erectile dysfunction) or female sexual dysfunction, premature labor, and dysmenorrhoea. In certain embodiments they can be used in combination with a phosphodiesterase inhibitor.

The peptides and agonists of the invention can be used alone or in combination therapy to prevent and/or treat osteopenia disorders (bone loss disorders). "Bone loss disorders" include conditions and diseases wherein the inhibition of bone loss and/or the promotion of bone formation is desirable. Among such conditions and diseases are osteoporosis, osteomyelitis, Paget's disease (osteitis deformans), periodontitis, hypercalcemia, osteonecrosis, osteosarcoma, osteolyic metastases, familial expansile osteolysis, prosthetic loosening, periprostetic osteolysis, bone loss attendant rheumatoid arthritis, and cleiodocranial dysplasia (CCD). Osteoporosis includes primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Rile-Day syndrome) and osteoporosis due to immobilization of extremitiesosteomyelitis, or an infectious lesion in bone leading to bone loss. The peptides and agonists can be used alone or in combination therapy to stimulating bone regeneration. The bone regeneration may be following reconstruction of bone defects in cranio-maxillofacial surgery, or following an implant into bone, for example a dental implant, bone supporting implant, or prosthesis. The bone regeneration may also be following a bone fracture.

The peptides of the invention that have homology to ST peptides can be used as immunogens to treat and/or prevent one or more disease symptoms associated with traveler's diarrhea. The methods described in US20040146534, US4220584, US4285391, US5182109, US4603049, US4545931, US4886663, and WO08402700 can be similarly used to create immunogens comprising the peptides of the invention.

What is claimed is:

1. A peptide comprising the amino acid sequence of SEQ ID NO:3.

2. A method comprising chemically synthesizing the peptide of claim 1 and purifying the peptide.

3. The peptide of claim 1 wherein the peptide is recombinantly produced.

4. The peptide of claim 1 wherein the peptide is chemically synthesized.

5. An isolated nucleic acid molecule encoding the peptide of claim 1.

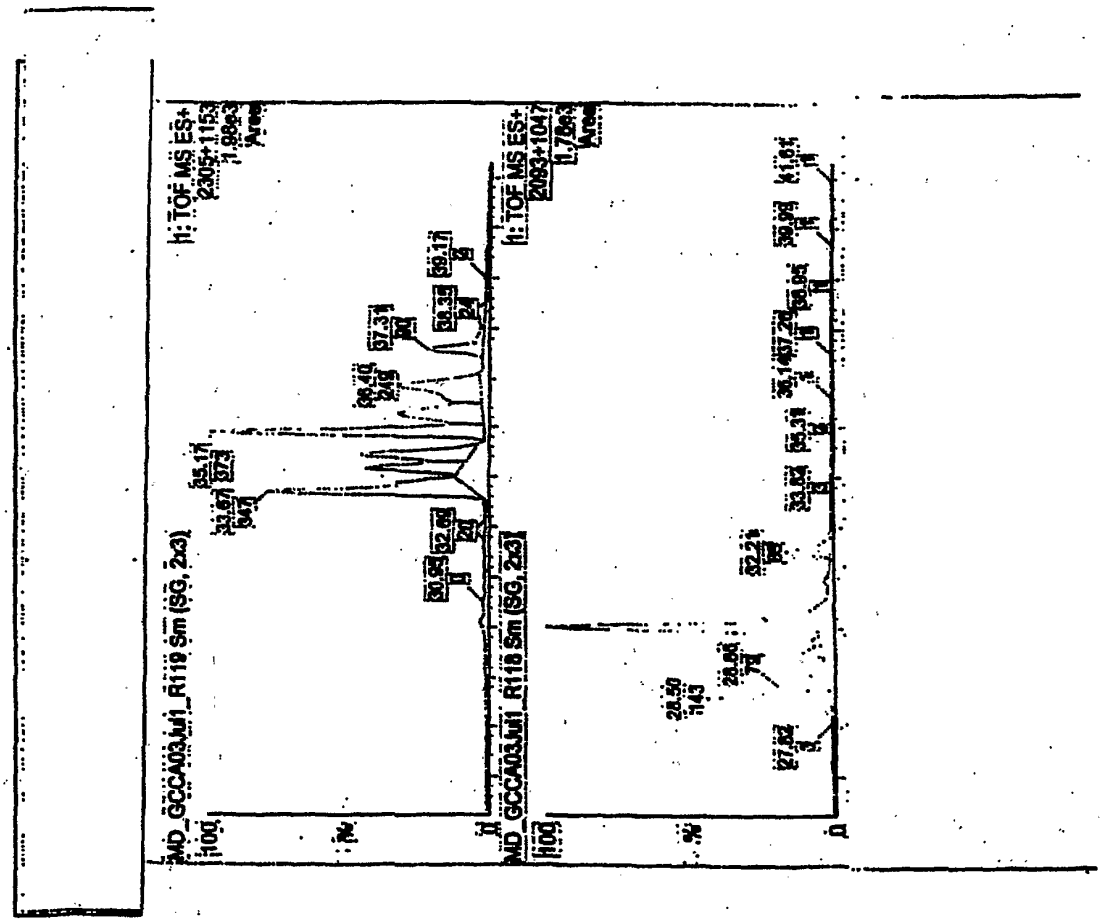
6. A vector comprising the nucleic acid molecule of claim 5

7. An isolated cell comprising the isolated nucleic acid molecule of claim 5.

8. A method for treating a patient for a gastrointestinal disorder comprising administering a composition comprising the peptide of claim 1.

9. A method for increasing GCC receptor activity comprising adiministering the peptide of claim 1.

Figure 1. LCMS analysis of recombinant peptide variants




SEQ ID NO: 4

SEQ ID NO: 5

Figure 1b: LCMS analysis of synthetic SEQ ID NO: 3 (Total Ion Chromatograph (TIC) )

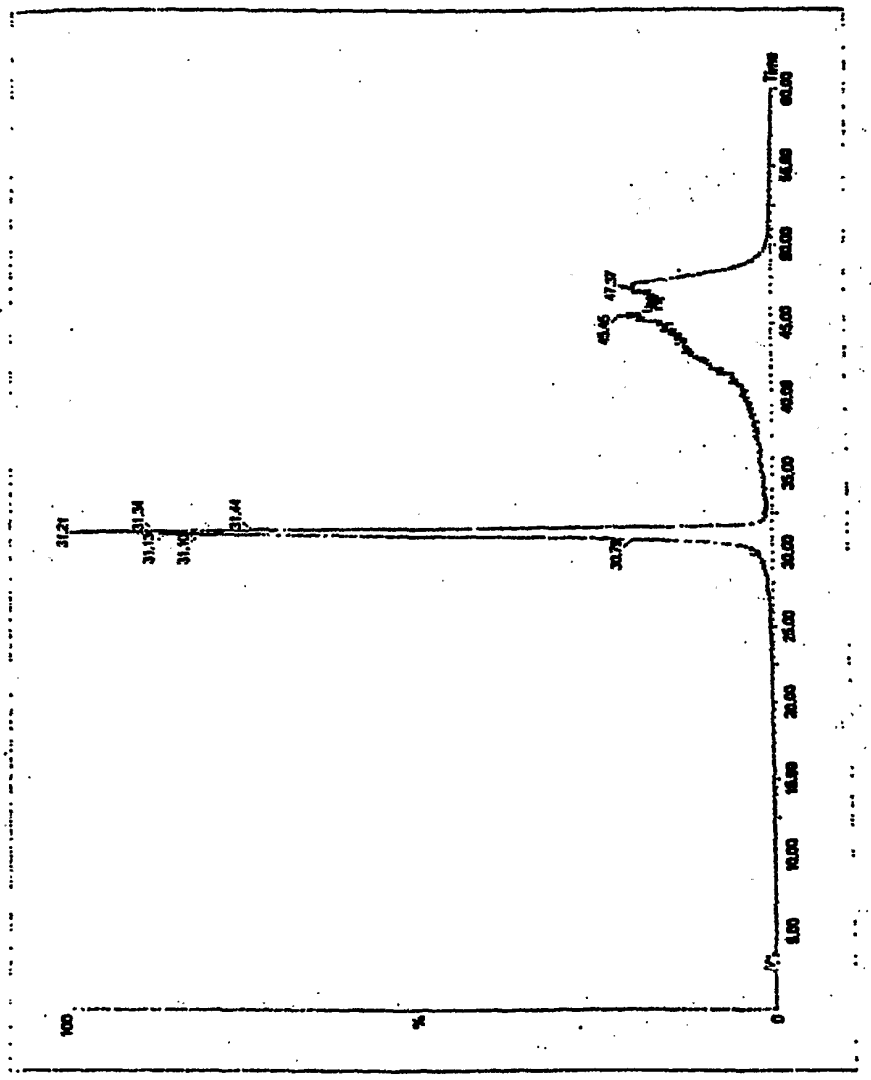


Figure 1c: LCMS analysis (Total Ion Chromatograph of blank used in SEQ ID NO:3 analysis)

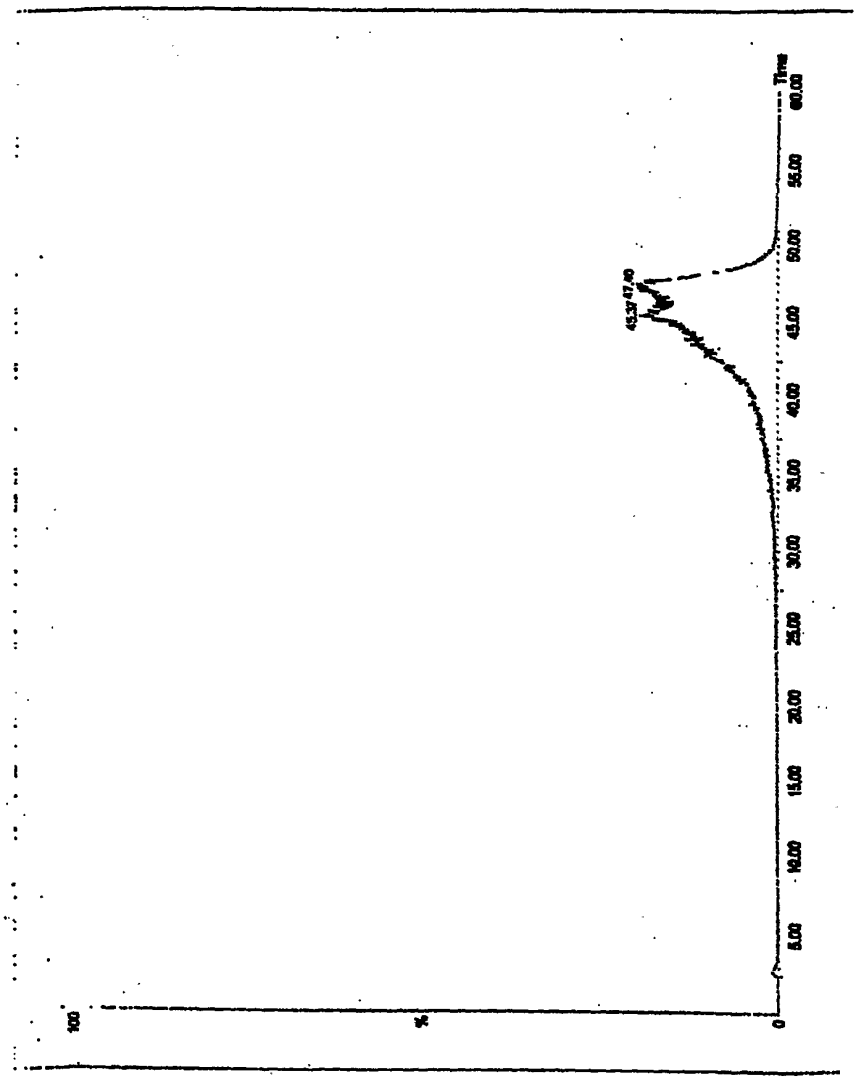


Figure 2: Chemically synthesized peptides in the Intestinal GC-C Receptor Activity Assay

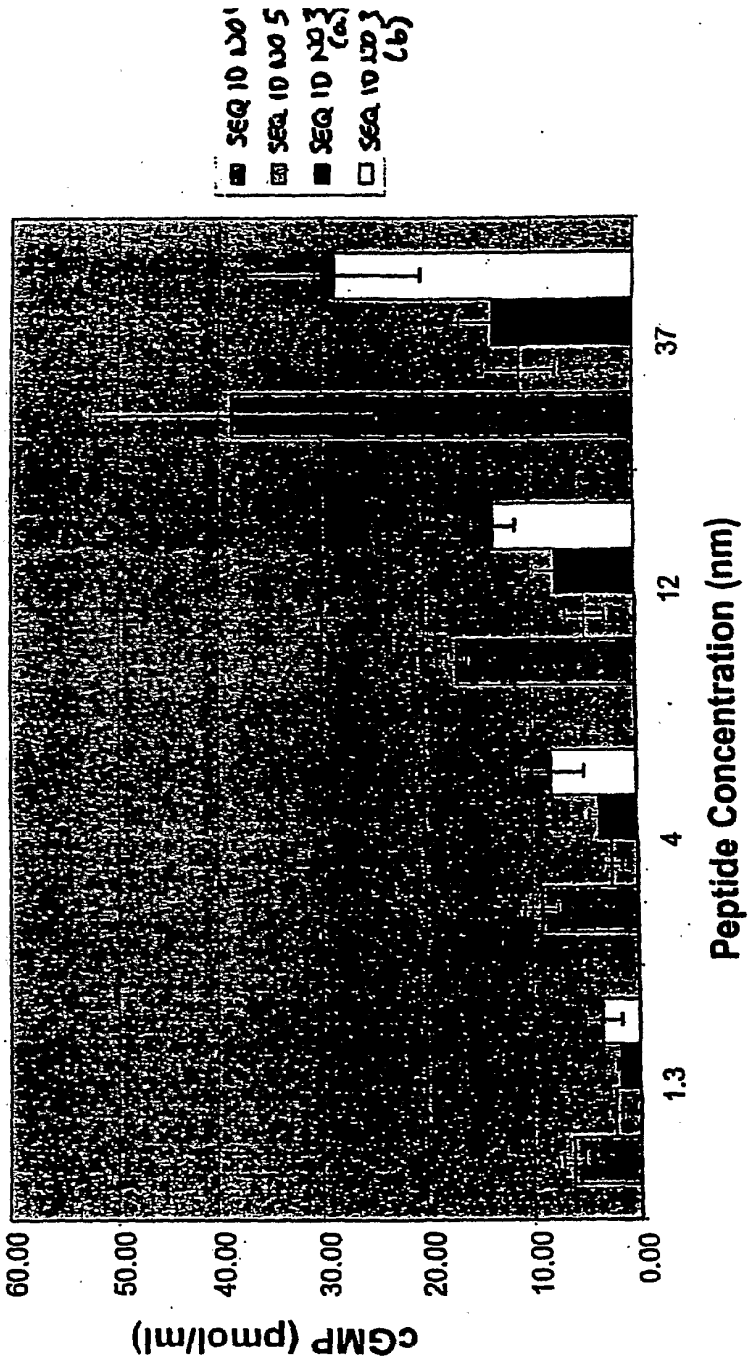


Figure 3a. Seq ID NO:4 vs Zelnorm® in an acute Mouse Gastrointestinal Transit Model (GIT)

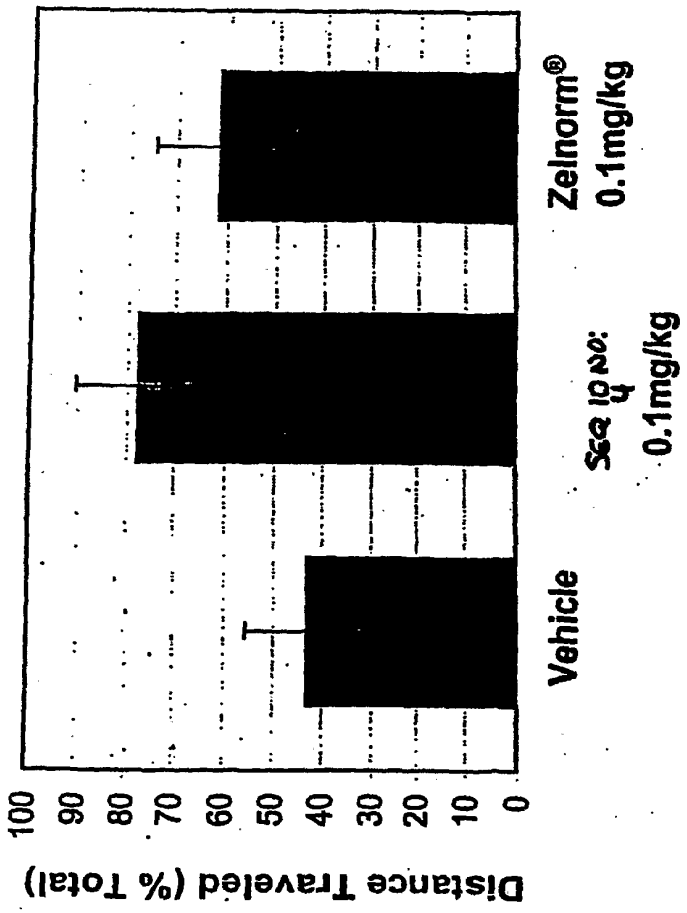


Figure 3b: Seq ID NO: 3 vs. Zelnorm® in an acute Mouse Gastrointestinal Transit Model

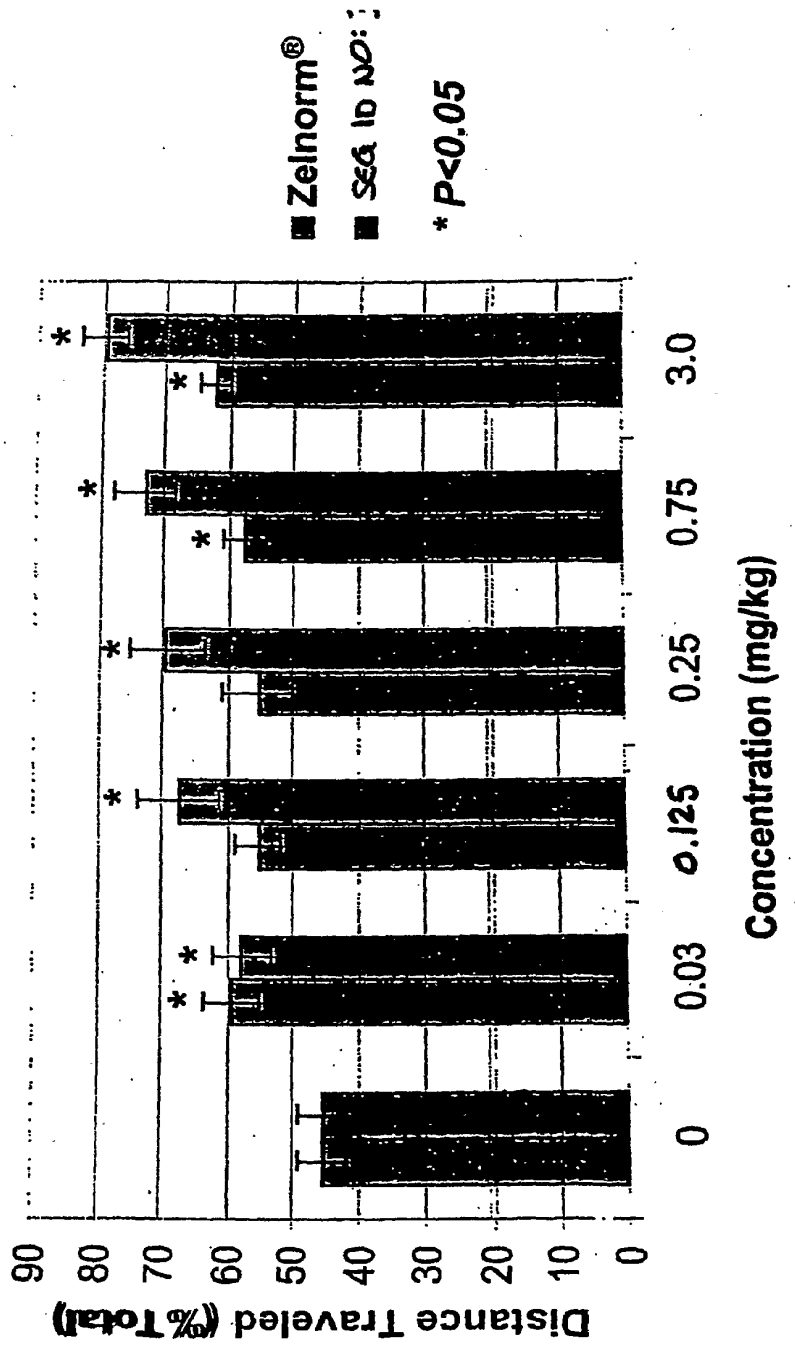


Figure 4a. Purified MD-915 and MM-416776 in GIT Model

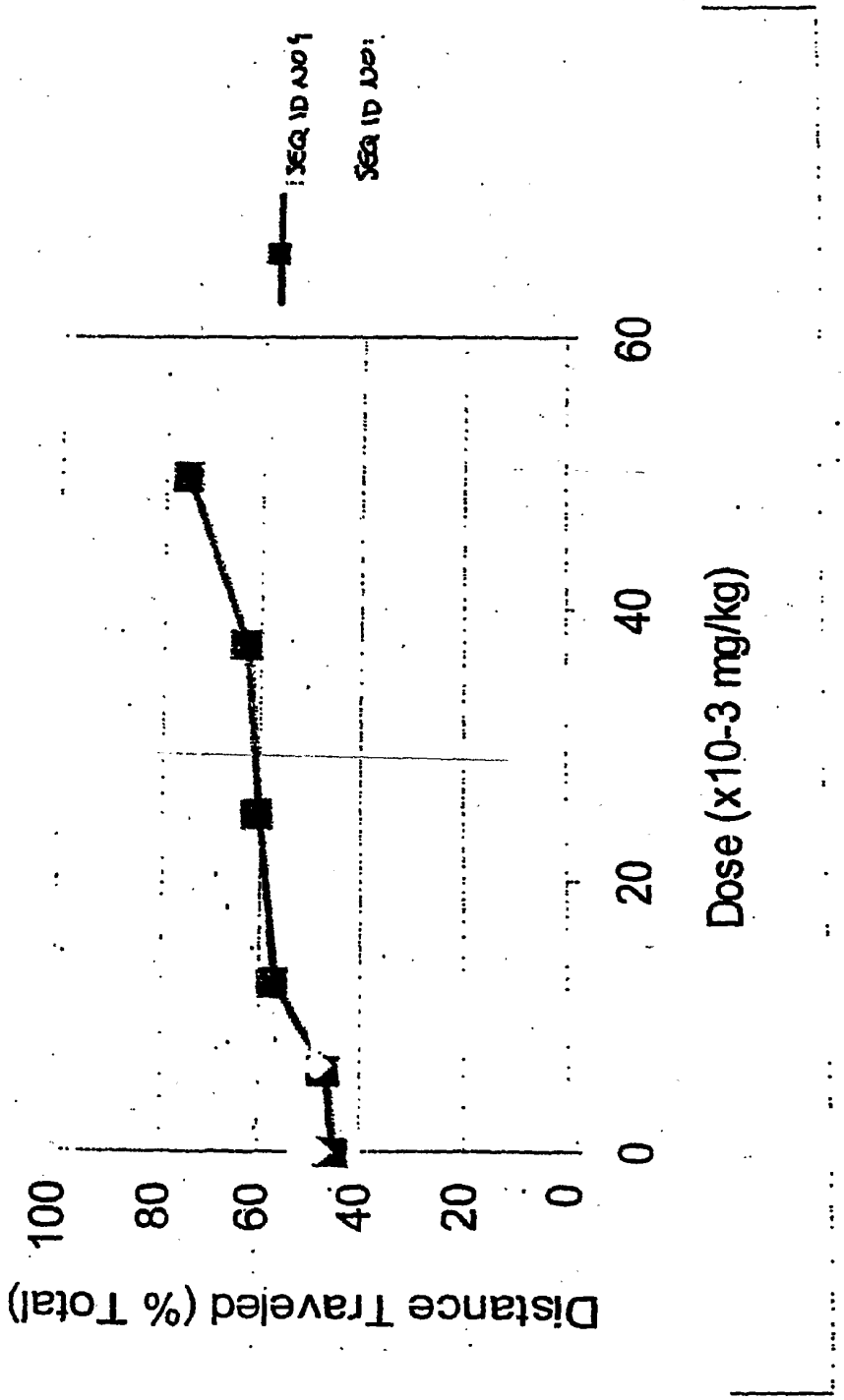
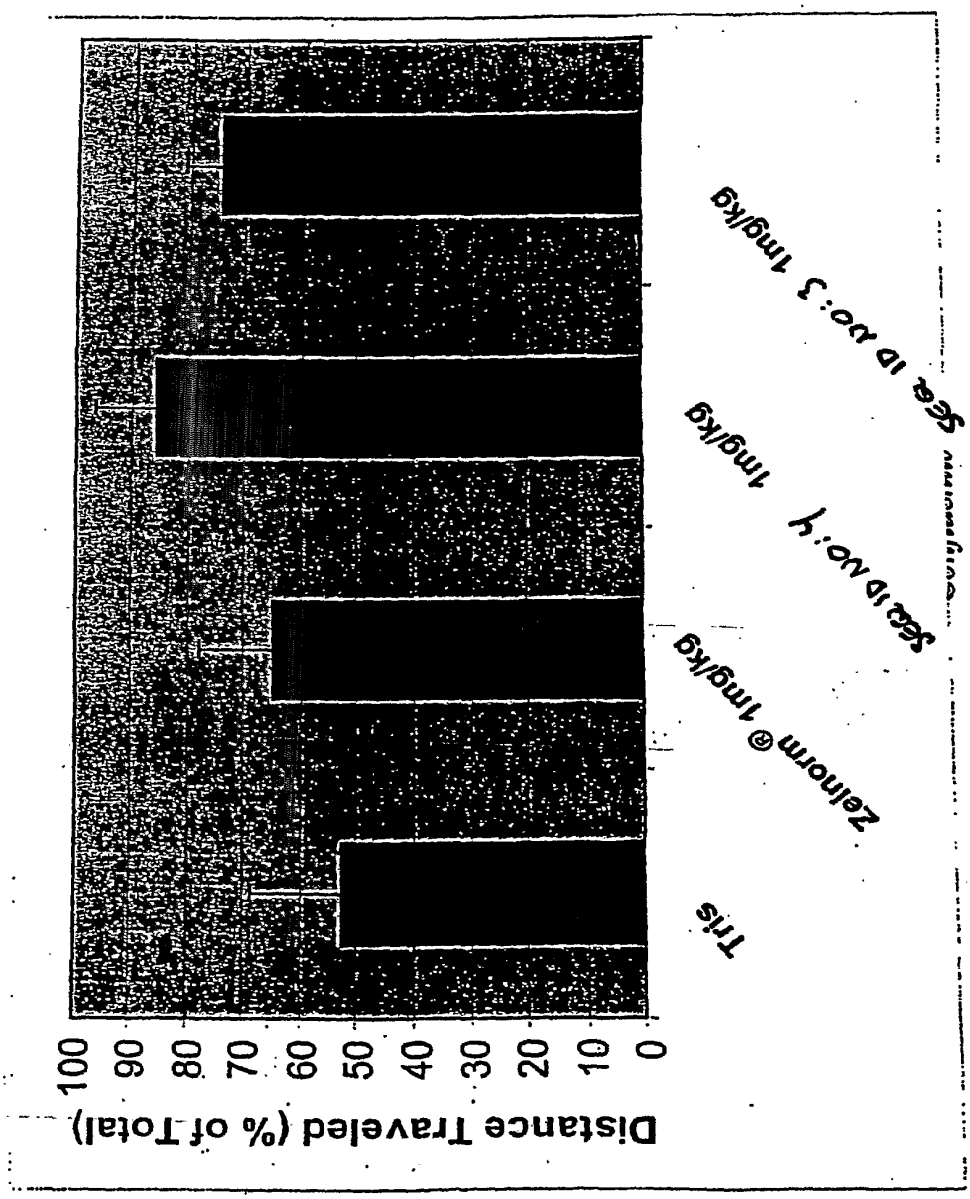


Figure 4b. Chemically Synthesized Peptides in GIT Model



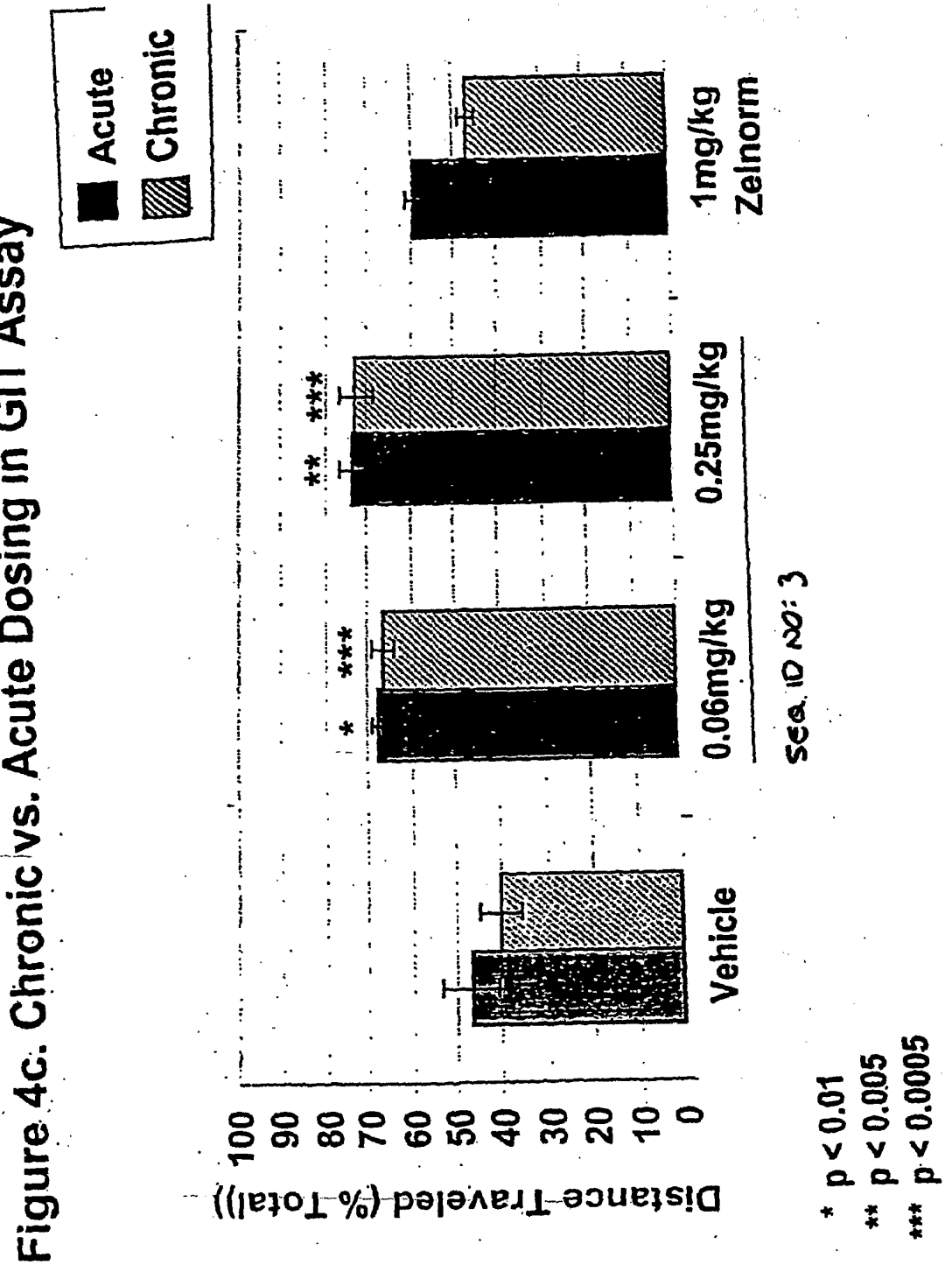


Figure 4c. Chronic vs. Acute Dosing in GIT Assay
Acute
Chronic
Distance Traveled (% Total))
100
90
80
70
60
50
40
30
20
10
0
* ***
** ***
Vehicle
0.06mg/kg
0.25mg/kg
1mg/kg Zelnorm
Seq. ID NO: 3
* p < 0.01
** p < 0.005
*** p < 0.0005

Figure 5a. Seq ID NO:4 vs Zelnorm® in a Mouse Intestinal Secretion Model

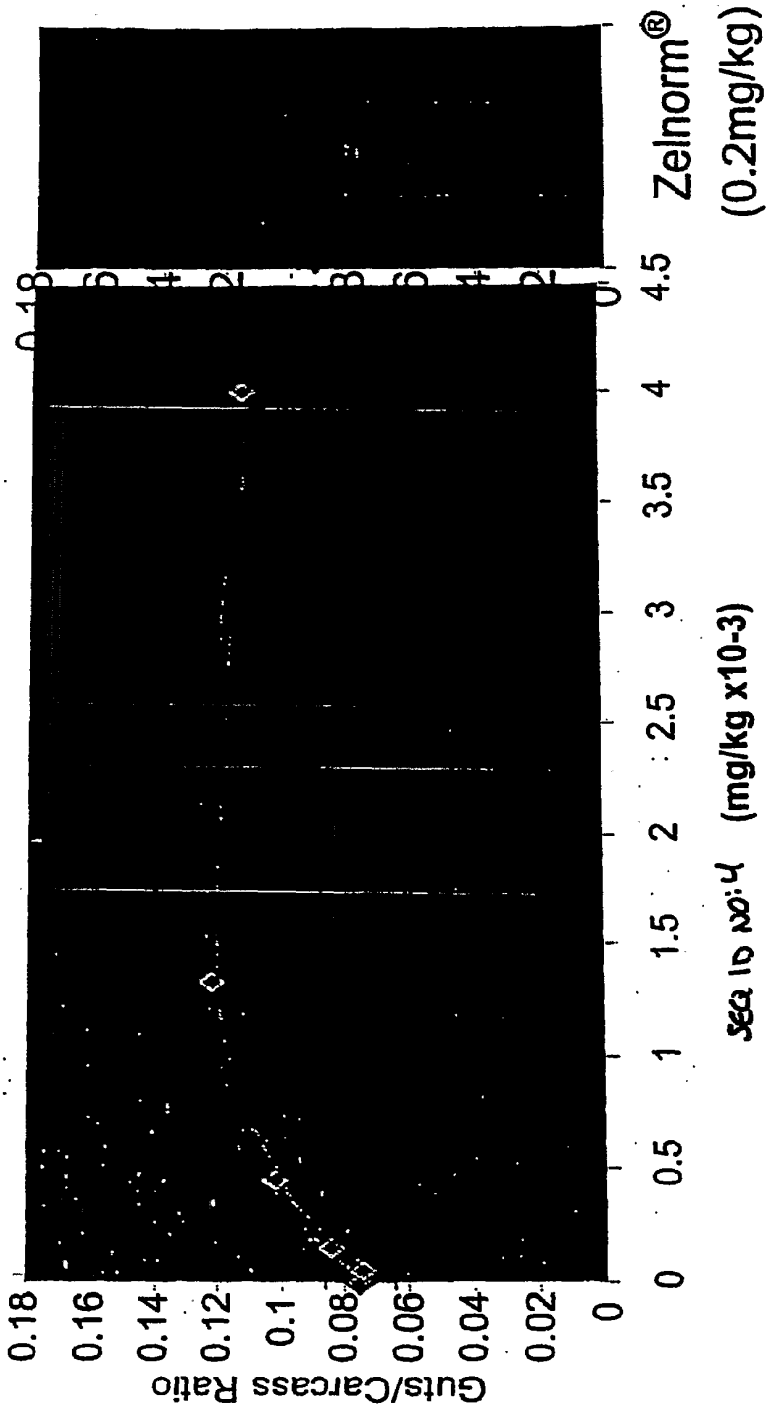


Figure 5b: SEQ ID NO: 3 vs Zelnorm® in Mouse Intestinal Secretion Model

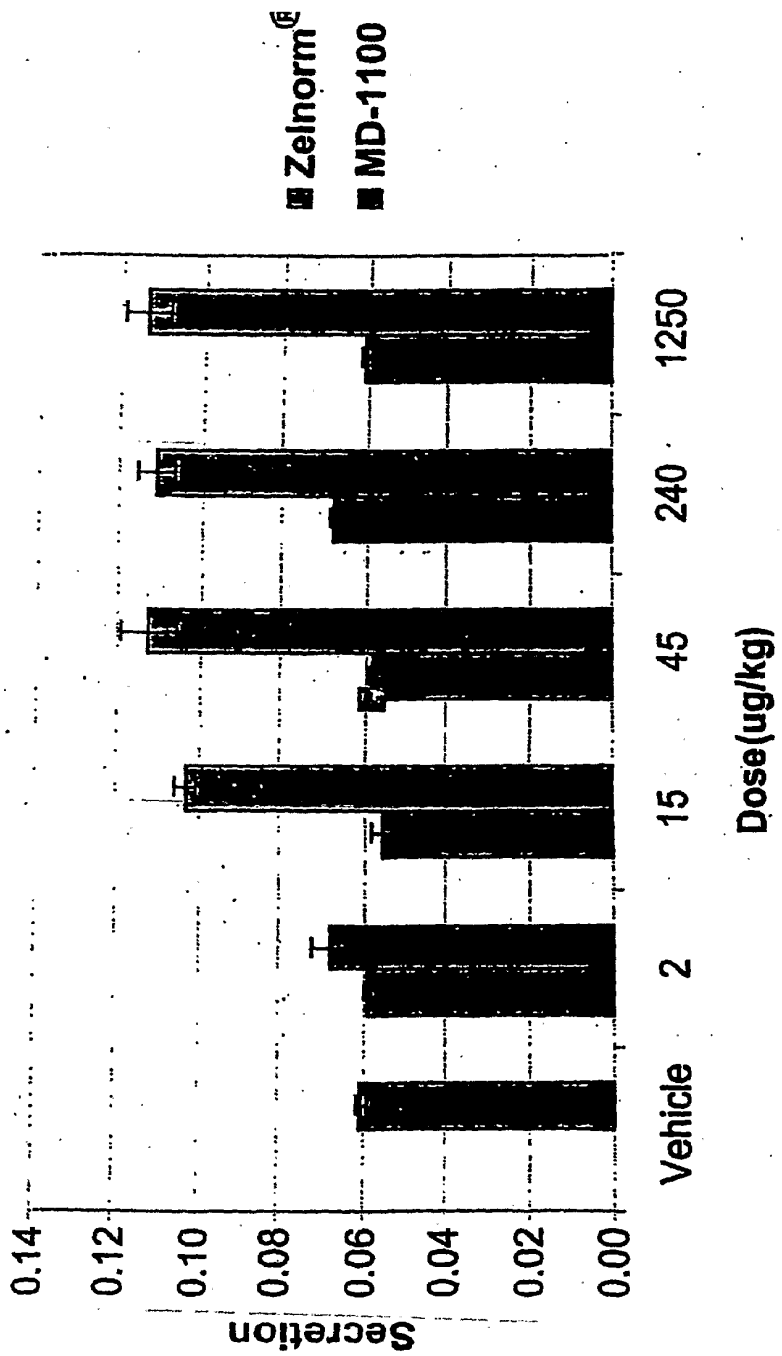


Figure 6a. Recombinantly generated SEQ ID NO:5 and SEQ ID NO:4 in Mouse Intestinal Secretion Model

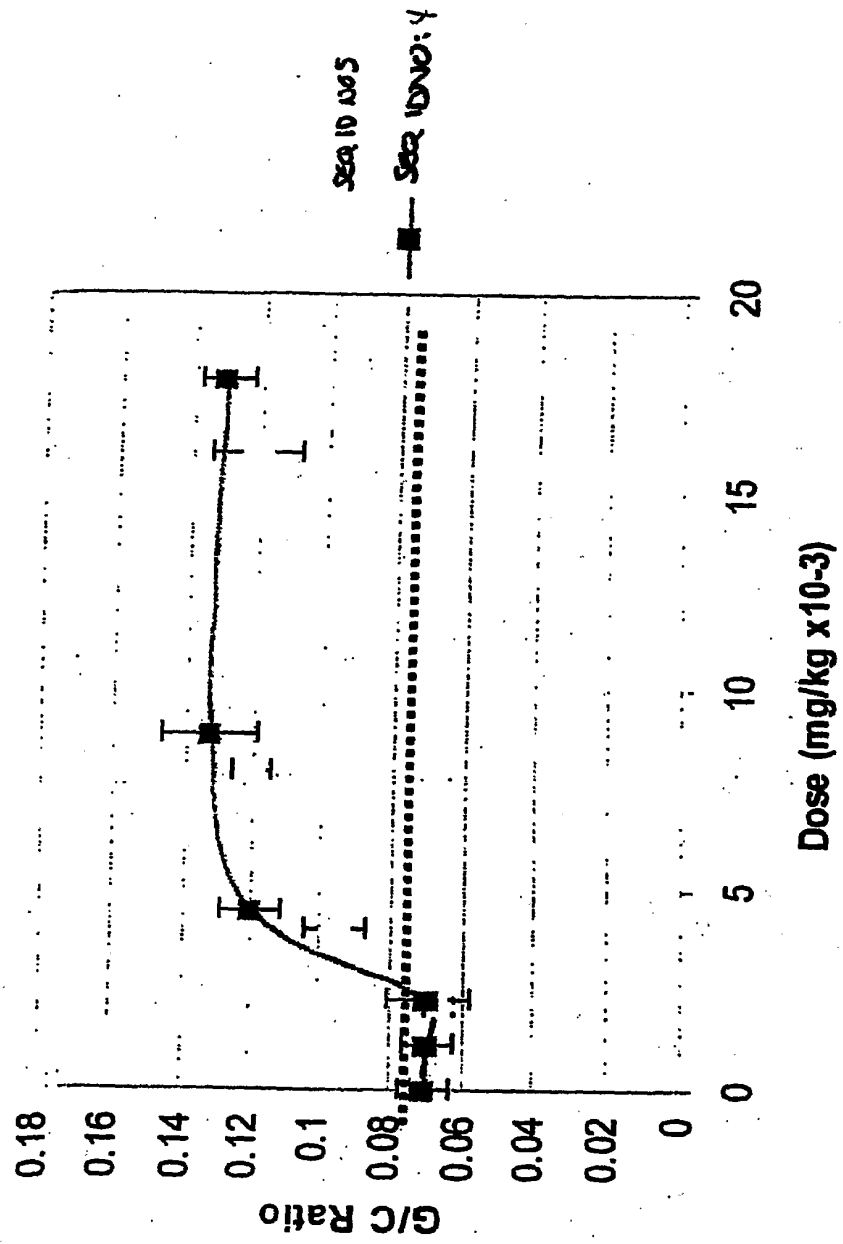


Figure 6b. Chemically synthesized peptides in Mouse Intestinal Secretion Model

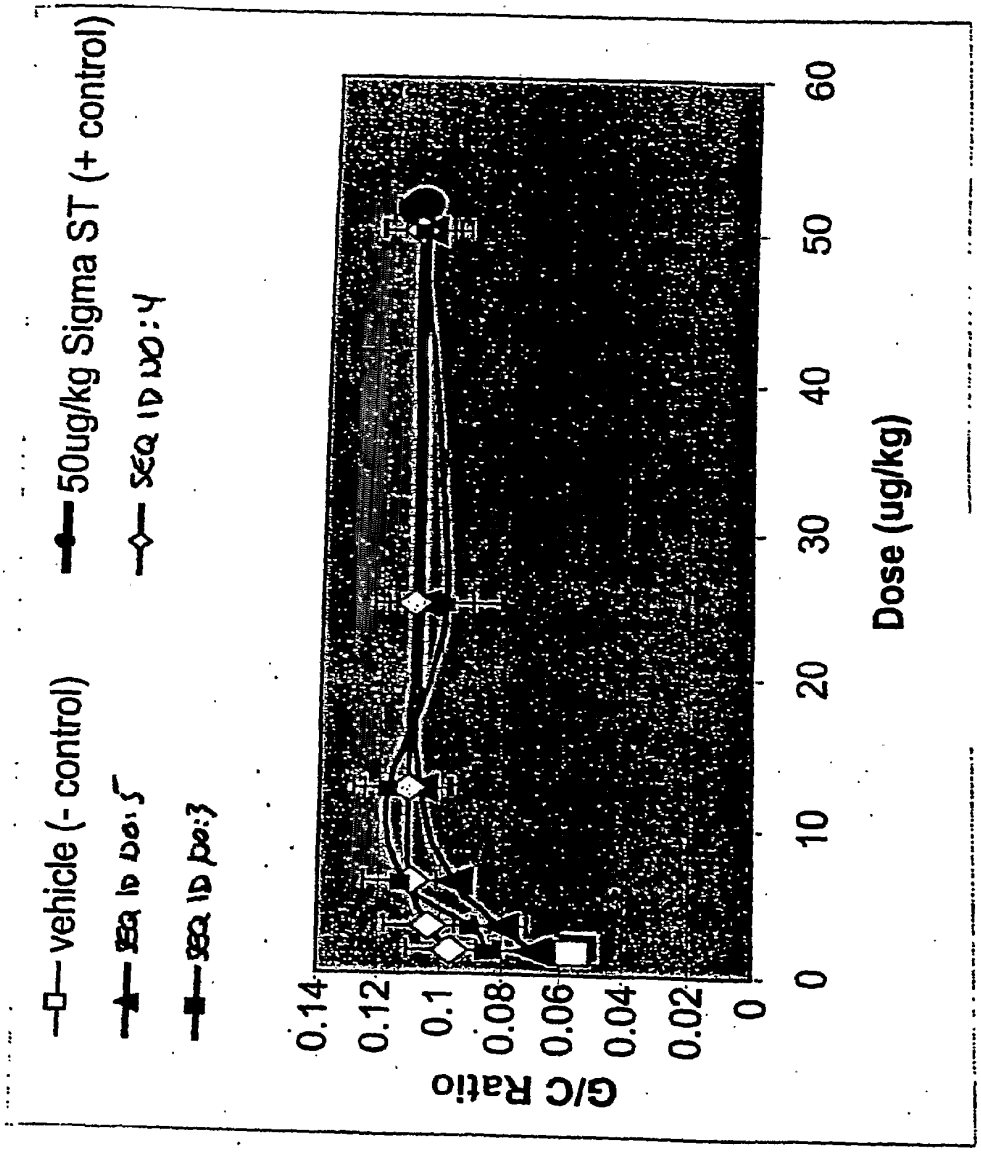
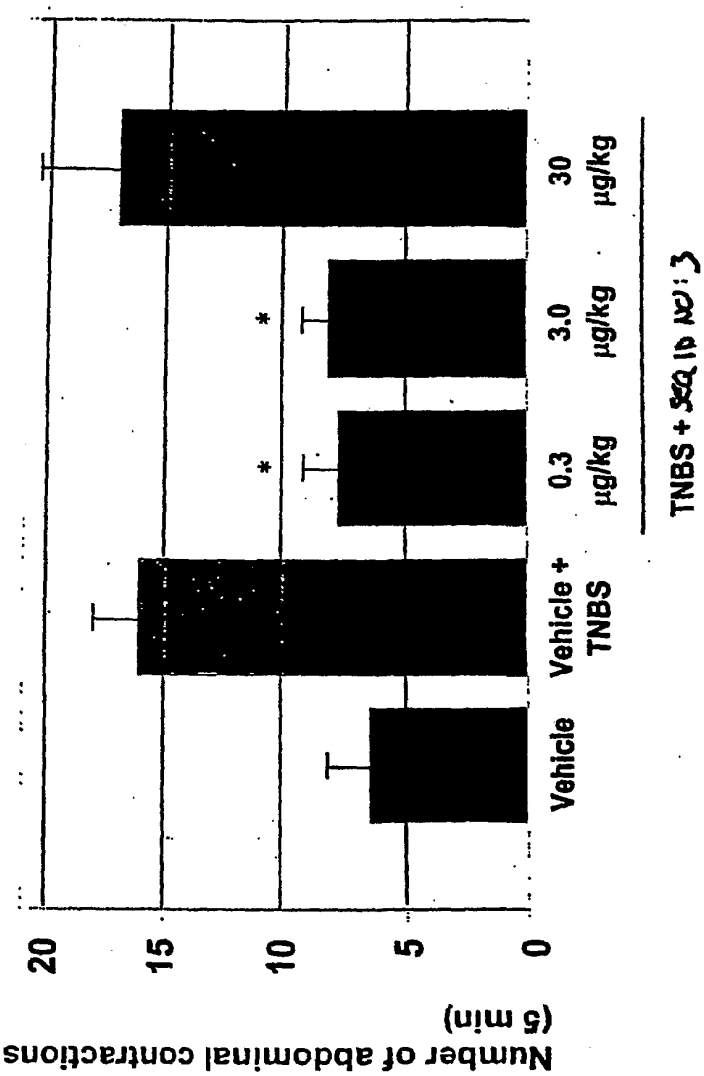


Figure 7: Effect of SEQ ID NO: 3 in a rat TNBS Colorectal Distention Assay




* p<0.05 as compared to "vehicle" value

Figure 8a: Visceral Antinociceptive Effects of SEQ ID NO: 5 in a Mouse Writhing Assay

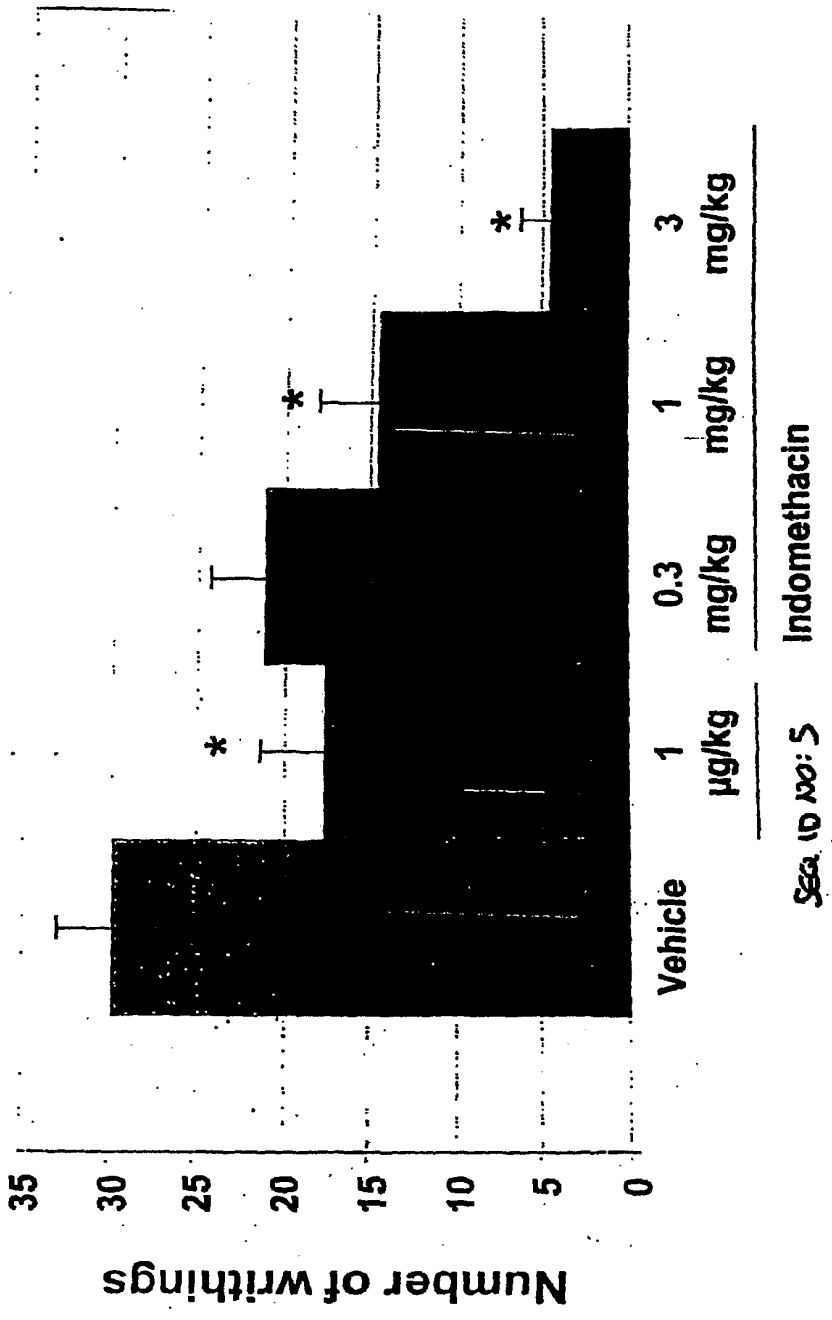


Figure 8b: Visceral Antinociceptive Effects of SEQ ID NO: 3 in a Mouse Writhing Assay

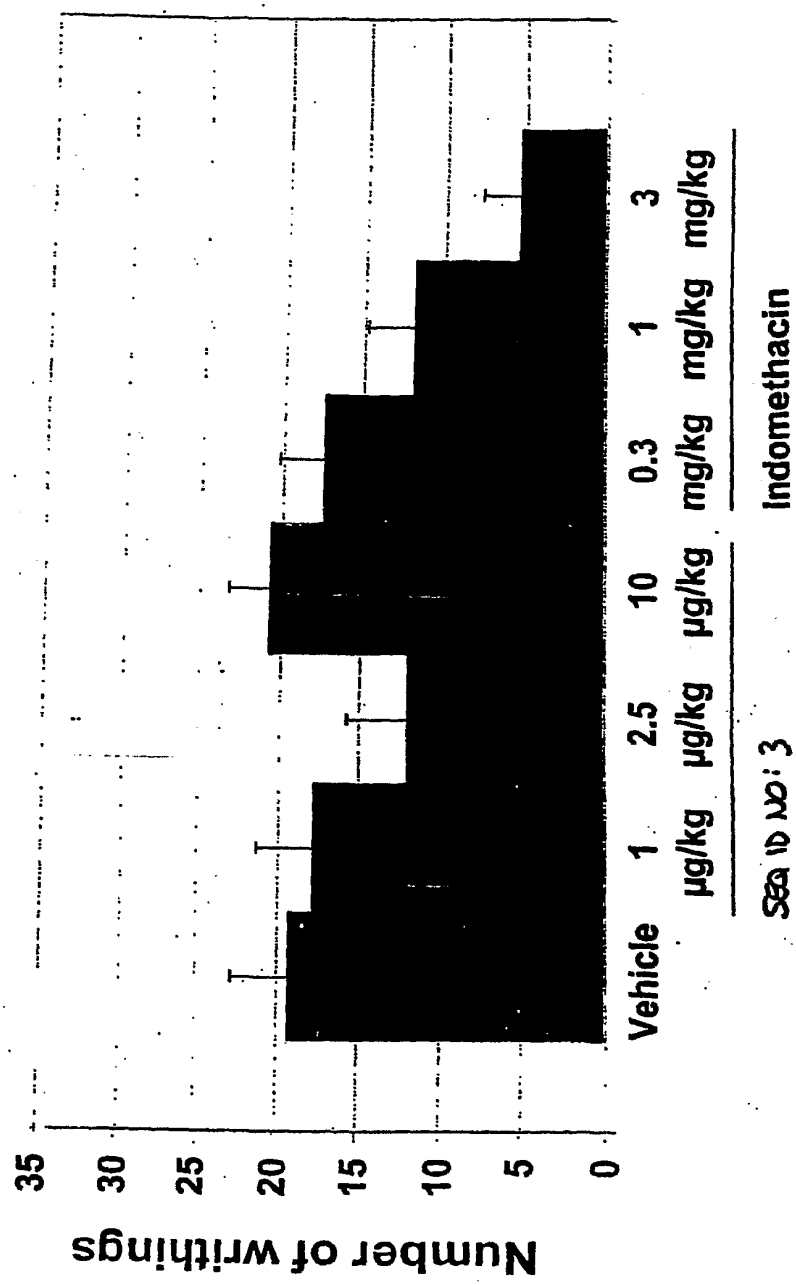


Figure 9: Competitive Radioligand Binding of SEQ ID NO:3

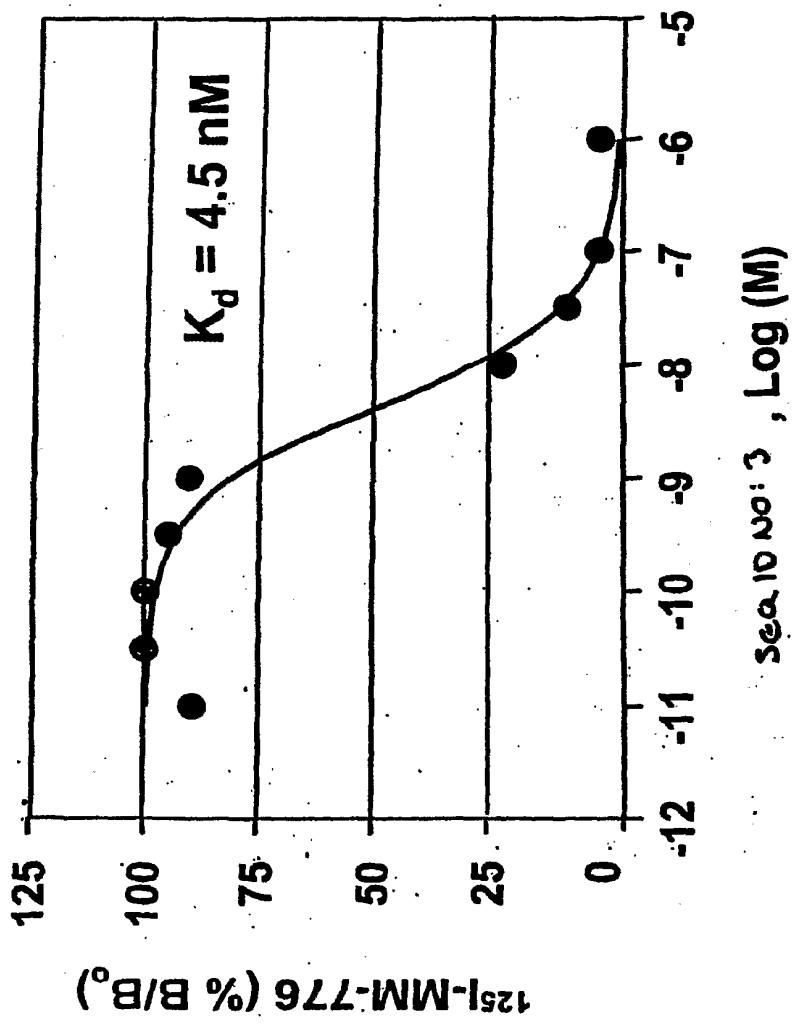
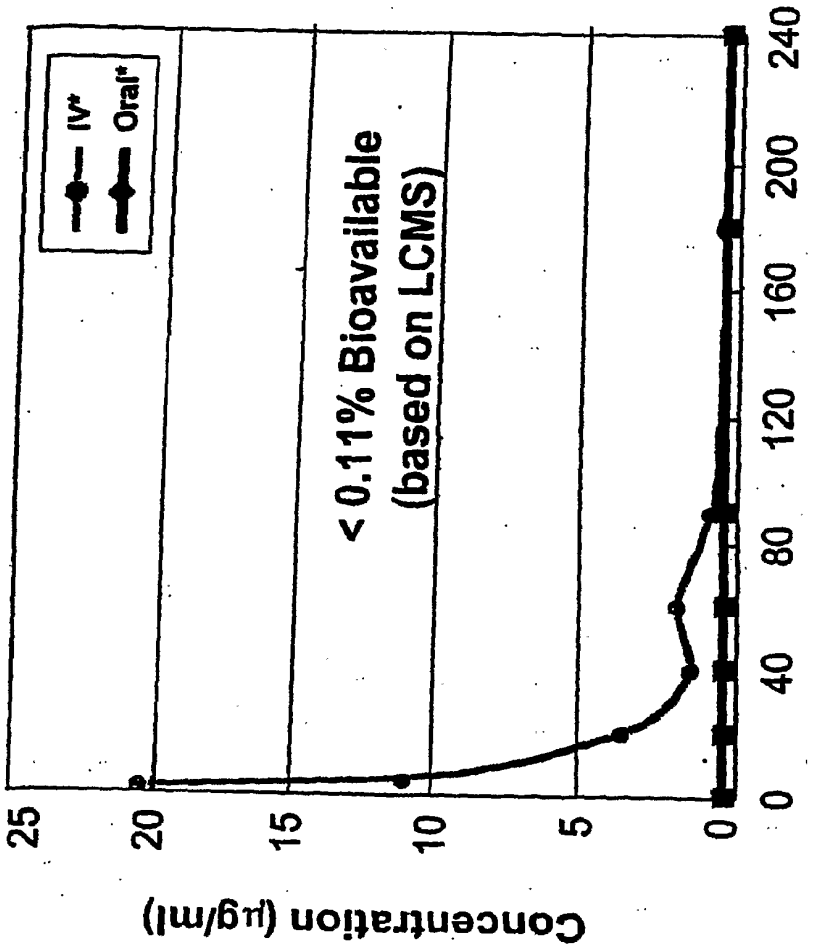
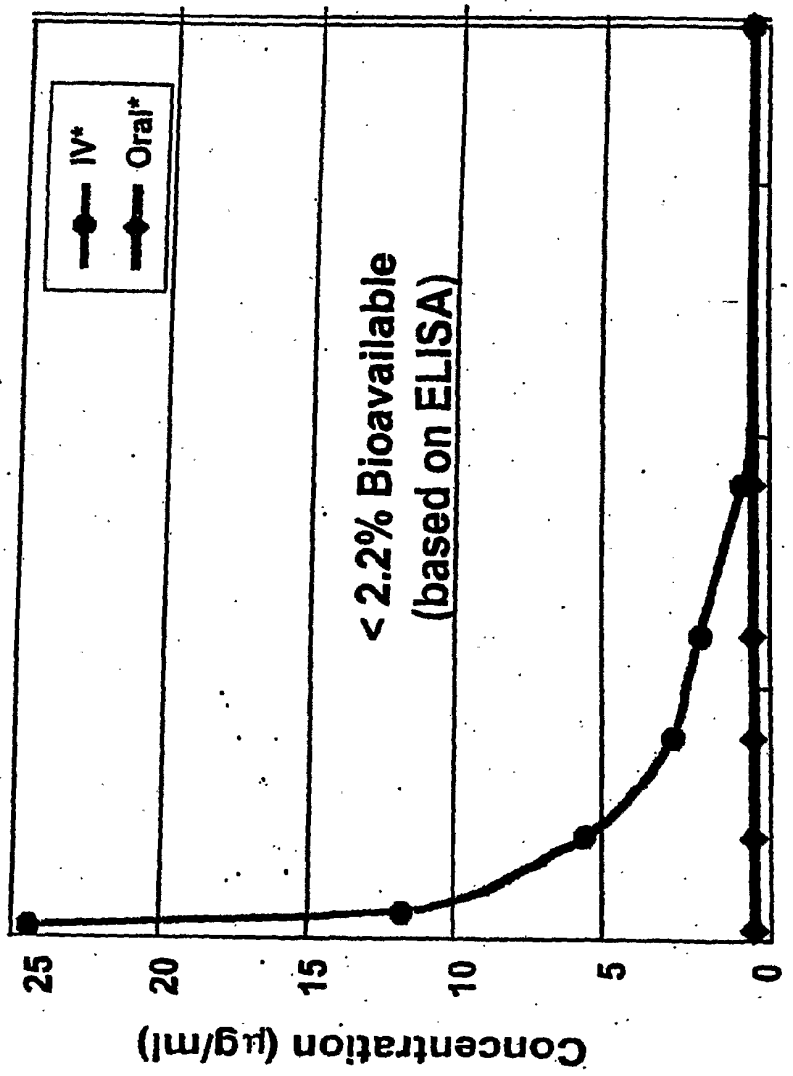


Figure 10a: Minimum Systemic Absorption of SEQ ID NO: 3 (based on LCMS)




• Limit of detection 0.00063 µg/mL (0.6 nM)
• Dosing at 10 mg/kg

Figure 10b: Minimum Systemic Absorption of SEQ ID NO:3 (based on ELISA)




* Limit of detection 0.061 µg/ml (40 nM)
Dosing at 10 mg/kg

**FIG. 11 (sheet 1 of 3)**

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO:3)

Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys --- Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys --- --- Cys Cys Asn Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys --- Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )

**FIG. 11 (sheet 2 of 3)**

Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys --- Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys --- Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
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Cys Cys Glu --- Cys Cys Asn --- Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu --- Cys Cys Asn Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys --- Gly Cys --- (SEQ ID NO: )

**FIG. 11 (sheet 3 of 3)**

Cys Cys Glu Tyr Cys Cys --- Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys --- Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn --- Ala Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro --- Cys Thr Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys --- Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys --- --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys --- --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys --- Gly Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr --- Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr --- Cys --- (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys --- (SEQ ID NO: )

FIG. 12 (sheet 1 of 33)

Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr. (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 2 of 33)

Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 3 of 33)

Cys Cys Xaa' Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 4 of 33)

Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 5 of 33)

Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 6 of 33)

Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 7 of 33)

Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 8 of 33)

Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 9 of 33)

Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Xaa' Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 10 of 33)

Cys Cys Glu Xaa' Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

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| | |
|---|---|
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr | (SEQ ID NO: ) |
| Cys Cys Glu Xaa' Tyr Xaa' Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr | (SEQ ID NO: ) |

FIG. 12 (sheet 13 of 33)

Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )

FIG. 12 (sheet 14 of 33)

Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )

FIG. 12 (sheet 15 of 33)

Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Xaa' Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Gly Cys Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )
Cys Cys Glu Tyr Xaa' Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' (SEQ ID NO: )

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Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 23 of 33)

Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 24 of 33)

Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Xaa' Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Xaa' Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Xaa' Asn Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 26 of 33)

Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Xaa' Pro Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Xaa' Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )

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Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Xaa' Cys Xaa' Tyr  (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Xaa' Tyr       (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Xaa' Thr Gly Cys Xaa' Xaa' Tyr  (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Tyr            (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Cys Tyr      (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Cys Xaa' Tyr      (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr      (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Tyr           (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Cys Tyr     (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr     (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Xaa' Xaa' Tyr         (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Xaa' Pro Ala Cys Thr Gly Cys Xaa' Tyr          (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Gly Cys Tyr               (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Tyr          (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr     (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Xaa' Ala Cys Thr Gly Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Xaa' Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Xaa' Cys Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Gly Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Xaa' Cys Thr Gly Cys Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 30 of 33)

Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Xaa' Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Xaa' Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Xaa' Xaa' Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Xaa' Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )

FIG. 12 (sheet 31 of 33)

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Xaa' Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Xaa' Xaa' Thr Gly Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Xaa' Cys Thr Gly Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 32 of 33)

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Xaa' Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Xaa' Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Xaa' Xaa' Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Tyr (SEQ ID NO: )

FIG. 12 (sheet 33 of 33)

Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Xaa' Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Xaa' Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Cys Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Tyr (SEQ ID NO: )
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Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Xaa' Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Tyr (SEQ ID NO: )
Cys Cys Glu Tyr Cys Cys Asn Pro Ala Cys Thr Gly Cys Xaa' Xaa' Xaa' Tyr (SEQ ID NO: )

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/07752

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : CO7K 7/08
US CL : 514/14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
West and STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US2004/0266989 A(Currie et al) 30 December 2004 (30.12.2004), abstract, page 27, and page 46, claim 17 | 1-9 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703)305-3230

Authorized officer
Roy Teller

Telephone No. 571-272-1600

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(71) Applicant *(for all designated States except US)*: **NOBEX CORPORATION** [US/US]; P.O. Box 13940, Research Triangle Park, NC 27709 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JAMES, Kenneth, D.** [US/US]; 3908 Long Meadow Drive, Mebane, NC 27302 (US). **RADHAKRISHNAN, Balasiugam** [US/US]; 209 Faison Road, Chapel Hill, n 27517 (US). **MALKAR, Navdeep, B.** [IN/US]; 831 Berwick Valley Lane, Cary, NC 27513 (US). **MILLER, Mark, A.** [US/US]; 1820 Avent Ridge Road, Apt. 201, Raleigh, NC 27606 (US). **EKWURIBE, Nnochiri, N.** [NG/US]; 216 Coltsgate Drive, Cary, NC 27511 (US).

(74) Agent: **BARRETT, William, A.**; Moore & Van Allen, P.O. Box 13706, 430 Davis Drive, Research Triangle Park, NC 27709 (US).

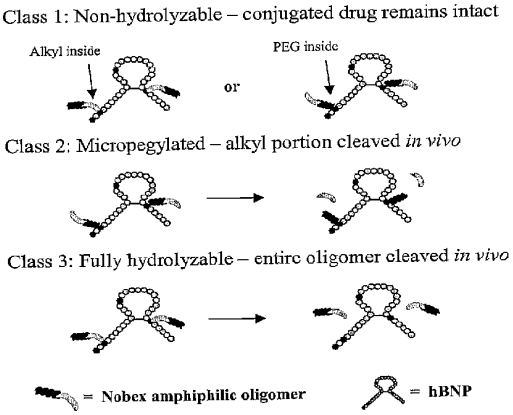
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv)) for US only*

*[Continued on next page]*

(54) Title: NATRIURETIC COMPOUNDS, CONJUGATES, AND USES THEREOF




(57) Abstract: Modified natriuretic compounds and conjugates thereof are disclosed in the present invention. In particular, conjugated forms of hBNP are provided that include at least one modifying moiety attached thereto. The modified natriuretic compound conjugates retain activity for stimulating cGMP production, binding to NPR-A receptor, and in some embodiments an improved half-life in circulation as compared to unmodified counterpart natriuretic compounds. Oral, parenteral, enteral, subcutaneous, pulmonary, and intravenous forms of the compounds and conjugates may be prepared as treatments and/or therapies for heart conditions particularly congestive heart failure. Modifying moieties comprising oligomeric structures having a variety of lengths and configurations are also disclosed. Analogs of the natriuretic compound are also disclosed, having an amino acid sequence that is other than the native sequence.

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**Published:**
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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# NATRIURETIC COMPOUNDS, CONJUGATES, AND USES THEREOF

By:

Kenneth D. JAMES

Balasingham RADHAKRISHNAN

Navdeep B. MALKAR

Mark A. MILLER

Nnochiri N. EKWURIBE

# NATRIURETIC COMPOUNDS, CONJUGATES, AND USES THEREOF

## 1. Related Applications

[01] This application is based on and claims priority to, United States provisional patent application serial no. 60/574,436, filed May 26, 2004, pending, entitled "NATRIURETIC COMPOUNDS, CONJUGATES, AND USES THEREOF". The entire disclosure of each of these applications is incorporated here by reference in its entirety, and the benefit of the filing date of each such patent application is hereby claimed for all purposes that are legally served by such claim

## 2. Statement of Government Support

[02] The subject invention was made with government support under a research project supported by NIH Grant #1 R43 HL074529-01. The United States government has certain rights in this invention.

## 3. Field of the Invention

[03] The present invention relates to the field of natriuretic compound conjugates and variant natriuretic compounds, and uses of these in the treatment of congestive heart disease and conditions related to this condition. For example, the compositions of the present invention provide a pharmacologically active natriuretic agent and prodrug that may be used in a formulation suitable for oral, nasal, intravenous, or subcutaneous administration. The invention also provides methods of preparing the natriuretic compound conjugates, compounds, and formulations containing them, as well as methods of using these conjugates and compounds. By way of example, the natriuretic compound conjugates comprise a natriuretic peptide including an NPR-A binding motif, at least one modifying moiety conjugation site, and also include at least one modifying moiety attached to the modifying moiety conjugation site. In some embodiments, the compound conjugates have retained pharmacological activity of the native natriuretic peptide, and have enhanced characteristics, such as improved bioavailability, enhanced resistance to proteolytic activity, and/or prolonged activity in the blood stream. In other embodiments, the compound conjugates are provided as hydrolysable prodrugs, which may have reduced pharmacological activity in the prodrug form relative to the native natriuretic peptide, and upon hydrolysis of the prodrug *in vivo*, an active natriuretic compound is released.

[04] The present invention is also related to the field of recombinant peptides and proteins, as well as methods for preparing these recombinant peptides and proteins. In particular, analogs of natriuretic peptides and proteins are disclosed herein. The analog natriuretic compounds of the invention may be described in some embodiments as having an amino acid sequence that has at least one substituted amino acid relative to the native sequence of the corresponding natriuretic peptide. In some embodiments, the analog natriuretic compounds of the invention may be described as having a pharmacological activity of native forms of brain-

type natriuretic peptides (BNP), especially human BNP (hBNP), urodilatin, canine brain natriuretic peptide (cBNP), atrial natriuretic peptide (ANP), especially human ANP (hANP), dendroaspis natriuretic peptide (DNP), or C-type natriuretic peptide (CNP), particularly human CNP (hCNP).

## 4. Background of the Invention

[05] Cardiovascular diseases constitute the leading cause of death in the United Sates regardless of gender or ethnicity. Of these diseases, congestive heart failure (CHF) is the only one that is increasing in prevalence (Massie and Shah 1997; Packer and Cohn 1999). According to the American Heart Association, the number of hospital discharges and the number of deaths due to CHF both rose roughly 2.5-fold from 1979 to 1999. Currently, about 5 million Americans have been diagnosed with CHF, and about 550,000 new cases occur annually (American Heart Association 2001). This life-threatening condition is accompanied by great financial impact. In fact, it is the single largest Medicare expense (Kayser 2002). Direct and indirect costs for treating CHF have been estimated as high as $56 billion (Hussar 2002). Hospital expenses for the treatment of HF are more than double those for all forms of cancer combined (O'Connell and Bristow 1994).

[06] CHF is a common cause of death, is accompanied by high indirect costs for treatment, and has a high mortality rate. Once a patient has been diagnosed with CHF, the one-year mortality rate is about 20% (American Heart Association 2001). The probability for readmission for the same condition is very high, and several studies of readmission have recently been performed (Chin and Goldman 1997; Krumholz, Parent et al. 1997; Krumholz, Chen et al. 2000). Readmission rates in excess of 35% within one year of diagnosis are typical (Tsuchihashi, Tsutsui et al. 2001). Such frequent recurrence results in multiple emergency care visits and inpatient hospitalizations (Krumholz, Parent et al. 1997). Multiple hospitalizations and inadequate therapeutics define the current situation faced by those who suffer from CHF.

[07] A recent randomized study indicated that home-based intervention can potentially decrease the rate of readmission, prolong survival, and improve the quality of life for patients with CHF (Stewart, Marley et al. 1999). In an independent study that looked at socioeconomic factors, Tsuchihashi, et. al. concluded that both outpatient and home-based care are needed in order to reduce the mortality rate and lower the overall costs associated with CHF (Tsuchihashi, Tsutsui et al. 2001). Clearly, new therapies with broad application that can be used on an outpatient basis are desperately needed in this growing market.

[08] Brain type natriuretic peptide (BNP) is one of a family of peptides that are involved in cardiovascular, renal, and endocrine homeostasis. It was discovered in 1988 (Sudoh, Kangawa et al. 1988), almost a decade after the discovery of atrial natriuretic peptide (ANP). Although it was first isolated from porcine brain, it is known for its activity at receptors in vascular smooth muscle and endothelial cells. BNP

is an endogenous peptide produced by the heart. It is first produced as prepro-BNP and is subsequently shortened twice to the active form, a 32-amino acid peptide with one disulfide bond.

[09] As illustrated in Figure 1, BNP binds to the natriuretic peptide receptor A (NPR-A), a membrane bound protein on the cell surface. The binding event triggers the synthesis of cGMP in the cytosol by guanylate cyclase. It is through this secondary messenger that BNP accomplishes the cardio-vascular, renal, and endocrine effects with which it is associated. Regulation of BNP is accomplished by several different means. BNP molecules that bind to NPR-A and stimulate cGMP production are removed from circulation, but there are other means by which BNP is eliminated without invoking a response. The most common means of removal is through binding to the clearance receptor, natriuretic peptide receptor C (NPR-C). Upon binding to NPR-C, the peptide is taken into the cell and cleaved enzymatically. The next major means of clearance is degradation by neutral endopeptidase (NEP), which is a membrane-bound enzyme on the cell surface. Finally, BNP is removed to a small extent by renal filtration.

[10] Under normal conditions, BNP is produced in low amounts in the atria and ventricles. However, when the ventricles are stretched during cardiac decompensation, the amount of BNP that is produced increases greatly. Although the atria are still involved, the ventricles become the main site of production. The heart produces BNP in response to a stretching of the ventricles that occurs during decompensation at the outset of CHF. The effects of BNP include natriuresis, diuresis, vasodilation, and a lowering of diastolic blood pressure. These effects are brought about through the actions of a secondary messenger, cyclic guanosine monophosphate (cGMP). Production of cGMP is triggered when BNP interacts with the natriuretic peptide receptor A (NPR-A) which is a membrane-bound receptor located on the surface of endothelial cells in blood vessels, kidneys, and lungs. Plasma concentration of BNP incrementally increases with increased severity of CHF. Despite this increase, the beneficial effects of BNP are blunted in severe CHF, raising the possibility of a relative deficiency state in overt CHF. Alternatively, as the assays currently employed to measure plasma concentration of BNP do not specifically differentiate between pre-pro BNP and the mature form, this pro-hormone may not be adequately processed to its mature form in overt CHF. Therefore, either the amount of BNP that the heart can produce is overcome or prepro-BNP is not adequately converted into its active form, thus reducing its beneficial actions. Because of its early production at the onset of heart disease, BNP has become important as a diagnostic marker to detect patients who are at high risk of developing CHF (Yamamoto, Burnett et al. 1996; McDonagh, Robb et al. 1998; Richards, Nicholls et al. 1998; Nagaya, Nishikimi et al. 2000; Kawai, Hata et al. 2001; Maisel, Krishnaswamy et al. 2002; McNairy, Gardetto et al. 2002).

[11] BNP functions to relieve cardiac decompensation in several ways. As the name implies, BNP leads to the excretion of sodium and an increase in urine output, which lessen congestion. It also functions as a vasodilator, the effects of which are enhanced by several other actions. Most notable of these functions are the roles BNP plays in the interference of the renin-angiotensin-aldosterone system (RAAS). It leads to

inhibition of renin, which is a key enzyme in the generation of the vasoconstrictive peptide angiotensin. It inhibits the overgrowth of epithelial cells lining vascular tissue, which left unchecked, can greatly reduce blood flow. A final way that BNP functions to relieve cardiac decompensation is its lusitropic effects. It improves myocardial relaxation of the ventricles, resulting in lower diastolic blood pressure.

[12] Practical limitations exist in using peptides as drugs. Proteolysis, both in the gut and in the bloodstream, is a major barrier to using peptides as therapeutics. Another difficulty encountered with non-endogenous peptides is immunogenicity. As a result of these problems, the approach of the pharmaceutical industry has been to create small, non-peptide molecules using medicinal chemistry. While this approach has met with success, it is costly, time consuming, and fraught with uncertainty in terms of pharmacokinetics and toxicity. Furthermore, identification of small organic molecules with agonist activity at peptide receptors has proved exceptionally challenging.

[13] While the use of "PEGylated" proteins is well established to date, they have been confined to injectable use. The present invention provides orally available conjugates of polypeptides, such as human brain-type natriuretic peptide (hBNP). Specifically, the present invention provides conjugates comprising PAG linked to therapeutic peptides and proteins in a formulation in the treatment of congestive heart failure. These preparations then function to protect the hBNP against proteolytic enzymes, and thereby permit the effective use of this agent as an agonist of human natriuretic peptide receptor A. As a result of this agonistic activity, there is enhanced production of cGMP.

[14] In August 2001, hBNP (native peptide) was approved by the FDA under the trade name Natrecor® (Nesiritide) for the treatment of acute congestive heart failure. Natrecor® was the first drug approved for the treatment of CHF in over twelve years. It is administered by intravenous continuous infusion over a period of 48 hours. As the drug is expensive and requires hospitalization, Natrecor® is only used for the most acute cases. Despite this expense and inconvenience, Natrecor® may be considered less expensive than some other therapies by reducing the amount of time patients spend in intensive care units.

[15] Currently, almost 5 million Americans have CHF and over 550,000 new cases are reported each year (American Heart Association 2001). Currently, direct costs for the treatment of CHF are well over $20 billion (American Heart Association 2001). With diagnostic procedures now available to detect the onset of heart failure before cardiac damage occurs, there is great need for a drug with expanded utility that can be used in an outpatient or home-based setting.

## 5. Summary of the Invention

[16] The present invention broadly comprises variant and modified forms of several naturally occurring natriuretic peptides, proteins, analogs, and chemical conjugates of these natiruertic peptides that possess one or more advantages over their naturally occurring counterparts. By way of example, some of these

advantages include an increased resistance to proteolytic degradation, an improved time of persistence in the bloodstream, and/or an improved ability to traverse cell membrane barriers.

[17] Natriuretic compound conjugates according to some embodiments of the present invention comprise a natriuretic compound that includes a natriuretic protein receptor A binding motif (an NPR-A), at least one modifying moiety conjugation site, and at least one modifying moiety attached to said modifying moiety conjugation site. By virtue of the modifying moiety attached to said natriuretic compound as part of the conjugate, the natriuretic compound conjugate can have modified hydrophilic characteristics relative to the native natriuretic compound that does not include a modifying moiety as described herein. By way of example and not limitation, and as described more fully herein, the modifying moiety may take the form of an oligomer of any variety of sizes, shapes, substitutions, and configurations.

[18] In some cases, the natriuretic compound conjugate is characterized at least in part by its increased resistance to enzymatic degradation, such as proteolysis, relative to a corresponding unconjugated form of the native natriuretic compound. These compound conjugates may be even further characterized by a retained therapeutically significant percentage of biological activity, such as cGMP stimulating activity, relative to the corresponding unconjugated natriuretic compound. The retained cGMP stimulating activity is typically at least 30%, 40%, 50%, 60%, 70%, 90%, 95%, or even greater than 99% or 100% of the cGMP activity of an unconjugated form of the natriuretic peptide as measured *in vitro*. Other examples of improved characteristics of the natriuretic compound conjugates of the invention having a modifying moiety, relative to unmodified (unconjugated) natriuretic compound, include improved ability of the natriuretic compound to pass through the GI tract and enter the blood stream; improved hydrophilicity, hydrophobicity, or amphiphilicity of the natriuretic compound; improved solubility of the natriuretic compound in aqueous environments or organic solvents; improved ability of the natriuretic compound to cross cell membranes; improved ability of the natriuretic compound to traverse the blood-brain barrier; improved ability of the natriuretic compound to target a certain receptor, cell, tissue, or organ; and improved pharmacokinetic profile of the natriuretic compound. In a preferred embodiment, the degradation of the biologically active agent component of the natriuretic compound is less than the degradation of unmodified (unconjugated) biologically active natriuretic compound, at a pH of about 2 for less than about 2 hours. The natriuretic compound component of the natriuretic compound can, for example, be more stable as a component of the natriuretic compound conjugates than the unconjugated natriuretic compound in the presence of plasma, proteases, liver homogenate, acidic conditions and/or basic conditions.

[19] Natriuretic peptide conjugates of the invention may induce the anti-hypertensive, cardiovascular, renal, and/or endocrine effects that are associated with the native peptide. In some embodiments, the modification of the natriuretic peptide will protect the peptide, such as hBNP, from proteolysis and facilitate delivery into the systemic circulation through the gut wall, resulting in natriuresis, diuresis, and/or vasodilation. Natriuretic peptide conjugates of the invention can therefore be effectively delivered as an oral

formulation (instead of by continuous intravenous infusion for days in a hospital setting). This advantage is expected to reduce hospital costs associated with other CHF therapies by enabling self administration, which has not heretofore been possible, and is expected to expand the therapeutic use of natriuretic peptide, especially hBNP, to include early stage (e.g., class 1) and chronic CHF as well as acute CHF. A preferred embodiment of the present invention is a non-immunogenic peptide conjugate that has increased resistance to degradative enzymes and is suitable for oral delivery and transport across the intestinal epithelium.

[20] The invention also provides several methods for the preparation of the natriuretic compound conjugates. These modifying moieties, can for example, take the form of linear and branched PAG or other polymeric structure.

## 6. Definitions

[21] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. Headers are used for the convenience of the reader and are also not intended to be limiting of the invention. All publications, patent applications, patents, and other references mentioned herein are incorporated herein by reference in their entirety, as are the package inserts of any branded drugs referred to herein by their brand names.

[22] Singular forms "a", "an", and "the", are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[23] As used in the specification and the claims set forth herein, the following terms have the meanings indicated:

[24] "Amino acid" is defined herein as any naturally occurring, artificial, or synthetic amino acid in either its L or D stereoisomeric forms, unless otherwise specified. The term "residue" is used interchangeably with the term "amino acid", and is often designated as having a particular position in a given sequence of amino acids.

[25] All amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. § 1.822(b). The following one-letter amino acid designations are used in the description of the present invention. Xaa is used to designate an unknown or undesignated amino acid. The integers above specific residues of the structure provided herein define the residue position number. This residue number is used in conjunction with the one letter amino acid nomenclature, described below, to designate the residue at which a substitution is made in the natriuretic peptide analogs of, for example, hBNP and ANP.

[26] Thus for example, when a mutant hBNP is synthesized in which arginine (R) replaces lysine (K) at residue position number 3 of wild-type hBNP, the nomenclature "BNPK3R" or "hBNP(1-32)K3R" is used. Multiple substitutions are designated in the same manner with a comma separating each substitution as exemplified below.

[27] The term "hBNP(1-32)K3R, K14R, K27R" designates a triple mutant hBNP having that hBNP sequence defined above with the substitution of arginine for lysine at residue position 3 (i.e. K3R), the substitution of arginine for lysine at residue position 14 (i.e. K14R), and the substitution of arginine for lysine at position 27 (i.e. K27R). Other mutants are defined in an analogous manner.

[28] The term "hBNP(1-32)K3R, K14R" designates a double mutant having the lysine replaced with arginine at residue 3 and 14 of hBNP.

| | | |
|---|---|---|
| A=ala= alanine | L=leu= leucine | nle= Norleucine |
| R=arg= arginine | K=lys= lysine | cha= cyclohexylalanine |
| N=asn= asparagine | M=met= methionine | A*=har= hemoarginine |
| D=asp= aspartic acid | F=phe= phenylalanine | orn = ornithine |
| C=cys= cysteine | P=pro= proline | pen = penicillamine |
| Q=gln= glutamine | S=ser= serine | phg = phenyl glycine |
| E=glu= glutamic acid | T=thr= threonine | mpa = mercaptopropionic acid |
| G=gly= glycine | W=trp = tryptophan | a =ala* =D alanine |
| H=his= histidine | Y=tyr= tyrosine | C*= hemocysteine |
| I=ile= isoleucine | V=val=valine | |

[29] "Amphiphilic" means the ability to dissolve in both water and lipids and/or having hydrophilic and lipophilic characteristics, and the terms "amphiphilic moiety" and "amphiphile" mean a moiety which is amphiphilic and/or which, when attached to a polypeptide or non-polypeptide drug, increases the amphiphilicity of the resulting conjugate, e.g., PEG-fatty acid oligomer, sugar fatty acid oligomer.

[30] "Biologically active" refers to an agent having therapeutic or pharmacologic activity, such as an agonist, partial agonist or antagonist.

[31] "Effective amount" as provided herein refers to a nontoxic but sufficient amount to provide the desired therapeutic effect. As will be pointed out below, the exact amount required will vary from subject to subject, depending on age, general condition of the subject, the severity of the condition being treated, the particular biologically active agent administered, and the like. An appropriate "effective" amount in any

individual case may be determined by one of ordinary skill in the art by reference to the pertinent texts and literature and/or by using routine experimentation.

[32] "Hydrolyzable" refers to molecular bonds which are subject to hydrolysis under physiological conditions.

[33] "Hydrophilic" means the ability to dissolve in water, and the term "hydrophilic moiety" or "hydrophile" refers to a moiety which is hydrophilic and/or which when attached to another chemical entity, increases the hydrophilicity of such chemical entity. Examples include, but are not limited to, sugars and polyalkylene moieties such as polyethylene glycol.

[34] "Lipophilic" means having an affinity for fat, such as chemicals that accumulate in fat and fatty tissues, the ability to dissolve in lipids and/or the ability to penetrate, interact with and/or traverse biological membranes, and the term, "lipophilic moiety" or "lipophile" means a moiety which is lipophilic and/or which, when attached to another chemical entity, increases the lipophilicity of such chemical entity.

[35] "Lower alkyl" refers to substituted or unsubstituted alkyl moieties having 1, 2, 3, 4, 5, or 6 carbon atoms.

[36] "Monodispersed" refers to a mixture of compounds wherein about 100 percent of the compounds in the mixture have the same molecular weight.

[37] "Pharmaceutically acceptable" with respect to a component, such as a salt, carrier, excipient or diluent of a composition according to the present invention is a component that is compatible with the other ingredients of the composition, in that it can be combined with the natriuretic compound conjugates of the present invention without eliminating the biological activity of the biologically active agent and is suitable for use with subjects as provided herein without undue adverse side effects (such as toxicity, irritation, and allergic response). Side effects are "undue" when their risk outweighs the benefit provided by the pharmaceutical composition. Examples of pharmaceutically acceptable components include, without limitation, any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, microemulsions and various types of wetting agents.

[38] "Polyalkylene glycol" or PAG refers to linear or branched polyalkylene glycol polymers such as polyethylene glycol (PEG), polypropylene glycol (PPG), and polybutylene glycol (PBG), and combinations thereof (e.g., linear or branched polymers including combinations of two or more different PAG subunits, such as two or more different PAG units selected from PEG, PPG, PPG, and PBG subunits), and includes the monoalkylether of the polyalkylene glycol. In a particular embodiment, the polyalkylene glycol is polyethylene glycol or "PEG." The term "PEG subunit" refers to a single polyethylene glycol unit, i.e., -($CH_2CH_2O$)-. The term "PPG subunit" refers to a single polypropylene glycol unit, i.e., -($CH_2CH_2CH_2O$)-.

The term "PBG subunit" refers to a single polypropylene glycol unit, i.e., -($CH_2CH_2CH_2CH_2O$)-. PAG subunits may also include alkyl side chains, such as methyl, ethyl or propyl side chains.

[39] "Prodrug" refers to a biologically active agent that has been chemically derivitized such that, upon administration to a subject, the prodrug is metabolized to yield the biologically active agent.

[40] "Treat" or "treating" as used herein refers to any type of treatment that imparts a benefit to a subject afflicted with a disease or illness, including improvement in the condition of the subject (*e.g.,* in one or more symptoms), delay in the progression of the condition, prevention or delay of the onset of the disease or illness, enhancement of normal physiological functionality, etc.

## 7. Brief Description of the Drawings

[41] Figure 1 – illustrates the mode of action and the regulation of BNP.

[42] Figure 2 – A representative scheme for oligomer activation and conjugation following a three-tiered conjugation strategy. Class 1 modifying moieties are non-hydrolysable, Class 2 modifying moieties are microPAGylated, and Class 3 modifying moieties are fully hydrolysable.

[43] Figure 3 – Cyclic GMP production of HAEC cells as a function of concentration of hBNP or hBNP conjugate. (¦ = Native, ? = BN-002, ? = BN-021, ? = BN-022, ? = BN-024)

[44] Figure 4 – BNP and BNP conjugate trypsin digestion. (-?- = Time (min) vs % BN D1, •?• = Time (min) vs % BND2, ? = Time (min) vs % BND2, ? = Time (min) vs % BN034 D1, ¦ = Time (min) vs % BN034 D2)

[45] Figure 5 – Plasma levels of hBNP conjugates at various times after oral dosing in rats. (¦ = BN-002, ? = BN-021, ? = BN-022, ? = BN-024)

[46] Figures 6a-d – Data showing structure and results of work-up for conjugates in Classes 1, 2 and 3.

## 8. Detailed Description of the Invention

[47] Natriuretic compound conjugates according to some embodiments of the present invention comprise a natriuretic compound that includes a natriuretic peptide receptor A binding motif (NPR-A), at least one modifying moiety conjugation site, and at least one modifying moiety attached to said modifying moiety conjugation site. By virtue of the modifying moiety attached to said natriuretic compound as part of the conjugate, the natriuretic compound conjugate can have modified hydrophilic characteristics related to the native natriuretic compound that does not include a modifying moiety as described herein. By way of example and not limitation, and as described more fully herein, the modifying moiety may take the form of an oligomer of any variety of sizes, shapes, substitutions, and configurations.

### 8.1 Natriuretic Compound

[48] The natriuretic compound conjugates of the invention include a natriuretic compound which includes a binding site for a natriuretic peptide receptor, such as NPR-A, as well as a conjugation site for coupling a modifying moiety thereto.

#### 8.1.1 Native Natriuretic Peptide

[49] The natriuretic compound may have the amino acid sequence of a native natriuretic peptide such as ANP, BNP, CNP or DNP, urodilatin, from any of a variety of species, such as humans, canines, and rats. Preferred native natriuretic peptides are human BNP, rat BNP , canine BNP, or hANP. Native sequences are also intended to include pro-natriuretic peptides and pre-pro peptides.

#### 8.1.2 Natriuretic Compound Analogs

[50] The natriuretic compound may also be a biologically active analog of a native natriuretic peptide (a natriuretic analog). For example, a biologically active analog can be a native natriuretic compound with truncations, deletions, insertions, substitutions, replacements, side chain extensions, and fusion proteins, or combinations of the foregoing which do not eliminate the biological activity of the original compound. Preferably, the analog will include a native or artificial NPR-A binding motif and will retain some or all of the activity for binding NPR-A.

[51] Natriuretic polypeptide analogs can be obtained by various means. For example, certain amino acids can be substituted for other amino acids in the native natriuretic peptide structure without eliminating interactive binding capacity. In some cases, as have been described in the art, such modifications have resulted in increased affinity for NPR-A, relative to NPR-C, the clearance receptor, resulting in extended half life.

[52] Preferably, the analog will include a natriuretic molecule binding motif, such as an NPR-A binding motif.

[53] The natriuretic peptide may, for example, be defined by the sequence:

[54] CFGRXMDRISSSSGLGC (SEQ ID NO 2),

[55] wherein X is a compound, such as an amino acid residue, including a modifying moiety conjugation site. X in some embodiments comprises an amino acid to which a modifying moiety may attach. For example, X may comprise the amino acid Lys or Cys to which a modifying moiety may be attached. In another embodiment, X may be other than lysine; In these embodiments, the unconjugated peptide is also an aspect of the invention where X is arginine or and amino acid other then lysine to which a conjugation site may be created.

[56] An alternative structure for these embodiments is:

[57] CFGR$X^1$MDRI$X^2$GLGC (SEQ ID NO.__),

[58] where $X^1$ is lysine, $X^2$ is one to four amino acids. $X^2$ may be S, SS, SSS, SSSS, K, KS, KSS, or KSSS. Where K is included as $X^2$ or part of the sequences of $X^2$, a modifying moiety conjugation site.

[59] The natriuretic compound may, for example, have the structure:

[60] $X^1$-CFGR$X^3$MDRISSSSGLGC-$X^2$ (SEQ ID NO.__),

[61] wherein at least one of $X^1$ and $X^2$ is present, $X^1$ is a peptide of from 1 to 10 amino acids, wherein $X^2$ is a peptide of 1, 2, 3, 4, 5, or 6 amino acids, and wherein $X^3$ is other than lysine, such as arginine. For example, $X^1$ may include all or a C-terminal fragment of the 1-10 amino acid residue sequence from the N-terminus of hBNP. In one embodiment, $X^1$ includes SP$Z^1$MVQGSG- (SEQ ID NO: ), SP$Z^1$MVQG, SP$Z^1$MVQ, SP$Z^1$MV, SP$Z^1$M, SP$Z^1$, P$Z^1$MVQGSG, $Z^1$MVQGSG, where $Z^1$ is lysine or arginine. Where $Z^1$ is lysine, a modifying moiety conjugation site is provided. In another embodiment, $X^2$ includes all or an N-terminal fragment of the 1-6 amino acid residue sequence from the C-terminus of hBNP. In one embodiment, $X^2$ is sequence $Z^2$VLRRH (SEQ. ID. NO: ), $Z^2$VLRR(SEQ. ID. NO: ), $Z^2$VLR (SEQ. ID. NO: ), $Z^2$VL, K, R, RVLRR (SEQ. ID. NO: ), RVLR (SEQ. ID. NO: ), RVL, RV, or R, where $Z^2$ can be lysine or arginine. Where $Z^2$ is lysine, a modifying moiety conjugation site is provided. Where $Z^1$ is lysine, $Z^2$ may be other than lysine, and where $Z^2$ is lysine, $Z^1$ may be other than lysine. Alternatively, $X^1$ and $X^2$ may be any N-terminal and C-terminal tail amino acid sequence obtained from any natriuretic peptide. In some embodiments, an N-terminal and/or C-terminal tail sequence is present and is specifically not the N-terminal and C-terminal tails sequence of hBNP or any fragment thereof. It will be appreciated that the unconjugated natriuretic compound is also an aspect of the invention.

[62] In one embodiment, the natriuretic compound analog comprises an amino acid sequence:

[63] $X^1$MVQGSG$C^1$FGR$X^2$MDRISSSSGLG$C^2X^3$ (SEQ ID NO. __),

[64] wherein $X^1$, $X^2$ and $X^3$ are each independently selected from the group consisting of Lys and amino acids other than Lys, and wherein at least one of $X^1$, $X^2$ and $X^3$ is Lys and at least one of $X^1$, $X^2$ and $X^3$ is an amino acid other than Lys; and

[65] $C^1$ and $C^2$ are cysteines and may be coupled by a disulfide bond. It will be appreciated that the unconjugated peptide analog is also an aspect of the invention.

[66] In one embodiment, at least one of $X^1$, $X^2$ and $X^3$ is Arg. In another embodiment, $X^1$ is Lys, $X^2$ is Arg and $X^3$ is Arg. This embodiment may also include an amino acid sequence as described herein, N-

terminal to $X^1$ and/or C-terminal to $X^3$. For example, the N-terminal tail sequence, when present, may be S- or SP-, and the C-terminal tail, when present, may be -VLRRH, -VLRR, -VLR, -VL, or -V. In some embodiments, the N-terminal and/or C-terminal tail sequence is present and is specifically not N-terminal and C-terminal tail of hBNP or a fragment thereof.

[67] In another embodiment, the natriuretic peptide analog includes an amino acid sequence:

[68] $CFGRX^1MDRISSSSGLGCX^2$ (SEQ ID NO: ),

[69] wherein at least one of $X^1$ and $X^2$ is an amino acid comprising a modifying moiety conjugation site coupled to the modifying moiety and the other is any other amino acid or an unconjugated Lys. In one embodiment, $X^1$ is Lys coupled at its side chain to the modifying moiety and $X^2$ is another amino acid, for example Gly or Arg. Alternatively, $X^2$ is Lys coupled at its side chain to the modifying moiety and $X^1$ is another amino acid, for example Gly, Arg, or an amino acid other than lysine. In another embodiment, $X^1$ is Lys coupled at its side chain to the modifying moiety and $X^2$ is an unconjugated Lys. Alternatively, $X^2$ is Lys coupled at its side chain to the modifying moiety and $X^1$ is an unconjugated Lys. It will be appreciated that the unconjugated peptide is also an aspect of the invention.

[70] Virtually any natriuretic peptide may be modified according to the present invention. By way of example peptide/proteins that are suitable candidates for modification are described in PCTUS0217567, which is specifically incorporated herein by reference. BNP, for example, includes Lys residues in the native sequence that preferably serve as the conjugation sites for the oligomer. In some embodiments of the present invention in which BNP is the native peptide, it may be desirable to remove any conjugation sites from the binding region of the peptide or to eliminate a binding site. Where it is desired that an oligomer not attach at a particular Lys residue of the peptide sequence, the Lys may be replaced with another amino acid, such as arginine. For example, conjugation with non-hydrolysable oligomers in this region can be detrimental to activity, though the applicants have surprisingly discovered that conjugation at $Lys^{14}$ results in a significant amount of retained activity. Thus, it may be desirable to replace such conjugation sites with amino acids that have similar chemical properties but are not readily conjugated. For example, in the hBNP sequence, the $Lys^{14}$ may be substituted with Arg, and thereby favor conjugation of the peptide at the $Lys^3$ of the peptide sequence for native BNP. Amino acid substitutions can be selected to replace Lys with an amino acid that is not readily conjugatable.

[71] In some cases, it may be desirable to add an additional site for conjugation. For example, in some embodiments, a positively charged amino acid residue is replaced with a Lys residue, for example, in the ANP peptide (native sequence), $Arg^{27}$ can be replaced with Lys.

[72] Mutations to add a conjugation site can be selected so that mutation and conjugation do not eliminate the activity of the resulting peptide conjugate, and in particular it's affinity for NPR-A. In one

embodiment, the natriuretic compound is defined as the native hBNP amino acid sequence with one or more Lys residues are inserted within the hBNP sequence and/or added to an end of the hBNP sequence, and/or one or more native Lys residues deleted or replaced with conservative substitutions. Preferably such substitution or insertion is in one or more of the tail amino acid sequences of the natriuretic peptide.

[73] The conjugation site may in some embodiments be inserted, replaced or added at or near the N-terminal tail, e.g., an insertion or substitution within the N-terminal tail amino acid sequence, preferably at the N-terminus, or positioned 1, 2, 3, 4 or 5 amino acids from the N-terminus, or alternatively, positioned 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acids in an N-terminal direction from the N-terminal Cys that forms a part of the Cys bridge creating the loop. In a preferred embodiment, the natriuretic compound is defined as the native hBNP sequence with one or more mutations selected from the group consisting of $Lys_3 \rightarrow Arg$, $Lys_{14} \rightarrow Arg$, $Arg_{30} \rightarrow Lys$, and $Lys_{27} \rightarrow Arg$, which one or more mutations do not eliminate the biological activity of the natriuretic peptide compound. Addition of more than one modifying moiety, such as an oligomer, may improve enzyme stability and/or enhance absorption.

[74] Many of the natriuretic peptide analogs will include the loop component of a native natriuretic peptide, such as $Cys_{10}$-$Cys_{26}$ of hBNP in which $Cys_{10}$ and $Cys_{26}$ are coupled by a disulfide bond thereby forming a loop. In some cases, the loop may include substitutions, deletions, and/or insertions of amino acids differing from the native sequences, so long as such substitutions, deletions, and/or insertions do not eliminate the activity of the native sequences. Examples of such altered loop sequences can be found in Schoenfelda et al., "Mutations in B-type natriuretic peptide mediating receptor-A selectivity," FEBS Letters 414 (1997) 263-267, the entire disclosure of which is incorporated herein by reference, describes variants of BNP that preferentially bind natriuretic peptide receptor-A (NPR-A) compared to receptor-C (NPR-C). (U.S. Patent 6,525,022 and U.S. Patent 6,028,055). As an example, the natriuretic loop may include a native loop having one or more conservative substitutions which do not eliminate the natriuretic activity of the loop, e.g., in some cases that loop will have the sequence of the native loop (e.g., the native loop of hBNP) and have 1, 2, 3, 4, 5, 6, 7 or 8 conservative substitutions. In another embodiment the loop is shortened by removing a set of amino acids that does not eliminate biological activity. In one embodiment, the peptide analog includes the $Cys_{10}$-$Cys_{26}$ loop of hBNP in which Lys14 is replaced with Gly or Arg. In another embodiment, the SSSS component of the loop is altered or deleted.

[75] In addition, the natriuretic peptide loops or analogs of the native loops may include an N-terminal tail and/or a C-terminal tail, such as the tails of native natriuretic peptides, e.g., $hBNP_{1-9}$ and $hBNP_{27-32}$. The tails are single amino acids or peptides that do not eliminate biological activity. In some cases, the tails may include substitutions, deletions, and/or insertions of amino acids differing from the native sequences, so long as such substitutions, deletions, and/or insertions do not eliminate the beneficial activity of the native sequences. In one embodiment the tail or tails are based on native sequences, but truncated by one or more amino acids. For example, the N-terminal tail, when present, may selected from the following hBNP

segments 8-9, 7-9, 6-9, 5-9, 4-9, 3-9, 2-9, and 1-9; and any of the foregoing segments in which one or more Lys residues is replaced with a Gly or Arg residue. Similarly, a C-terminal tail, when present, may be selected from: hBNP segments 27-28, 27-29, 27-30, 27-31, and 27-32; and any of the foregoing hBNP segments wherein one or more Lys residues is replaced with a Gly or an Arg residue. Examples of preferred loop-plus-tail natriuretic peptides include hBNP segment 1-29; hBNP segment 1-26; and either of the foregoing hBNP segments in which one or more Lys residues are replaced with a Gly or an Arg.

[76] In addition to the foregoing analogs, a wide variety of analogs suitable for use in the invention have been described in the art. For example U.S. Patent 5,114,923, issued May 19, 1992, the entire disclosure of which is incorporated herein by reference, describes a peptide having natriuretic activity of the formula $R^1$-Cys-Phe-Gly-Arg-Lys-Met-Asp-Arg-Ile-Ser-Ser-Ser-Ser-Gly-Leu-Gly-Cys-$R^2$ wherein $R^1$ is selected from (H); Gly-; Ser-Gly-; Gly-Ser-Gly-; Gln-Gly-Ser-Gly-; Val-Gln-Gly-Ser-Gly-; Met-Val-Gln-Gly-Ser-Gly-; Lys-Met-Val-Gln-Gly-Ser-Gly-; Pro-Lys-Met-Val-Gln-Gly-Ser-Gly; Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly-; and $R^3$-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly- wherein $R^3$ is the 102 amino acid sequence of positions 1-99 for the human protein or a C-terminal portion thereof, and $R^2$ is (OH), NH2, NHR' or wherein the modifying moiety' and the modifying moiety" are independently lower alkyl (1-4C) or R2 is Lys; Lys-Val; Lys-Val-Leu; Lys-Val-Leu-Arg; Lys-Val-Leu-Arg-Arg; Lys-Val-Leu-Arg-Arg-His; or the amides ($NH_2$, NHR' or NR' the modifying moiety") thereof.

[77] U.S. Patent, 4,904,763, issued February 2, 1990, the entire disclosure of which is incorporated herein by reference, also describes natriuretic peptide analogs suitable for use in the present invention, such as X-Cys Phe Gly Arg Lys Met Asp Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His-OH, wherein X is H, H-Gly-Ser-Gly-, or H-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly.

[78] U.S. Patent 4,904,763, issued February 27, 1990 (the entire disclosure of which is incorporated herein by reference) describes other natriuretic peptide analogs suitable for use in the present invention, including X-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg-Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys-Y (where the 2 cysteines are bridged by a disulfide bond) wherein X means H or H-Asp-Ser.-Gly- and Y denotes -Asn-Val-Leu-Arg-Arg-Tyr-OH, -Asn-Val-Leu-Arg-Arg-OH, -Asn-Val-Leu-Arg-Tyr-OH, -Asn-Val-Leu-Arg-OH, -Asn-Val-Leu-OH or Asn-Ser-Phe-Arg-Tyr-OH, or a salt thereof. Another set of analogs suitable for use in PCT Publication No. WO8912069, published December 14, 1989.

[79] A further set of natriuretic peptide analogs suitable for use in the present invention is described in U.S. Patent Publication No. 20030109430, published on June 12, 2003, the entire disclosure of which is incorporated herein by reference. This publication describes a peptide having natriuretic activity of the formula: $R^1$-Cys-Phe-Gly-Arg-Arg/Lys-Leu/Met-Asp-Arg-Ile-Lys-Met-Gly/Ser-Ser-Leu/Ser-Ser-Gly-Leu-Gly-Cys-R2, wherein $R^1$ is selected from the group consisting of: (H); Gly-; Ser-Gly-; Asp/Lys/Gly-Ser-Gly-; Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; Met/Val-Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; Thr/Met-Met/Val-

Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; Lys-Thr/Met-Met/Val-Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; Pro-Lys-Thr/Met-Met/Val-Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; Ser-Pro-Lys-Thr/Met-Met/Val-Arg/His/Gln-Asp/Lys/Gly-Ser-Gly-; or a 10 to 109-amino acid sequence of the native upstream sequence for porcine, canine or human BNP, or a composite thereof; $R^2$ is (OH), NH2, or NR'R" wherein R' and R" are independently lower alkyl (in this case, 1-4 C) or is Asn/Lys; Asn/Lys-Val; Asn/Lys-Val-Leu; Asn/Lys-Val-Leu-Arg; Asn/Lys-Val-Leu-Arg-Arg/Lys; Asn/Lys-Val-Leu-Arg-Arg/Lys-Tyr/His; or the amides ($NH_2$ or NR'R") thereof, with the proviso that if the formula is $R^1$-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg- Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys-$R^2$, and $R^1$ is Asp-Ser-Gly-, $R^2$ cannot be Asn-Val-Leu-Arg-Arg-Tyr.

[80] Still another set of analogs is described in Scios, European Patent EP0542863B1, issued November 26, 1997, which describes a fusion protein which comprises from N-terminal to C-terminal: a carrier protein of about 10 to about 50 kDa which does not contain Glu residues or Asp-Gly sequences as a Staph V8 cleavage site; a Staph V8 cleavage comprising a Glu residue or Asp-Gly sequence positioned at the C-terminal of said carrier; and ; and a peptide not containing a Staph V8 cleavage site fused to said cleavage site; wherein said fusion protein exhibits a pI of about 8.0 or greater. The patent also describes the use of an N-terminal leader of 6 to 20 amino acids.

[81] Other natriuretic peptide analogs suitable for use in the present invention are described in Daiichi's U.S. Patent Publication No. 20020086843, published on July 4, 2002 (the entire disclosure of which is incorporated herein by reference), which describes a physiologically active polypeptide X-Cys-Phe-Gly-Arg-Lys-Met-Asp-Arg-Ile-Ser-Ser-Ser-Ser-Gly-Leu-Gly-Cys-Lys-Val-Leu-Arg-Arg-His-OH [where the 2 cysteines are bridged] wherein X is H, H-Gly-Ser-Gly-, or H-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly-.

[82] In making such substitutions, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant polypeptide, which in turn defines the interaction of the protein with other molecules. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagines (-3.5); Lys (-3.9); and Arg (-4.5). As will be understood by those skilled in the art, certain amino acids can be substituted by other amino acids having a similar hydropathic index or score and still result in a polypeptide with similar biological activity, *i.e.*, still obtain a biological functionally equivalent polypeptide. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 of each other is preferred, those which are within ±1 of each other are particularly preferred, and those within ±0.5 of each other are even more particularly preferred.

[83] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, the disclosure of which is incorporated herein in its entirety, provides that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correletates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: Arg (+3.0); Lys (±3.0); aspartate (±3.0 ± 1); glutamate (±3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). As is understood by those skilled in the art, an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 of each other is preferred, those which are within ±1 of each other are particularly preferred, and those within ±0.5 of each other are even more particularly preferred.

[84] As outlined above, amino acid substitutions/insertions can be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions (*i.e.*, amino acids that can be interchanged without significantly altering the biological activity of the polypeptide) that take various of the foregoing characteristics into consideration are well known to those skill in the art and include, for example Arg and Lys; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

[85] As will be understood by those skilled in the art, natriuretic peptide (e.g., BNP) analogs can be prepared by a variety of recognized peptide synthesis techniques including, but not limited to, classical (solution) methods, solid phase methods, semi-synthetic methods, and recombinant DNA methods.

### 8.1.3 Multi-BNP Peptide

[86] The natriuretic compound may also be a multipeptide having two or more natriuretic compound units in sequence and optionally including spacer sequences between the natriuretic compound units. The compounds may also optionally comprise a leader and/or extension sequence at either or both ends of the natriuretic peptide compound. For example, by way of example and not limiting and without limiting the structure and/or formula of each multipeptide may, have the following structures:

$NP\text{-}[NP]_n$;

$NP\text{-}[Spacer\text{-}NP]_n$;

$Leader\text{-}NP\text{-}[NP]_n$;

$Leader\text{-}NP\text{-}[Spacer\text{-}NP]_n$;

Leader-[Spacer-NP]$_n$;

Leader-[Spacer-NP]$_n$-Extension;

Leader-NP-[Spacer-NP]$_n$-Extension;

[87] where

[88] n may, for example be 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10;

[89] NP is a natriuretic peptide or natriuretic peptide analog;

[90] Spacer may be an amino acid residue or a series of amino acid residues that are cleaved by an enzyme or enzyme cocktail, preferably an amino acid residue or sequence of amino acids not present in NP (e.g., Glu-Asp-Ala-Gly-Glu; Arg-Thr-Arg-Arg; Arg-X-Lys-Arg; Arg-Val; Asp-Lys; Lys-Ile; Arg-Thr; Arg-Ile);

[91] Leader may for example be a single amino acid, an amino acid sequence, a peptide (e.g., leader peptide or signal peptide), or a protein; and Leader is selected to block the N-terminus from conjugation, assists in purification of the multipeptide (e.g., $(His)_6$-Ala-Asp-Gly-Glu – cleavable by enzyme cocktail: V8 protease (endoproteinase Glu-C), (His)6-Ala-Asp-Arg-Thr-Arg-Arg- or (His)6-Ala-Asp-Arg-X-Lys-Arg where X can be any amino acid or (His)6-Ala-Asp-Arg-Glu-Arg-Arg -cleavable by Furin; (His)6-Ala-Asp-Arg-Val-cleavable by Urokinase; (His)6-Ala-Asp-Lys or (His)6-Ala-Asp-Lys-Ile-Cleavable by Enterokinase; (His)6-Ala-Asp-Arg-Thr or (His)6-Ala-Asp-Arg-Ile-Cleavable by Factor Xa or Factor 10a), improves solubility and/or assists in excretion from the cell (e.g., Ala-Asp-Gly-Glu), and/or purification (e.g., $(His)_6$-Ala-Asp-Gly-Glu); and Leader is preferably cleavable from the multipeptide by enzymatic or chemical cleavage, preferably by an enzyme that would not cleave the NP; the Leader can be a leader peptide from a native NP;

[92] Extension may for example be a single amino acid, an amino acid sequence, a peptide (e.g., leader peptide or signal peptide), or a protein; and Extension is selected to block the C-terminus from conjugation, assist in purification of the multipeptide (e.g., $(His)_6$-Ala-Asp-Gly-Glu), improves solubility, and/or assists in excretion from the cell, (e.g., Ala-Asp-Gly-Glu – cleavable by enzyme cocktail: V8 protease (endoproteinase Glu-C) and endoproteinase (Asp-N), Arg-Thr-Arg-Arg- or Arg-X-Lys-Arg where X can be any amino acid or Arg-Glu-Arg-Arg -cleavable by Furin; Arg-Val-cleavable by enzyme cocktail Urokinase and carboxypeptidase B; Asp-Lys or Lys-Ile-Cleavable by Enterokinase; Arg-Thr or Arg-Ile-Cleavable by Factor Xa or Factor 10a); and Extension is preferably cleavable from the multipeptide by enzymatic or chemical cleavage preferably by an enzyme that would not cleave the NP.

[93] Enzyme cleavage sites, such those included in the Spacer, or separating the leader and/or extension from the NP, are preferably selected so that a single enzyme or enzyme cocktail will cleave all of the cleavage sites. For example, the cleavage site Arg-Thr-Arg-Arg can be cleaved by Furin.

[94] In one embodiment, Leader is $(His)_6$-Ala-Asp-Gly-Glu, Extension is $(His)_6$-Ala-Asp-Gly-Glu, Spacer is GluAla-Asp-Gly-Glu. This embodiment can be cleaved by V8 protease (endoproteinase Glu-C). The resulting product is NP-Glu or NP-Glu conjugate.

[95] In another embodiment, Leader is $(His)_6$-Ala-Asp-Gly-(-Arg-Thr-Arg-Arg or Arg-X-Lys-Arg) (where X is be any amino acid), Extension is $(His)_6$-Ala-Asp-Gly-(-Arg-Thr-Arg-Arg or Arg-X-Lys-Arg) (where X can be any amino acid), and Spacer is Arg-Thr-Arg-Arg or Arg-X-Lys-Arg (where X is any amino acid). This embodiment can be cleaved by Furin. The resulting product is NP or NP conjugate.

[96] In another embodiment, Leader is $(His)_6$-Ala-Asp-Gly-Arg-Val, Extension is $(His)_6$-Ala-Asp-Gly-Arg-Val, and Spacer is Arg-Val. This embodiment can be cleaved by a Urokinase/Carboxypeptidase B cocktail. The resulting product is NP or NP conjugate.

[97] In another embodiment, Leader is $(His)_6$-Ala-Asp-Gly-(-Asp-Lys or -Lys-Ile), Extension is $(His)_6$-Ala-Asp-Gly-(-Asp-Lys or -Lys-Ile), and Spacer is Asp-Lys or Lys-Ile). This embodiment can be cleaved by a Enterokinase. The resulting product is NP or NP conjugate.

[98] In another embodiment, Leader is $(His)_6$-Ala-Asp-Gly-(-Arg-Thr or -Arg-Ile), Extension is $(His)_6$-Ala-Asp-Gly-(-Arg-Thr or -Arg-Ile), and Spacer is Arg-Thr or Arg-Ile. This embodiment can be cleaved by a Factor Xa or Factor 10a / Carboxypeptidase B enzyme cocktail. The resulting product is NP or NP conjugate.

[99] In another embodiment, Spacer is Arg-Arg-Asp-Ala-Glu-Asp-Pro-Arg, Leader is Glu-Gly-Asp-Arg-Arg, and Extension is $(His)_6$-Glu-Gly-Asp-Arg-Arg. In this embodiment, the NP or NP conjugate can be released using a trypsin and carboxypeptidse B enzyme cocktail under controlled conditions.

[100] In another embodiment, Spacer is Arg-Arg-Asp-Ala-Glu-Asp-Arg-Arg, Leader is Glu-Gly-Asp-Arg-Arg, Extension is $(His)_6$-AAA-Glu-Gly-Asp-Arg-Arg, where AAA is an amino acid sequence from 3 to 40 amino acid residue in length, preferably 3-15. In this embodiment, the NP or NP conjugate can be released using a trypsin and carboxypeptidse B enzyme cocktail under controlled conditions.

[101] In another embodiment, Spacer is Arg-Gly-Asp-Ala-Glu-Asp-Pro-Arg, Leader is Glu-Gly-Asp-Pro-Arg, and Extension is $(His)_6$-Glu-Gly-Asp-Pro-Arg. In this embodiment, the NP or NP conjugate can be released using a thrombine and Carboxypeptidse B enzyme cocktail.

[102] In another embodiment, Spacer is Ala-Arg-Gly-Asp-Ala-Glu-Asp-Pro-Arg, Leader is Glu-Gly-Asp-Pro-Arg, and Extension is $(His)_6$-Glu-Gly-Asp-Pro-Arg. In this embodiment, the NP or NP conjugate can be released using a thrombine and carboxypeptidse A enzyme cocktail.

[103] In another embodiment, Spacer is Met-Met, Leader is Met-Met, and extension is $(His)_6$-AAA-Met-Met, where AAA is any amino acid sequence from 3 to 40 amino acid residues in length. In this embodiment, the NP or NP conjugate can be released using CNBr.

[104] In another embodiment, Spacer is Asp-Asp-Ala-Gly-Glu, Leader is Ala-Asp-Gly-Glu, and Extension is $(His)_6$-Ala-Asp-Gly-Glu. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) and endoproteinase Asp-N coctail.

[105] In another embodiment, Spacer is Glu-Ala-Gly-Glu, Leader is Ala-Asp-Gly-Glu, and Extension is $(His)_6$-Ala-Asp-Gly-Glu. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) to yield NP-Glu, a novel NP analog or a conjugate of NP Glu.

[106] In another embodiment, Spacer is Glu-Glu, Leader is Glu-Gly-Asp-Ala at the C-terminus and Extension is Glu-Gly-Asp-Ala$(His)_6$-Glu, where the C-terminus is linked with a fusion partner, an appropriate fusion protein, which can, for example, be cleavable via enterokinase. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) to yield NP-Glu, a novel NP analog or a conjugate of NP Glu.

[107] In another embodiment, Spacer is Glu-Glu, Leader is Ala-Asp-Gly-Glu and Extension is $(His)_6$-Ala-Asp-Gly-Glu where the N-terminus is linked with a fusion partner, an appropriate fusion protein, which can, for example, be cleavable via enterokinase. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) to yield NP-Glu, a novel NP analog or a conjugate of NP Glu.

[108] In another embodiment, Spacer is Glu-Glu, Leader is Glu-Gly-Asp-Ala-Glu and the Extension is Glu-Gly-Asp-Ala-Glu, and the C-terminus is linked with a fusion partner, an appropriate fusion protein. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) to yield NP-Glu, a novel NP analog, or a conjugate of NP Glu.

[109] In another embodiment, Spacer is Glu-Glu, Leader is Ala-Asp-Gly-Glu and Extension is Glu-$(His)_6$-Ala-Asp-Gly-Glu where the N-terminus is linked with a fusion partner, an appropriate fusion protein. In this embodiment, the NP or NP conjugate can be released using a V8 protease (endoproteinase Glu-C) to yield NP-Glu, a novel NP analog or a conjugate of NP Glu.

### 8.2 Modifying Moieties

[110] Modifying moieties are moieties that modify the natriuretic compound, such as a BNP peptide compound, and provide the compound with desired properties as described herein. For example, the modifying moiety can reduce the rate of degradation of the natriuretic compound in various environments (such as the GI tract, and/or the bloodstream), such that less of the natriuretic compound is degraded in the modified form than would be degraded in the absence of the modifying moiety in such environments. Preferred modifying moieties are those which permit the natriuretic compound conjugate to retain a therapeutically significant percentage of the biological activity of the parent natriuretic compound.

#### 8.2.1 Moieties that Effect Stability, Solubility, and/or Biological Activity

[111] There are numerous moieties that can be attached to the natriuretic compound to form the natriuretic compound conjugates described herein that modify the stability, solubility, and/or biological activity of the parent natriuretic compound. Examples include hydrophilic polymers or oligomers, amphiphilic polymers or oligomers, and lipophilic polymers or oligomers.

[112] The polymers (or shorter chain oligomers) can include weak or degradable linkages in their backbones. For example, the polyalkylene glycols can include hydrolytically unstable linkages, such as lactide, glycolide, carbonate, ester, carbamate and the like, which are susceptible to hydrolysis. This allows the polymers to be cleaved into lower molecular weight fragments. Examples of such polymers are described, for example, in U.S. Patent No. 6,153,211 to Hubbell et al.

[113] Representative hydrophilic, amphiphilic, and lipophilic polymers and oligomers are described in more detail below.

#### 8.2.2 Hydrophilic Moieties

[114] The hydrophilic moiety may be various hydrophilic moieties as will be understood by those skilled in the art including, but not limited to, polyalkylene glycol moieties, other hydrophilic polymers, sugar moieties, polysorbate moieties, and combinations thereof.

##### 8.2.2.1 Polyalkylene Glycol Moieties

[115] Polyalkylene glycols are compounds with repeat alkylene glycol units. In some embodiments, the units are all identical (e.g., polyethylene glycol or polypropylene glycol). In other embodiments, the alkylene units are different (e.g., polyethylene-co-propylene glycol, or PLURONICS®). The polymers can be random copolymers (for example, where ethylene oxide and propylene oxide are co-polymerized) or branched or graft copolymers.

[116] Polyethylene glycol, or PEG, is a preferred polyalkylene glycol, and is useful in biological applications because it has highly desirable properties and is generally regarded as safe (GRAS) by the Food

and Drug Administration. PEG has the formula $-(CH_2CH_2O)_n-$, where n can range from about 2 to about 4000 or more. PEG typically is colorless, odorless, water-soluble or water-miscible (depending on molecular weight), heat stable, chemically inert, hydrolytically stable, and generally nontoxic. PEG is also biocompatible, and typically does not produce an immune response in the body. Preferred PEG moieties of the invention include a number of PEG subunits selected from the following ranges shown in order of increasing preference: 2-50, 2-40, 2-30, 2-25, 2-20, 2-15, 2-10. In certain embodiments, the modifying moieties will include 2, 3, 4, 5, 6, 7, 8, 9, or 10 subunits.

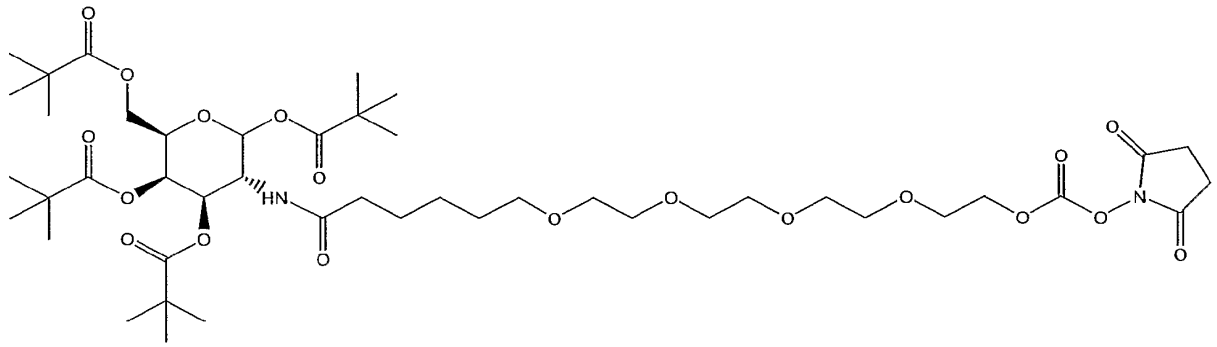
[117] The PEG may be monodispersed (e.g., as previously described by the applicants in U.S. Patent Application Nos. 09/873,731 and 09/873,797, both filed June 4, 2001 the entire disclosures of which are incorporated herein by reference) or polydispersed as commonly supplied on the market. By monodispersed, it is meant that the polyalkylene glycol can have a single molecular weight, or a relatively narrow range of molecular weights. One advantage of using the relatively low molecular weight, monodispersed polymers is that they form easily defined conjugate molecules, which can facilitate both reproducible synthesis and FDA approval.

[118] The PEG can be a linear polymer with a hydroxyl group at each terminus (before being conjugated to the remainder of the natriuretic compound). The PEG can also be an alkoxy PEG, such as methoxy-PEG (or mPEG), where one terminus is a relatively inert alkoxy group, while the other terminus is a hydroxyl group (that is coupled to the natriuretic compound). The PEG can also be branched, which can in one embodiment be represented as $R(-PEG-OH)_m$ in which R represents a central (typically polyhydric) core agent such as pentaerythritol or glycerol, and m represents the number of arms. Each branch can be different and can be terminated, for example, with ethers and/or esters. The number of arms m can range from three to a hundred or more, and one or more of the terminal hydroxyl groups can be coupled to the remainder of the natriuretic compound, or otherwise subject to chemical modification. Other branched PEG include those represented by the formula $(CH_3O-PEG-)_pR-Z$, where p equals 2 or 3, R represents a central core such as Lys or glycerol, and Z represents a group such as carboxyl that is subject to ready chemical activation. Still another branched form, the pendant PEG, has reactive groups, such as carboxyls, along the PEG backbone rather than, or in addition to, the end of the PEG chains. Forked PEG can be represented by the formula $PEG(-LCHX_2)_n$ is another form of branched PEG, where L is a linking group and X is an activated terminal group. The term polyethylene glycol or PEG represents or includes all forms of linear or branched PEG, and polyalkalene glycol or PEG includes all forms of linear or branched PEG.

#### 8.2.2.2 Sugar Moieties

[119] The natriuretic compounds described herein can include sugar moieties, as such as known by those skilled in the art. In general, the sugar moiety is a carbohydrate product of at least one saccharose group. Representative sugar moieties include, but are not limited to, glycerol moieties, mono-, di-, tri-, and

oligosaccharides, and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include $C_6$ and above (preferably $C_6$ to $C_8$) sugars such as glucose, fructose, mannose, galactose, ribose, and sedoheptulose; di- and trisaccharides include moieties having two or three monosaccharide units (preferably $C_5$ to $C_8$) such as sucrose, cellobiose, maltose, lactose, and raffinose. An example of a modifying moiety including a sugar moiety is as follows:



[120] Conjugation using sugar moieties is described in US Patents 5,681,811, 5,438,040, and 5,359,030, the entire disclosures of which are incorporated herein by reference.

#### 8.2.2.3 Polysorbate Moieties

[121] The polysorbate moiety may be various polysorbate moieties as will be understood by those skilled in the art including, but are not limited to, sorbitan esters, and polysorbate derivatized with polyoxyethylene. Conjugation using polysorbate moieties is described in US Patents 5,681,811, 5,438,040, and 5,359,030, the entire disclosures of which are incorporated herein by reference.

#### 8.2.2.4 Biocompatible Water-Soluble Polycationic Moieties

[122] In some embodiments, biocompatible water-soluble polycationic polymers can be used. Biocompatible water-soluble polycationic polymers include, for example, any polymer having protonated heterocycles attached as pendant groups. "Water soluble" means that the entire polymer is soluble in aqueous solutions, such as buffered saline or buffered saline with small amounts of added organic solvents as cosolvents, at a temperature between 20 and 37°C. In some embodiments, the polymer itself is not sufficiently soluble in aqueous solutions per se but is brought into solution by grafting with water-soluble polymers such as PEG chains. Examples include polyamines having amine groups on either the polymer backbone or the polymer sidechains, such as poly-L-Lys and other positively charged polyamino acids of natural or synthetic amino acids or mixtures of amino acids, including poly(D-Lys), poly(ornithine), poly(Arg), and poly(histidine), and nonpeptide polyamines such as poly(aminostyrene), poly(aminoacrylate), poly (N-methyl aminoacrylate), poly (N-ethylaminoacrylate), poly(N,N-dimethyl aminoacrylate), poly(N,N-diethylaminoacrylate), poly(aminomethacrylate), poly(N-methyl amino-

methacrylate), poly(N-ethyl aminomethacrylate), poly(N,N-dimethyl aminomethacrylate), poly(N,N-diethyl aminomethacrylate), poly(ethyleneimine), polymers of quaternary amines, such as poly(N,N,N-trimethylaminoacrylate chloride), poly(methyacrylamidopropyltrimethyl ammonium chloride), and natural or synthetic polysaccharides such as chitosan.

#### 8.2.2.5 Other Hydrophilic Moieties

[123] Other hydrophilic polymers can also be used. Examples include poly(oxyethylated polyols) such as poly(oxyethylated glycerol), poly(oxyethylated sorbitol), and poly(oxyethylated glucose); poly(vinyl alcohol) ("PVA"); dextran; carbohydrate-based polymers and the like. The polymers can be homopolymers or random or block copolymers and terpolymers based on the monomers of the above polymers, linear chain or branched.

[124] Specific examples of suitable additional polymers include, but are not limited to, poly(oxazoline), difunctional poly(acryloylmorpholine) ("PAcM"), and poly(vinylpyrrolidone)("PVP"). PVP and poly(oxazoline) are well known polymers in the art and their preparation should be readily apparent to the skilled artisan. PAcM and its synthesis and use are described in U.S. Patents 5,629,384 and 5,631,322, the disclosures of which are incorporated herein by reference in their entirety.

### 8.2.3 Bioadhesive Polyanionic Moieties

[125] Certain hydrophilic polymers appear to have potentially useful bioadhesive properties. Examples of such polymers are found, for example, in U.S. Patent No. 6,197,346 to Mathiowitz, et al. Those polymers containing carboxylic groups (e.g., poly(acrylic acid)) exhibit bioadhesive properties, and also are readily conjugated with the natriuretic compounds described herein. Rapidly bioerodible polymers that expose carboxylic acid groups on degradation, such as poly(lactide-co-glycolide), polyanhydrides, and polyorthoesters, are also bioadhesive polymers. These polymers can be used to deliver the natriuretic compounds to the gastrointestinal tract. As the polymers degrade, they can expose carboxylic acid groups to enable them to adhere strongly to the gastrointestinal tract, and can aid in the delivery of the natriuretic compound conjugates.

### 8.2.4 Lipophilic Moieties

[126] In some embodiments, the modifying moiety comprises a lipophilic moiety. The lipophilic moiety may be various lipophilic moieties as will be understood by those skilled in the art including, but not limited to, alkyl moieties, alkenyl moieties, alkynyl moieties, aryl moieties, arylalkyl moieties, alkylaryl moieties, fatty acid moieties, adamantantyl, and cholesteryl, as well as lipophilic polymers and/or oligomers.

[127] The alkyl moiety can be a saturated or unsaturated, linear, branched, or cyclic hydrocarbon chain. In some embodiments, the alkyl moiety has at least 1, 2, 3, or more carbon atoms. In other embodiments, the alkyl moiety is a linear, saturated or unsaturated alkyl moiety having between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,

11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 carbon atoms. Examples include saturated, linear alkyl moieties such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, octadecyl, nonadecyl and eicosyl; saturated, branched alkyl moieties such as isopropyl, *sec*-butyl, *tert*-butyl, 2-methylbutyl, *tert*-pentyl, 2-methyl-pentyl, 3-methylpentyl, 2-ethylhexyl, 2-propylpentyl; and unsaturated alkyl moieties derived from the above saturated alkyl moieties including, but not limited to, vinyl, allyl, 1-butenyl, 2-butenyl, ethynyl, 1-propynyl, and 2-propynyl. In other embodiments, the alkyl moiety is a lower alkyl moiety. In still other embodiments, the alkyl moiety is a $C_1$ to $C_3$ lower alkyl moiety. In some embodiments, the modifying moiety specifically does not consist of an alkyl moiety, or specifically does not consist of a lower alkyl moiety, or specifically does not consist of an alkane moiety, or specifically does not consist of a lower alkane moiety.

[128] The alkyl groups can either be unsubstituted or substituted with one or more substituents, and such substituents preferably either do not interfere with the methods of synthesis of the conjugates or eliminate the biological activity of the conjugates. Potentially interfering functionality can be suitably blocked with a protecting group so as to render the functionality non-interfering. Each substituent may be optionally substituted with additional non-interfering substituents. The term "non-interfering" characterizes the substituents as not adversely affecting any reactions to be performed in accordance with the process of this invention.

[129] The fatty acid moiety may be various fatty acid moieties including natural or synthetic, saturated or unsaturated, linear or branched fatty acid moieties. In some embodiments, the fatty acid moiety has at least 2, 3, 4, or more carbon atoms. In other embodiments, the fatty acid moiety has 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 carbon atoms.

[130] When the modifying moiety is an aryl ring, the ring can be functionalized with a nucleophilic functional group (such as OH, SH, or NHR') that is positioned so that it can react in an intramolecular fashion with the carbamate moiety and assist in its hydrolysis. In some embodiments, the nucleophilic group is protected with a protecting group capable of being hydrolyzed or otherwise degraded *in vivo*, with the result being that when the protecting group is deprotected, hydrolysis of the conjugate, and resultant release of the parent natriuretic compound, is facilitated.

### 8.2.5 Amphiphilic Moieties

[131] In some embodiments, the modifying moiety includes an amphiphilic moiety. Many polymers and oligomers are amphiphilic. These are often block co-polymers, branched copolymers or graft co-polymers that include hydrophilic and lipophilic moieties, which can be in the form of oligomers and/or polymers, such as linear chain, branched, or graft polymers or co-polymers.

[132] The hydrophilic polymers or oligomers described may include combinations of any of the lipophilic and hydrophilic moieties described herein. Such polymers or oligomers typically include at least one reactive functional group, for example, halo, hydroxyl, amine, thiol, sulfonic acid, carboxylic acid, isocyanate, epoxy, ester, and the like, which are often at the terminal end of the polymer. These reactive functional groups can be used to attach a lipophilic linear or branched chain alkyl, alkenyl, alkynyl, arylalkyl, or alkylaryl group, or a lipophilic polymer or oligomer, thereby increasing the lipophilicity of the hydrophilic polymers or oligomers (and thereby rendering them generally amphiphilic).

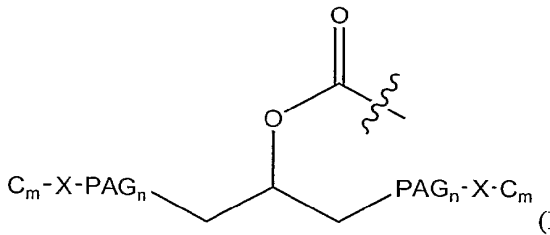
[133] The lipophilic groups can, for example, be derived from mono- or di-carboxylic acids, or where appropriate, reactive equivalents of carboxylic acids such as anhydrides or acid chlorides. Examples of suitable precursors for the lipophilic groups are acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, trimethylacetic acid, caproic acid, caprylic acid, heptanoic acid, capric acid, pelargonic acid, lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, lignoceric acid, ceratic acid, montanoic acid, isostearic acid, isononanoic acid, 2-ethylhexanoic acid, oleic acid, ricinoleic acid, linoleic acid, linolenic acid, erucic acid, soybean fatty acid, linseed fatty acid, dehydrated castor fatty acid, tall oil fatty acid, tung oil fatty acid, sunflower fatty acid, safflower fatty acid, acrylic acid, methacrylic acid, maleic anhydride, orthophthalic anhydride, terephthalic acid, isophthalic acid, adipic acid, azelaic acid, sebacic acid, tetrahydrophthalic anhydride, hexahydrophthalic anhydride, succinic acid and polyolefin carboxylic acids.

[134] The terminal lipophilic groups need not be equivalent, i.e., the resulting copolymers can include terminal lipophilic groups that are the same or different. The lipophilic groups can be derived from more than one mono or di-functional alkyl, alkenyl, alkynyl, cycloalkyl, arylalkyl or alkylaryl group as defined above.

#### 8.2.5.1 PEG/Alkyl Modifying Moieties

[135] The modifying moiety may be a linear or branched polymeric moiety comprising one or more linear or branched polyalkylene glycol moieties and/or one or more linear or branched, substituted or unsubstituted alkyl moieties. However, in certain embodiments, the modifying moiety specifically does not consist of an alkyl moiety and in other embodiments, the modifying moiety specifically does not consist of an alkane moiety. The polyalkylene glycol moieties in some embodiments include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 PAG subunits, preferably PEG or PPG subunits or combinations thereof. The alkyl moieties are saturated or unsaturated and are preferably 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms. The alkyl moieties are preferably alkane moieties.

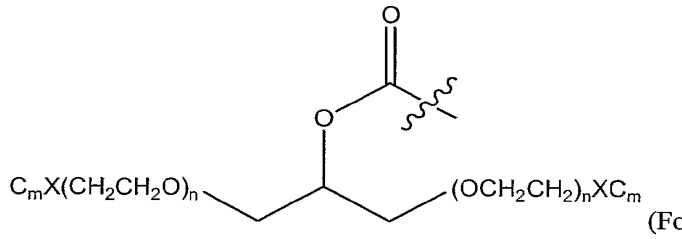
[136] The modifying moiety may, for example, have a formula:




(Formula I)

wherein each C is independently selected and is an alkyl moiety having m carbons and m is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and each PAG is independently selected and is a polyalkylene glycol moiety having n subunits and n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25; each X is independently selected and is a linking moiety coupling PAG to C, and is preferably -C-, -O-, -C(O)-, -C(O)O-, -OC(O)-, -NH-, -NHC(O)-, or -C(O)NH-. In some embodiments the Cm-X moiety is absent, and the $PAG_n$ moiety is terminated with a capping group, such as an -OH moiety or an -OCH3 moiety. For example, the PAG may be methoxy-terminated or hydroxy-terminated PEG, having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 PEG subunits.

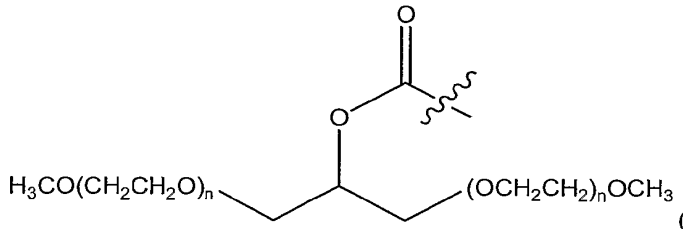
[137] In a preferred embodiment, the modifying moiety has a formula:




(Formula 1A)

wherein C, m, X and n are as described above for Formula I. Preferably n is 2, 3, 4, 5, 6, 7, 8, 9 or 10. X is preferably 0, and m is preferably 1.

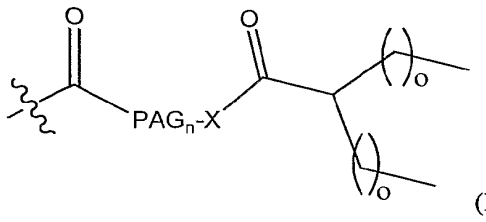
[138] In another preferred embodiment, the modifying moiety has a formula:




(Formula 1B)

wherein n is as described above for Formula I. Preferably n is 2, 3, 4, 5, 6, 7, 8, 9 or 10.

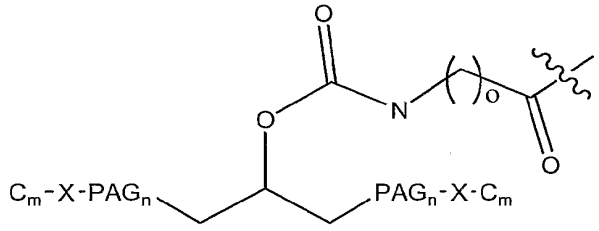
[139] In another aspect, the modifying moiety may have a formula:




(Formula II)

wherein PAG is a polyalkylene glycol moiety having n subunits and n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25; X is -O-, or -NH-; each o is independently selected and is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

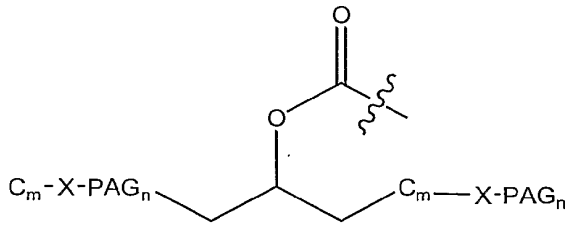
[140] In another aspect, the modifying moiety may have a formula:




(Formula III)

wherein each C is independently selected and is an alkyl moiety having m carbons and m is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and each PAG is independently selected and is a polyalkylene glycol moiety having n subunits and n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25; each X is independently selected and is a linking moiety coupling PAG to C, and is preferably -C-, -O-, -C(O)-, -C(O)O-, -OC(O)-, -NH-, -NHC(O)-, or -C(O)NH-; o is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. The $C_m$-X moiety may be absent, and the $PAG_n$ moiety terminated with an -OH moiety or an -$OCH_3$ moiety. For example, the PAG may be methoxy-terminated or hydroxy-terminated PEG, having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 subunits.

[141] In another aspect, the modifying moiety may have a formula:



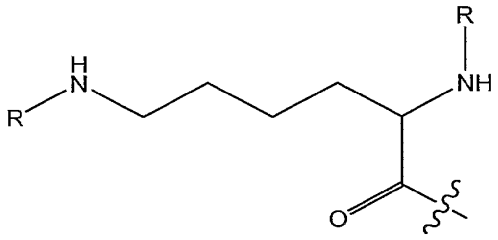

(Formula IV)

wherein each C is independently selected and is a saturated or unsaturated alkyl moiety having m carbons and m is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and each PAG is independently selected and is a polyalkylene glycol moiety having n subunits and n is 1, 2, 3, 4, 5, 6, 7, 8,

9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25; each X is independently selected and is a linking moiety coupling PAG to C, and is preferably -C-, -O-, -C(O)-, -C(O)O-, -OC(O)-, -NH-, -NHC(O)-, or -C(O)NH-.

[142] It will be appreciated that the oligomers of formulas I, IA, IB, II, III, and IV are themselves aspects of the invention. The oligomers may be provided, for example, as primary alcohols or as activated oligomers (see Section 8.7.1 for examples), and may be used to conjugate biologically active compounds, other than BNP, such as insulin, calcitonin, interferons, growth hormones, etc.

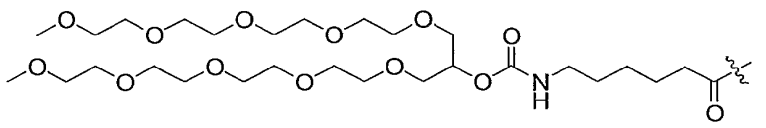
[143] In another aspect, the modifying moiety may have a formula:



(Formula V)

wherein each R is independently selected and is any of Formulas I, II, III or IV above.

[144] In particularly preferred embodiments, the modifying moiety is selected from the following:



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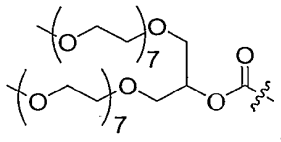

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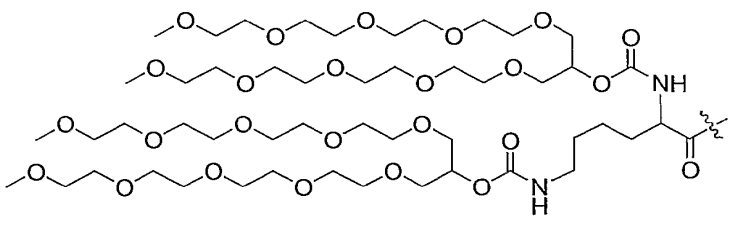
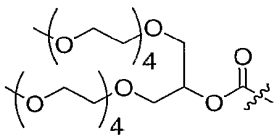
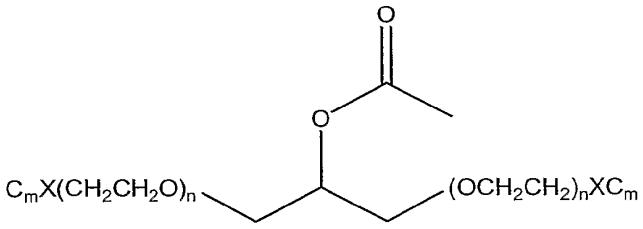


, or



(when this last modifying moiety is coupled to Lys3, of human BNP as a monoconjugate, the resulting moiety is referred to as BN-054).

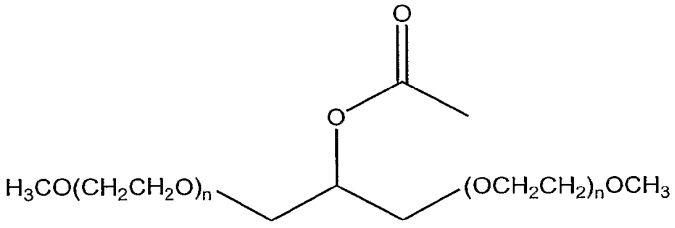
[145] In a preferred embodiment, the modifying moiety has a formula:



(Formula IA)

wherein C, m, X and n are as described above for Formula I.

[146] In another preferred embodiment, the modifying moiety has a formula:



(Formula IB)

[147] wherein n is as described above for Formula I.

[148] The pharmaceutical characteristics, such as hydrophilicity/lipophilicity of the conjugates can be varied by adjusting the number of PEG monomers, the type and length of alkyl chain, the nature of the PEG-peptide linkage, and the number of conjugation sites. The exact nature of the PEG-peptide linkage can be varied such that it is stable and/or sensitive to hydrolysis at physiological pH or in plasma.

### 8.2.6 Salt-Forming Moieties

[149] In some embodiments, the modifying moiety comprises a salt-forming moiety. The salt-forming moiety may be various suitable salt-forming moieties as will be understood by those skilled in the art including, but not limited to, carboxylate and ammonium. In some embodiments wherein the modifying moiety includes a salt forming moiety, the natriuretic compound conjugate is provided in salt form. In these

embodiments, the natriuretic compound conjugate is associated with a suitable pharmaceutically acceptable counterion as will be understood by those skilled in the art including, but not limited to, negative ions such as chloro, bromo, iodo, phosphate, acetate, carbonate, sulfate, tosylate, and mesylate, or positive ions such as sodium, potassium, calcium, lithium, and ammonium.

[150] The modifying moiety can include any hydrophilic moieties, lipophilic moieties, amphiphilic moieties, salt-forming moieties, and combinations thereof. In preferred embodiments, the modifying moiety is selected from the group consisting of $(CH_2CH_2O)_pCH_3$ where p is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20; $(CH_2)_qCH_3$ where q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20; $CH_2CH_2(OCH_2CH_2)_rOH$ where r is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20; $C(CH_2OH)_3$; $CH(CH_2OH)_2$; $C(CH_3)_3$; $CH(CH_3)_2$; $CH_2CH_2(OCH_2CH_2)_sC(O)(CH_2)_tCH_3$ where s is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 and t is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20; and $(CH_2CH_2O)_yC(O)(CH_2)_zCH_3$ where y is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 and z is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

[151] The foregoing examples of modifying moieties for specific purposes is intended as illustrative of the invention and should not be taken as limiting in any way. One skilled in the art will recognize that suitable moieties for conjugation to achieve particular functionality will be possible within the bounds of the chemical conjugation mechanisms disclosed and claimed herein. Accordingly, additional moieties can be selected and used according to the principles of the invention as disclosed herein.

### 8.3 Conjugation Strategies

[152] The natriuretic compound conjugates of the invention can have a different level of biological activity relative to the corresponding unconjugated natriuretic compound conjugates. In some embodiments, the natriuretic compound retains some or all of the activity of the unmodified form, but by virtue of factors such as the degree of conjugation with modifying moieties, selection of conjugation sites on the molecule and selection of modifying moieties, is less susceptible to *in vivo* degradation, and thus, has an increased plasma half life. For example, the natriuretic compounds of the invention may be modified to include a modifying moiety at one, two, three, four, five, or more sites on the natriuretic compound structure at appropriate attachment (i.e., modifying moiety conjugation) sites suitable for facilitating the association of a modifying moiety thereon. By way of example, such suitable conjugation sites may comprise an amino acid residue, such as a Lys amino acid residue.

[153] In many embodiments, for example, the biologically active agent functions, in part, by binding to an active site in a receptor. Often, when a functional group, such as an amino acid residue is modified, the agent no longer binds in the active site. In the case of BNP, for example, the peptide has a particular affinity for binding NPR-A. Depending on the site at which the natriuretic molecule is modified to include the

modifying group, the affinity that the BNP has for the receptor may be the same, or may be reduced. In some embodiments, the natriuretic compound conjugates have less activity than the native, unconjugated natriuretic compound conjugates, but retain improved characteristics relative to unconjugated natriuretic compound conjugates, such as increased resistance to proteolysis and plasma half life or ability to cross a cell membrane. It is envisioned that reduced activity can be preferred, for example, when long term release of the natriuretic compound is desirable.

[154] In some embodiments, the natriuretic compound conjugates are monoconjugates. In other embodiments, the natriuretic compound conjugates are multi-conjugates, such as di-conjugates, tri-conjugates, tetra-conjugates, penta-conjugates and the like. The number of modifying moieties on the natriuretic compound is limited only by the number of conjugation sites on the natriuretic compound. In still other embodiments, the natriuretic compound conjugates of the present invention are a mixture of mono- , di-, tri, tetra, and/or penta-modifying moiety conjugates. For example, in some embodiments, the biologically active natriuretic compound is hBNP, which includes within its 32 native amino acid sequence includes four preferred conjugation sites, including the N-terminus, $Lys^3$, $Lys^{14}$ and $Lys^{27}$. The work of the inventors points to monoconjugates conjugated at the N-terminus, $Lys^3$, $Lys^{14}$ or $Lys^{27}$, and diconjugates at $Lys^3/Lys^{14}$ and $Lys^3/Lys^{27}$ as highly preferred strategies for hBNP and related natriuretic peptides and analogs.

[155] The modifying moiety is preferably covalently coupled to the natriuretic compound. More than one moiety on the modifying moiety may be covalently coupled to the natriuretic compound. Coupling may employ hydrolysable or non-hydrolysable bonds or mixtures of the two (i.e., different bonds at different conjugation sites).

[156] In some embodiments, the natriuretic compound is coupled to the modifying moiety utilizing a hydrolysable bond (e.g., an ester, carbonate or carbamate bond). Use of a hydrolysable coupling will provide a natriuretic compound conjugate that acts as a prodrug. A prodrug approach may be desirable where the natriuretic compound-modifying moiety conjugate is inactive (i.e., the conjugate lacks the ability to affect the body through the natriuretic compound's primary mechanism of action), such as when the modifying moiety conjugation site is in a binding region of natriuretic compound. Use of a hydrolyzable coupling can also provide for a time-release or controlled-release effect, administering the natriuretic compound over a given time period as one or more modifying moieties are cleaved from their respective natriuretic compound-modifying moiety conjugates to provide the active drug.

[157] In other embodiments, the natriuretic compound is coupled to the modifying moiety utilizing a non-hydrolyzable bond (e.g., a carbamate, amide, or ether bond). Use of a non-hydrolyzable bond may be preferable when it is desirable to allow the natriuretic compound-modifying moiety conjugate to circulate in the bloodstream for an extended period of time, preferably at least 2 hours. Bonds used to covalently couple

the natriuretic compound to the modifying moiety in a non-hydrolysable fashion are typically selected from the group consisting of covalent bond(s), ester moieties, carbonate moieties, carbamate moieties, amide moieties and secondary amine moieties.

[158] In still other embodiments, a partial prodrug approach may be used, in which a portion of the modifying moiety is hydrolyzed. For example, U.S. Patent 6,309,633 (the entire disclosure of which is incorporated herein by reference) describes modifying moieties comprising hydrophilic and lipophilic components in which the lipophilic components hydrolyze *in vivo* to yield a microPAGylated conjugate.

[159] More than one modifying moiety (i.e., a plurality of modifying moietys) may be coupled to the natriuretic compound. The modifying moieties in the plurality are preferably the same. However, it is to be understood that the modifying moieties in the plurality may be different from one another, or, alternatively, some of the modifying moieties in the plurality may be the same and some may be different. When a plurality of modifying moieties are coupled to the natriuretic compound, it may be preferable to couple one or more of the modifying moieties to the natriuretic compound with hydrolyzable bonds and couple one or more of the modifying moieties to the natriuretic compound with non-hydrolyzable bonds. Alternatively, all of the bonds coupling the plurality of modifying moieties to the natriuretic compound may be hydrolyzable, but have varying degrees of hydrolyzability such that, for example, one or more of the modifying moieties may be rapidly removed from the natriuretic compound by hydrolysis in the body and one or more of the modifying moieties is slowly removed from the natriuretic compound by hydrolysis in the body.

[160] The modifying moiety may be coupled to the natriuretic compound at various nucleophilic residues of the drug including, but not limited to, nucleophilic hydroxyl functions and/or amino functions. Nucleophilic hydroxyl functions may be found, for example, at serine and/or tyrosine residues, and nucleophilic amino functions may be found, for example, at histidine and/or Lys residues, and/or at the one or more N-terminus of the polypeptide. When a modifying moiety is coupled to the N-terminus of the natriuretic peptide, coupling preferably forms a secondary amine.

### 8.4 Synthesis of the Conjugates

[161] Exemplary syntheses are described in the examples set forth below. The reaction conditions (e.g., selected molar ratios, solvent mixtures and/or pH) may be controlled according to known principles. For example, conjugation at the amino functionality of Lys may be suppressed by maintaining the pH of the reaction solution below the $pK_a$ of Lys.

[162] The mixture of natriuretic compound conjugates may be separated and isolated utilizing, for example, HPLC to provide natriuretic compound conjugates, for example mono-, di-, or tri-conjugates. The degree of conjugation (e.g., whether the isolated molecule is a mono-, di-, or tri-conjugate) of a particular isolated conjugate may be determined and/or verified utilizing various techniques as will be understood by

those skilled in the art including, but not limited to, mass spectroscopy. The particular conjugate structure (e.g., $Lys^3$, $Lys^{14}$, $Lys^{27}$, or the N-terminus of hBNP monoconjugate) may be determined and/or verified utilizing various techniques as will be understood by those skilled in the art including, but not limited to, sequence analysis, peptide mapping, selective enzymatic cleavage, and/or endopeptidase cleavage.

[163] One or more of the reaction sites on the natriuretic compound may be blocked by, for example, reacting the natriuretic compound with a suitable blocking reagent such as N-tert-butoxycarbonyl (t-BOC), or N-(9-fluorenylmethyloxycarbonyl) (N-FMOC). This process may be preferred, for example, when it is desired to form an unsaturated natriuretic compound conjugate (i.e., a conjugate wherein not all nucleophilic residues are conjugated) having a modifying moiety at one or more of the N-terminus of the polypeptide. Following such blocking, the substantially monodispersed mixture of blocked natriuretic compounds may be reacted with the substantially monodispersed mixture of activated modifying moieties to provide a mixture of natriuretic compound conjugates having modifying moiety(s) coupled to one or more nucleophilic residues and having blocking moieties coupled to other nucleophilic residues. After the conjugation reaction, the natriuretic compound-modifying moiety conjugates may be de-blocked as will be understood by those skilled in the art. If necessary, the mixture of natriuretic compound conjugates may then be separated as described above to provide a mixture of natriuretic compound conjugates. Alternatively, the mixture of natriuretic compound-modifying moiety conjugates may be separated prior to de-blocking.

[164] In a surprising aspect of the invention, the inventors discovered that synthesis of an hBNP conjugate using a PEG-alkyl moiety with the alkyl moiety adjacent to the natriuretic compound (i.e., positioned between the natriuretic compound and the PEG moiety) results in preferential conjugation at the highly desirable $Lys^3$ conjugation site. Thus, in one aspect, the invention provides a method of preferentially conjugating hBNP at $Lys^3$ comprising activating the alkyl component of a PEG-alkyl oligomer and coupling the activated PEG-alkyl oligomer to the hBNP.

### 8.5 Pharmaceutical Compositions

[165] Pharmaceutical compositions including the natriuretic compound conjugates described herein can be prepared. Such compositions typically include the modified natriuretic compound in combination with, or in admixture with, a pharmaceutically acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the pharmaceutical composition and should not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the prodrug as a unit-dose formulation, for example, a tablet, which may contain from about 0.01 or 0.5% to about 95% or 99% by weight of the natriuretic compound conjugate. The pharmaceutical compositions may be prepared by any of the well-known techniques of pharmacy including, but not limited to, admixing the components, optionally including one or more accessory ingredients.

[166] The pharmaceutical compositions according to embodiments of the present invention include those suitable for oral, rectal, nasal, topical, inhalation (e.g., via an aerosol) buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular, intradermal, intraarticular, intrapleural, intraperitoneal, intracerebral, intraarterial, or intravenous), topical (i.e., both skin and mucosal surfaces, including airway surfaces) and transdermal administration, although the most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular prodrug which is being used.

[167] Pharmaceutical compositions suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the prodrug; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Such formulations may be prepared by any suitable method of pharmacy that includes the step of bringing into association the prodrug and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the pharmaceutical composition according to embodiments of the present invention are prepared by uniformly and intimately admixing the prodrug with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the prodrug, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the mixture in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

[168] Pharmaceutical compositions suitable for buccal (sub-lingual) administration include lozenges comprising the prodrug in a flavored base, usually sucrose and acacia or tragacanth; and pastilles comprising the prodrug in an inert base such as gelatin and glycerin or sucrose and acacia.

[169] Pharmaceutical compositions according to embodiments of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the prodrug, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the composition isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The compositions may be presented in unit\dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. For example, an injectable, stable, sterile composition comprising a prodrug in a unit dosage form in a sealed container may be provided. The prodrug is provided in the form of a lyophilizate which is capable of being reconstituted with a suitable pharmaceutically acceptable carrier to

form a liquid composition suitable for injection thereof into a subject. The unit dosage form typically comprises from about 10 mg to about 10 grams of the prodrug. When the prodrug is substantially water-insoluble, a sufficient amount of emulsifying agent which is physiologically acceptable may be employed in sufficient quantity to emulsify the prodrug in an aqueous carrier. One such useful emulsifying agent is phosphatidyl choline.

[170] Pharmaceutical compositions suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include petroleum jelly, lanolin, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

[171] Pharmaceutical compositions suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Compositions suitable for transdermal administration may also be delivered by iontophoresis *(see,* for example, *Pharmaceutical Research* 3 **(6)**:318 (1986)) and typically take the form of an optionally buffered aqueous solution of the prodrug. Suitable formulations comprise citrate or bis\tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2M active ingredient.

### 8.6 Methods of Administration and Treatment

[172] The natriuretic compound conjugates and pharmaceutical formulations of the invention exhibit one or more improved characteristics relative to the unmodified (unconjugated) biologically active natriuretic compound, the addition of the modifying moiety can protect the biologically active natriuretic compound, from degradation in various environments (such as the gastrointestinal tract (GI tract)), such that less of it is degraded in the unmodified form than would be degraded in the absence of the modifying moiety in such environments. In particular, certain modified forms of the invention can be orally administered in a dosage that ultimately provides a pharmaceutically acceptable amount of the biologically active natriuretic compound in systemic circulation. That is to say, a sufficient amount of natriuretic compound can survive in the GI tract and enter the bloodstream such that the biologically active natriuretic compound is systemically present in a pharmacologically active amount sufficient to trigger production of cGMP. Preferably, the addition of the modifying moiety improves the delivery of orally administered unconjugated natriuretic compound into the bloodstream upon oral administration relative to the delivery of orally administered unconjugated natriuretic compound into the bloodstream. More preferably, the improvement of the delivery of active compound into the bloodstream for orally administered natriuretic compound conjugates is at least 2 times the delivery of orally administered unconjugated parent biologically active natriuretic compound, into the bloodstream. Still more preferably, the improvement of the delivery of active compound into the bloodstream for orally administered natriuretic compound conjugates is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, or 500 times the delivery of

orally administered unmodified (unconjugated) biologically active natriuretic compound, into the bloodstream. Thus, administration of the natriuretic compound conjugates of the invention can provide greater bioavailability of the biologically active natriuretic compound relative to administration of unmodified biologically active natriuretic compound. An oral route of administration (instead of by continuous intravenous infusion for days in a hospital setting) may reduce hospital costs associated with other CHF therapies and/or expand the therapeutic use of hBNP to include early stage and chronic CHF as well as acute CHF.

[173] Thus, in one aspect, the invention provides a method of treating a disease condition susceptible to treatment using a natriuretic peptide compound by administering to a subject in need thereof a therapeutically effective amount of a natriuretic compound conjugate of the invention. The natriuretic compound conjugate may be suitably administered by a variety of routes, including for example, parenteral and enteral routes. Examples of preferred routes include oral, subcutaneous, sublingual, buccal, nasal, intravenous and intramuscular.

[174] Several approaches may be used in the use of the present natriuretic compound conjugates for the treatment of heart failure. For example, it is envisioned that the natriuretic compound conjugates can be presented as a monotherapy, preferably in an oral dosage form alone. Alternatively, the natriuretic compound conjugates may be used together with more conventional therapeutic agents as part of a combination therapy. The primary categories of drugs that are currently used include the following:

[175] Diuretics – alleviate the fluid accumulation and resultant stretching of the heart associated with CHF.

[176] Vasodilators – expand arteries and veins, allowing for increased blood flow.

[177] Inotropic agents – increase the force of contraction of cardiac muscle.

[178] Digitalis drugs – increase force of contraction of the heart and reduce heart rate.

[179] Angiotensin converting enzyme (ACE) inhibitors – inhibit the production of the vasoconstrictor angiotensin II in the last stage of its synthesis.

[180] Angiotensin receptor blockers (ARB's) – permit angiotensin to be produced, but inhibit its arterial activity.

[181] Calcium channel blockers – inhibit calcium influx, resulting in vascular and smooth muscle relaxation.

[182] Nitrates – relax smooth muscles and dilate veins and arteries.

[183] Beta-blockers – block the action of catecholamines, resulting in less stress on the heart and lower force and rate of contraction.

[184] Some of the advantages of the natriuretic compound conjugates can be considered first in relation to the other approaches to treat CHF and compared to the current use of the natriuretic peptide in its unmodified form, that is continuously infused. The oral natriuretic compound conjugates of the invention exhibit natriuretic and diuretic properties that may be expected to relieve congestion through the elimination of sodium and excess water. Such functions are currently addressed with diuretics and potassium supplements. The natriuretic compound conjugates of the invention are expected to possess vascular and myocardial relaxant properties that are currently effected using vasodilators, calcium channel blockers, and nitrates. The natriuretic compound conjugates of the invention are expected to inhibit the renin-angiotensin-aldosterone system (RAAS) currently effected using ACE inhibitors and ARB's. Moreover, the natriuretic compound conjugates are expected to lack the negative effects and risk of sudden death associated with the inotropic and digitalis drugs. The natriuretic compound conjugates may have many, if not all, of the benefits of several groups of cardiovascular drugs while having a reduced amount of or lacking the negative effects of conventional therapies.

[185] The natriuretic compound-conjugates of the invention also have advantages over NATRECOR® (nesiritide, made by Scios, Inc., Sunnyvale, CA). Some of the advantages can be attributed to the enhanced pharmacokinetic profile that amphiphilic oligomers according to embodiments of the present invention provide. For example, resistance to degradation by proteases (such as NEP) may lead to a longer circulating half-life as compared to the unconjugated peptide. A significant advantage may result from the ability of BNP or ANP conjugated with such oligomers to be delivered orally. For instance, NATRECOR® is dosed by continuous infusion over 48 hours and carries a high cost per dose plus hospital costs. An oral hBNP compound conjugate according to embodiments of the present invention may be dosed at an overall lower cost, and may be available on an outpatient basis and may be self-administered. Instead of being limited to use with inpatients having the most acute cases of CHF, oral conjugates according to embodiments of the present invention can be used for those suffering with the gradual onset of chronic CHF. The ease of administration, reduced demand on hospital resources, and/or lower cost support the utility of an the natriuretic compound conjugates as a preventative therapy, self administered (e.g., at home) for those patients who are at high risk of heart failure. Oral preparations of the hBNP compound conjugate according to embodiments of the present invention are thus expected to have many, if not all the benefits of Natrecor®, with the advantages of an improved pharmacokinetic profile, greater ease of administration, reduced hospitalization expenses, expansion of indication to include chronic CHF, and/or utility in early-stage cardiovascular disease.

[186] Subjects taking or inclined to take the parent natriuretic compound can alternatively (or additionally) take the natriuretic compound preparation described herein. For example, patients suffering

from disorders that are conventionally treated using a parenterally administered natriuretic compound, such as NATRECOR®, can be treated using an effective amount of the modified form of that agent described herein. Advantageously, where such agents were previously only administrable via injection or intraveneous administration, the natriuretic compound can be administered via inhalation or, more preferably, oral administration.

[187] In one embodiment, the invention provides a method of delivering a biologically active agent to a subject, wherein the biologically active agent is orally administered as a component of a modified natriuretic compound of the invention, a portion of the orally administered natriuretic compound survives intact in the GI tract and traverses the intestinal wall to enter the bloodstream, and after leaving the GI tract, some or all of the natriuretic compound is hydrolyzed *in vivo* to yield a pharmaceutically acceptable amount of the biologically active agent. The hydrolysis may, for example, take place in the bloodstream or in the liver. In this method, the modified forms of the natriuretic compound enhances the oral bioavailability of the orally administered biologically active agent relative to the oral bioavailability of a corresponding orally administered unconjugated biologically active agent.

[188] The effective amount of any natriuretic the use of which is in the scope of present invention, will vary somewhat from agent to agent, and patient to patient, and will depend upon factors such as the age and condition of the patient and the route of delivery. Such dosages can be determined in accordance with routine pharmacological procedures known to those skilled in the art. As a general proposition, a dosage from about 0.1 to about 50 mg/kg will have therapeutic efficacy, with all weights being calculated based upon the weight of the patient. Toxicity concerns at the higher level may restrict intravenous dosages to a lower level such as up to about 10 mg/kg, with all weights being calculated based upon the weight of the active base. A dosage from about 10 mg/kg to about 50 mg/kg may be employed for oral administration. Typically, a dosage from about 0.5 mg/kg to 5 mg/kg may be employed for intramuscular injection. The frequency of administration is usually one, two, or three times per day or as necessary to control the condition. The duration of treatment depends on the type of condition being treated and may be for as long as the life of the patient.

[189] Suitable subjects to be treated according to the present invention include, but are not limited to, avian and mammalian subjects, preferably mammalian. Mammals according to the present invention include but are not limited to canine, felines, bovines, caprines, equines, ovines, porcines, rodents (*e.g.* rats and mice), lagomorphs, primates, humans, and the like, and encompass mammals *in utero*. Any mammalian subject in need of being treated according to the present invention is suitable. Human subjects are preferred. Human subjects of both genders and at any stage of development (*i.e.*, neonate, infant, juvenile, adolescent, adult) can be treated according to the present invention.

[190] Illustrative avians according to the present invention include chickens, ducks, turkeys, geese, quail, pheasant, ratites (*e.g.*, ostrich) and domesticated birds (*e.g.*, parrots and canaries), and include birds *in ovo*.

### 8.7 Assays

[191] Natriuretic peptide analogs of the invention may induce the cardiovascular, renal, and/or endocrine effects that are associated with the native peptide. Cell based assays may be used to show which conjugates are proficient agonists of the human natriuretic peptide receptor A, leading to the suitable production of cGMP. Biochemical assays may be used to show which conjugates offer the suitable protection against proteolytic enzymes. In vivo experiments may be used to show which conjugates afford a desirable bioavailability. Leading conjugates can be tested in established dog models. Desirable candidates may be subjected to detailed pharmacokinetic, pharmacodynamic, and toxicity studies in rats and dogs. BNP conjugates according to embodiments of the present invention will be useful for the treatment of early-stage, chronic, and acute congestive heart failure.

[192] The novel peptides and novel conjugates of the invention can be tested for agonist activity at the human natriuretic peptide receptor A (NPR-A) *in vitro*. The vasorelaxant, natriuretic, and diuretic properties of BNP are ascribed to a secondary messenger, cyclic GMP (cGMP). The production of cGMP is accomplished by guanylate cyclase, an enzyme that is activated when BNP binds to NPR-A. cGMP production can be measured in cultures of human aortic endothelial cells that endogenously express NPR-A. Thus, the relative activity of the natriuretic compound conjugates and natriuretic peptide analogs of the invention can be determined by the level of cGMP production in these cells.

[193] The conjugates of the invention can be tested for increased resistance to proteases. In general, drugs that are delivered orally are subjected to digestive enzymes such as pepsin, trypsin, and/or chymotrypsin. In the case of peptide drugs, these enzymes may be particularly problematic. However, peptide conjugation has been shown to increase resistance to these enzymes. Digestive enzyme cocktails can be used to test for increased resistance of hBNP conjugates and other conjugates of the invention to proteases of the digestive tract. Natriuretic compound conjugates are preferably less susceptible to proteolytic degradation than corresponding unconjugated natriuretic compounds, i.e., the conjugates digest more slowly than the corresponding unconjugated compound.

[194] The conjugates can be tested for oral bioavailability. Oral bioavailability of the conjugates can be tested in rats, for example. The conjugates can be administered to the gastrointestinal tract by oral gavage and the presence of hBNP conjugates in the bloodstream can be assayed using available radioimmunoassay procedures. Conjugates according to embodiments of the present invention may preferably be orally and/or perorally available, i.e., a therapeutically significant amount of the conjugate can be delivered by the oral and/or peroral routes.

[195] The conjugate may retain some or all of the activity of native natriuretic peptide (e.g., hBNP) with the additional benefits of oral administration. Such a compound may lower costs associated with treatment of acute CHF and/or expand the applicability of this therapeutic to include early stage and chronic CHF.

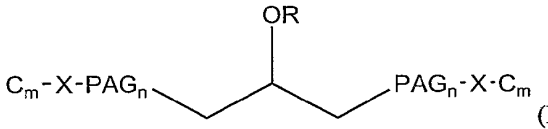
[196] In one aspect, the invention provides a method of generating data comprising assaying a natriuretic compound assaying a natriuretic compound conjugate of the invention or a series of such natriuretic compound conjugates, and compiling data resulting from such assaying. The data itself is therefore understood to constitute yet another embodiment of the invention, as well as the use of this data.

### 8.8 Branched Oligomeric Modifying Moieties

[197] The present invention also provides several PEG linear and branched, amine, microPAGylated and alkyl-PEG modifying moieties.

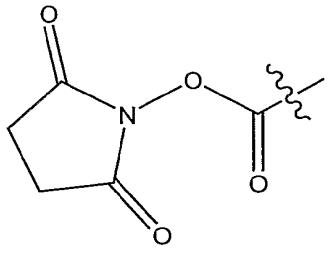
#### 8.8.1 Branched Oligomeric Modifying Moiety Formulas

[198] The present invention provides a compound having a formula:




(Formula VI)

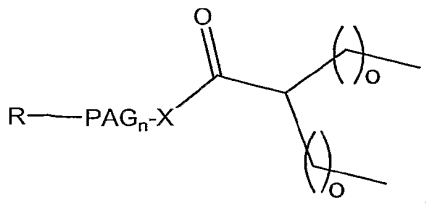
wherein R is -H, -C(O)OH or an activating moiety, such as C(O)X' (where X' is a halide (e.g., Cl, Br) or *p*-nitrophenol), or




,and

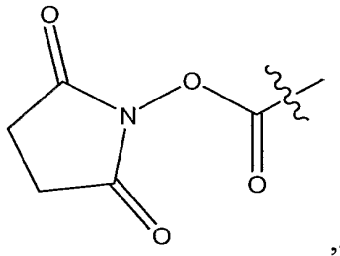
C, m, X, PAG, and n are as described above for Formula I.

[199] The present invention also presents a compound having a formula:




(Formula VII)

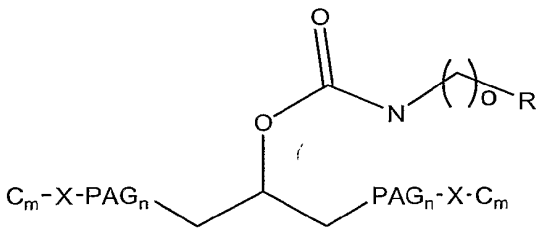
wherein R is H (it will be understood that the atom in PAG coupled to R is O, and therefore that the terminating moiety is OH), C(O)OH or an activating moiety, such as C(O)X' (where X' is a halide (e.g., Cl, Br) or *p*-nitrophenol), or




,and

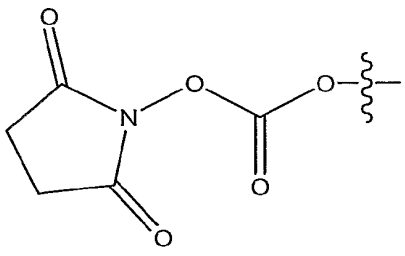
PAG, n, X, and o are as described above for Formula II.

[200] In yet another embodiment, a compound is provided having a formula:

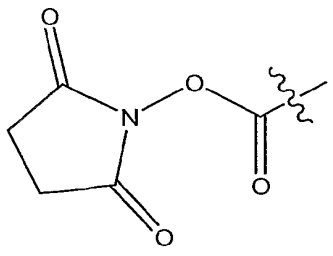



(Formula VIII)

[201] wherein R is -H, -OH, -C(O)OH or an activating moiety, such as C(O)X' or OC(O)X' (where X' is a halide (e.g., Cl, Br) or *p*-nitrophenol), or



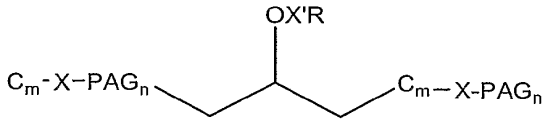

or




,and

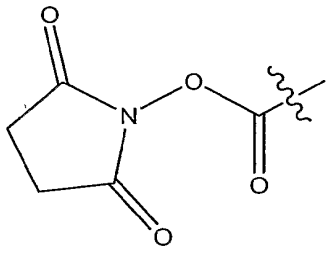
C, m, X, PAG, n and o are as described above for Formula III.

[202] In another aspect, the modifying moiety may have a formula:



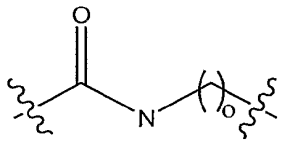

(Formula IX)

wherein R is H, C(O)OH or an activating moiety, such as C(O)X'' (where X'' is a halide (e.g., Cl, Br) or *p*-nitrophenol), or




,and

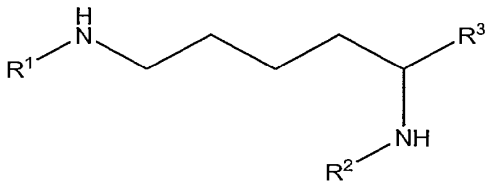
X' is




. and

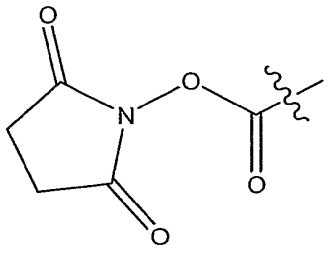
C, m, PAG, n, and X are as described above for Formula IV.

[203] In another aspect, the modifying moiety may have a formula:




(Formula X)

wherein $R^3$ is -OH, -C(O)OH or an activating moiety, such as

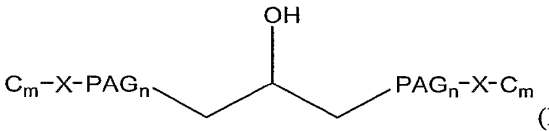


,and

[204] $R^1$ and $R^2$ are each independently selected and are any of Formulas I, II, III or IV above.

### 8.8.2 Methods of Making Oligomeric Modifying Moieties

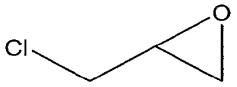
[205] The present invention also provides several methods for preparing the modifying moieties disclosed herein. A method of making a compound of the formula:



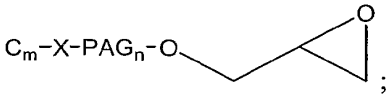
(Formula XI)

is provided, wherein C, m, X, PAG, and n are as described above for Formula I. This method may be described as comprising the steps of reacting a compound of formula:

with a compound of formula:

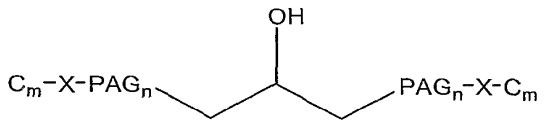


(note that Cl may be replaced with another leaving group, such as another halide) in the presence of a base and a solvent to yield a product of a formula:



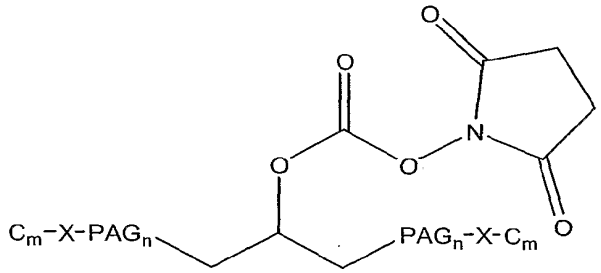
reacting the product a with a compound of formula:

in the presence of a Lewis acid and a solvent to yield:



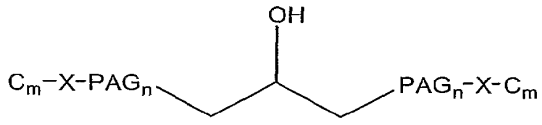

wherein C, m, X, PAG, and n as defined above for Formula I. Cl may be replaced with another halogen, such as Br. By way of example, the base may be further defined as NaH, and the solvent may be further defined as tetrahydrofuran. The Lewis acid to be used in this method may also be further defined as $BF_3OEt_2$.

[206] Another method of the invention is also disclosed for making a compound of the formula:



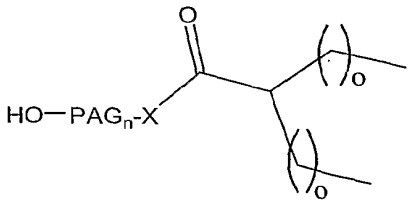

(Formula XII)

whereinC, m, X, PAG and n are as defined above for Formula I. This method may be further defined as comprising the steps of reacting a product

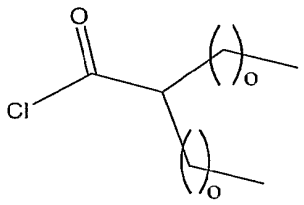



wherein C, m, X, PAG , and n defined as above, with paranitrochloroformate or disuccimidyl carbonate.

[207] Yet another embodiment of the invention is provided in a method of making a compound of the formula:



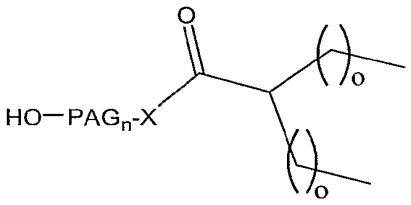

wherein PAG, n, X, and o are as defined above for Formula II. This method may be further described as comprising the steps of reacting a compound of formula:



(note that Cl may be replaced with another leaving group, such as another halide) wherein o is as defined above for Formula I, with a compound of formula:

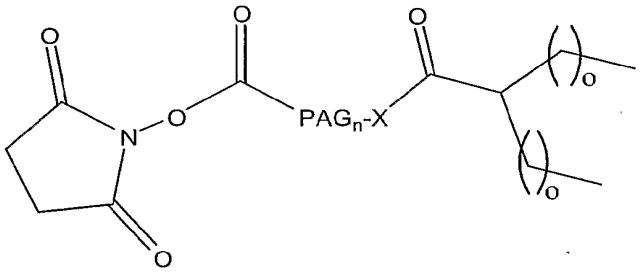
$$HO{-}PAG_n{-}X$$

wherein X is –NH or –OH, in a solvent, to yield a compound of formula:



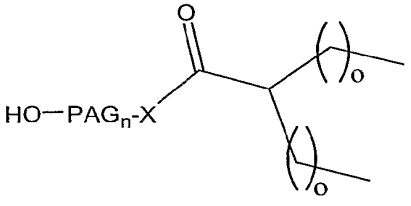
wherein PAG, n, X, and o are as defined above for Formula II.

[208] The invention also provides a method of making a compound of the formula:



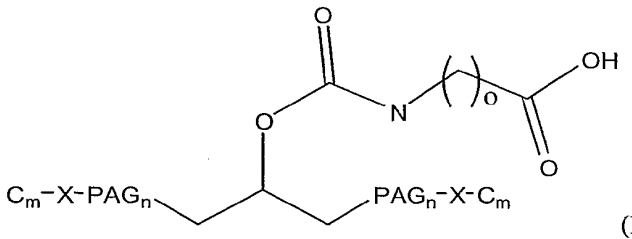
(Formula XIII)

wherein PAG, n, X, and o are defined above for Formula II. This method may be described as comprising the steps of activating a product



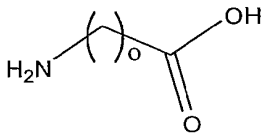
(wherein PAG, n, X, and o are defined above for Formula II using an activating agent, such as disuccinimidyl carbonate, paranitrochloroformate, phosgene and N-hydroxysuccinimide.

[209] Yet another embodiement of the invention provides a method of making a compound of the formula:



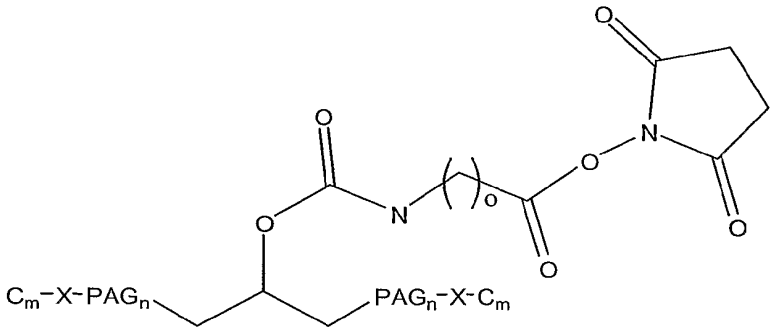

(Formula XIV)

wherein C, m, X, PAG, n, and o are as defined above for Formula III. This method may be described as comprising the steps of reacting the product identified here as Formula XII above with a compound of formula:




in the presence of a base in a solvent, wherein o is as defined above for Formula III. In preferred embodiments of this method, the base is $K_2CO_3$ and the solvent is an aqueous and/or organic solvent.

[210] In addition, the invention further provides a method of making a compound of the formula:




(Formula XV)

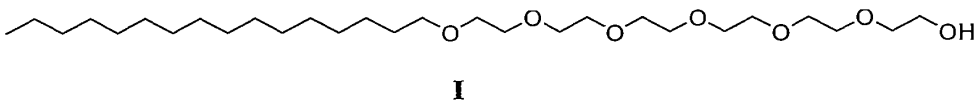
wherein C, m, PAG, n, and o are as defined above for Formula III. The method generally comprises reacting a compound produced according to the method of preparing the Formula XIV as defined above, with an activating agent such as N-hydroxysuccinimide.

## 9. Examples

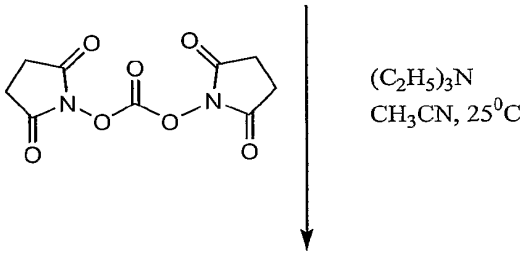
[211] The following examples have been included to illustrate models of the invention. Certain aspects of the following examples are described in terms of techniques and procedures found to demonstrate the best mode of practicing the invention. In light of the present disclosure and the general level of skill known in the relevant art of the present invention, those of skill will appreciate that the following examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the invention.

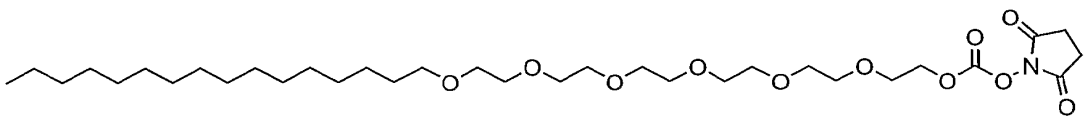
### 9.1 Activation of PEG-Alkyl Modifying Moiety (carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-[2-(2-{2-[2-(2-hexadecyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester (II))

[212] Hexaethyleneglycol monohexadecyl ether, **I** (0.202 g , 0.4 mmol) was dissolved in acetonitrile (5 mL) and disuccinimidyl carbonate (DSC, 0.157 g , 0.6 mmol) was added. Then triethylamine (0.12 g , 1.2 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~ 16 hours, the crude reaction was evaporated to dryness and then dissolved in saturated $NaHCO_3$ (10 mL), washed with ethyl acetate (2 x 20 mL), dried over $MgSO_4$, and evaporated to dryness. The crude product mixture was purified *via* column chromatography (silica, EtOAc/methanol, 10:1) to yield 0.258 g (81%) of the title compound **II** as an oil. ESI MS: *m/e* 648.84 $(M+H)^+$.



**I**



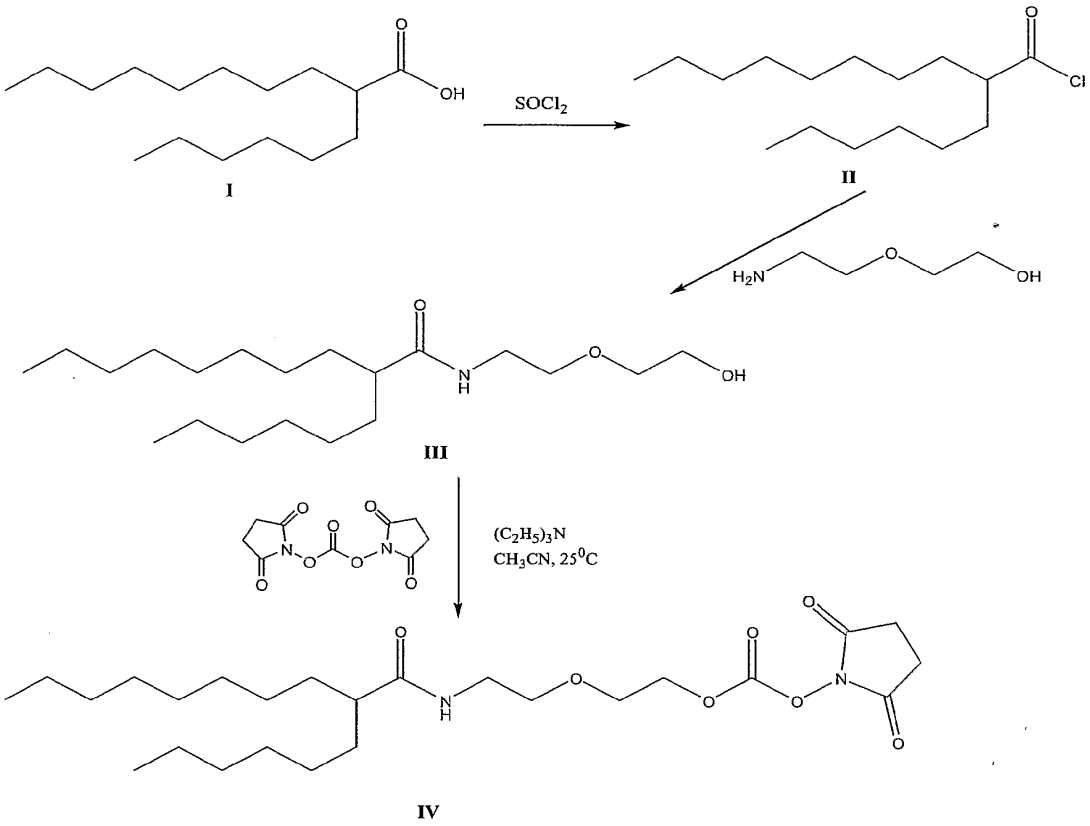
**II**

### 9.2 Synthesis of Branched PEG Amine Modifying Moiety (carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-[2-(2-hexyl-decanoylamino)-ethoxy]-ethyl ester (IV))

[213] Thionyl chloride (5.5 gm, 46.6 mmol) was added drop wise over a period of thirty minutes to a solution of 2-Hexyl-decanoic acid **I** (10 gm, 38.9 mmol) in 100 mL carbon tetrachloride. After the addition was complete, the reaction mixture was refluxed for 3 hours. After the reaction was complete, the carbon tetrachloride was removed by distillation and the reaction mixture was concentrated to get crude acid chloride. The crude acid chloride was purified by fractional distillation to obtain **II** as a clear liquid (10.1 gm, 91%). ESI MS: *m/e* 275.87 $(M+H)^+$.

[214] To a cooled solution of 2-(2-Amino-ethoxy)-ethanol (575 g, 5.47 mmol) in 10 ml dichloromethane, 2-hexyl-decanoyl chloride II (750 mg, 2.74 mmol) was added drop wise over a period of thirty minutes. After the addition was complete, the temperature of the reaction mixture temperature was increased to 25 °C. Reaction was stirred overnight at room temperature. After stirring for ~20 hours, the crude reaction was acidified with 1NHCl and diluted with 10 ml $H_2O$. The reaction mixture was then extracted with dichloromethane. The organic layer was then washed with 1N HCl, water, dried over $MgSO_4$, filtered and concentrated. Crude material was purified by flash chromatography (silica, gradient elution: 2-5 % methanol in $CHCl_3$), to yield 902 mg (96%) of the monodispersed compound **III** as an off-white solid. ESI MS: *m/e* 344.54 $(M+H)^+$.

[215] Monodispersed branched C16-PEG2 **III** (200 mg, 0.58 mmol) was dissolved in acetonitrile (5 mL) and disuccinimidyl carbonate (DSC, 0.224 g , 0.87 mmol). Then triethylamine (0.118 g , 1.17 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred at room temperature overnight. After stirring for ~16 hours, the crude reaction was evaporated to dryness and then dissolved in saturated $NaHCO_3$ (10 mL), washed with ethyl acetate (2 x 20 mL), dried over $MgSO_4$, and evaporated to dryness. The residue was purified *via* column chromatography (silica, EtOAc/methanol, 10:1) to yield 0.206 g (74%) of the oil **IV** (0.206 g, 74% yield). ESI MS: *m/e* 485.63 $(M+H)^+$.



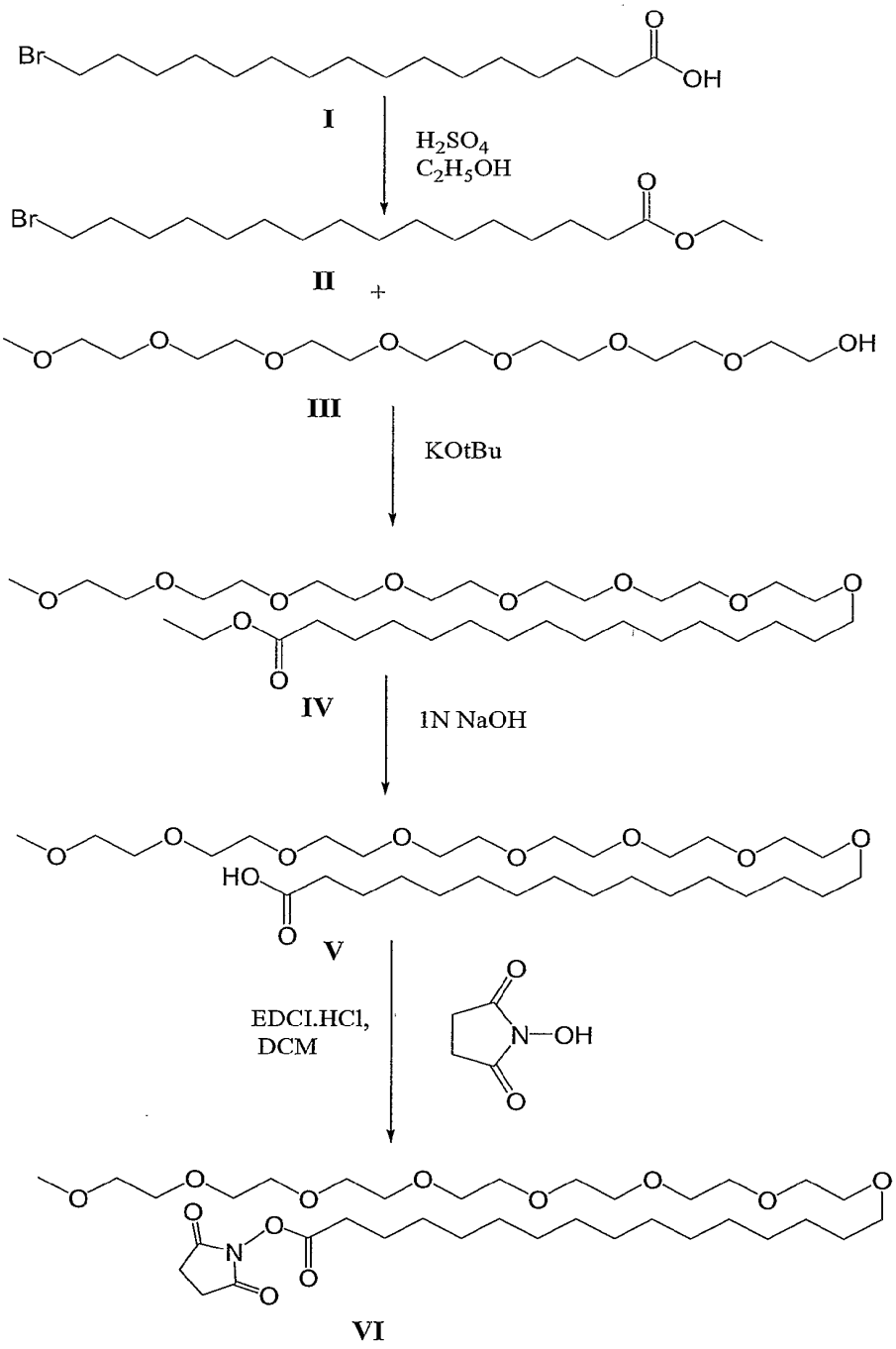

**9.3 Synthesis and Activation of PEG-Alkyl Modifying Moiety (16-(2-{2-[2-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-hexadecanoicacid 2,5-dioxo-pyrrolidin-1-yl ester)**

[216] To a solution of monodispersed 16-bromo-hexadecanoic acid (15.3 g, 45 mmol) in ethanol (300 mL) was added $H_2SO_4$ (1.5 mL, 31.25 mmol) and the reaction was stirred for 48 h. The crude reaction mixture was diluted with water and extracted with dichlormethane (2 x 300 mL). The organic layer was washed with $H_2O$ (300 mL), sat. $NaHCO_3$ (2 x 300 mL), $H_2O$ (300 mL), dried $MgSO_4$, and evaporated to dryness to afford a off-white solid **II** (16.03 g, 98% yield).

[217] To a solution of monodispersed heptaethylene glycol monomethyl ether (8.51 g, 25 mmol) in THF (250 mL) was added potassium t-butoxide (3.1 g, 27.5 mmol, small portions over ~30 min). The reaction mixture was then stirred for 1 h and then **II** (10 g, 27.5 mol) dissolved in THF (90 mL) was added dropwise and the reaction mixture was stirred overnight. The crude reaction mixture was filtered through Celite (washed $CH_2Cl_2$, ~200 mL) and evaporated to dryness to afford oil. The crude oil was purified via flash chromatography (silica, gradient elution: 2-5 % methanol in $CHCl_3$) to give clear yellow oil **IV**, 2.48 g (16 %).

[218] To the oil of the monodispersed compound **IV** (2.22 g, 3.56 mmol) was added 1N NaOH (50.0 mL), 25 mL methanol, 25 mL ethanol and the reaction mixture was stirred for 24 h. The crude reaction mixture was concentrated, acidified (pH~2), saturated with NaCl, and washed $CH_2Cl_2$ (3 x 75 mL). The organic layers were combined, washed sat. NaCl, dried $MgSO_4$, and evaporated to dryness to afford the monodispersed compound **V** as a white solid. The crude solid was purified via flash chromatography (silica, ethyl acetate) to give **V**, 858 mg (40 %).

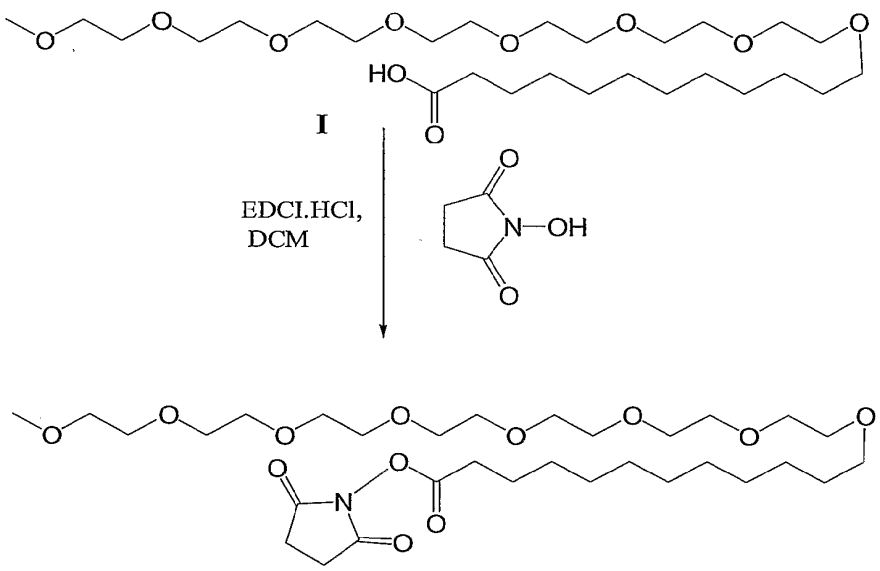
[219] Monodispersed mPEG7-C16-acid **V** (324 mg, 544 mmol) was dissolved in 15 ml of anhydrous methylene chloride and then solution of N-hydroxysuccinimide (94 mg, 816 mmol) and 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide. HCl (EDCI·HCl, 156 mg, 816 mmol) in anhydrous methylene chloride added. Reaction was stirred for 24 hours, then washed with 1N HCl, water, dried over $MgSO_4$, filtered and concentrated. Crude material was purified by flash chromatography (silica, gradient elution: 2-5 % methanol in $CHCl_3$), to afford monodispersed activated MPEG7-C16 **VI** as a clear oil (290 mg, 77%).




**9.4 Activation of PEG-Alkyl Modifying Moiety (12-(2-{2-[2-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-dodecanoic acid 2,5-dioxo-pyrrolidin-1-yl ester)**

[220] Monodispersed mPEG7-C12-acid **I** (500 mg, 0.78 mmol) was dissolved in 20 ml of anhydrous methylene chloride and then solution of N-hydroxysuccinimide (160 mg, 1.39 mmol) and 1-ethyl-3-(3'-

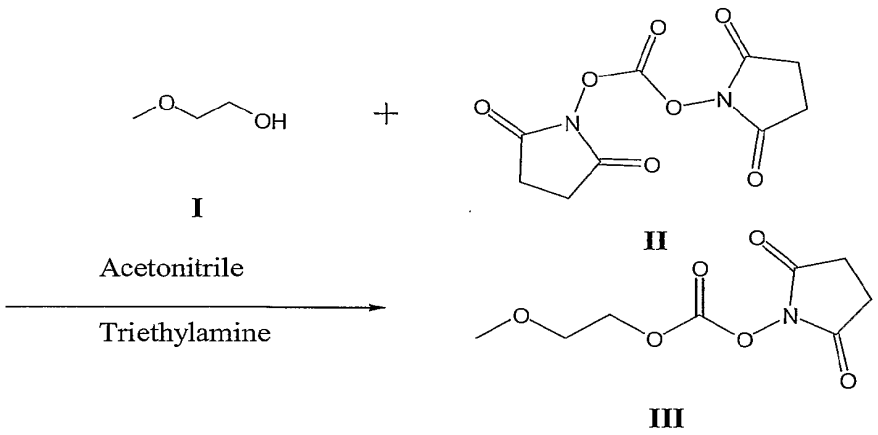
dimethylaminopropyl)carbodiimide. HCl (EDCI·HCl, 233 mg, 1.390 mmol) in anhydrous methylene chloride added. Reaction was stirred for 24 hours, then washed with 1N HCl, water, dried over $MgSO_4$, filtered and concentrated. Crude material was purified by flash chromatography (silica, gradient elution: 2-5 % methanol in $CHCl_3$), to afford monodispersed activated MPEG7-C16 **VI** as a clear oil (370 mg, 62%).




**II**

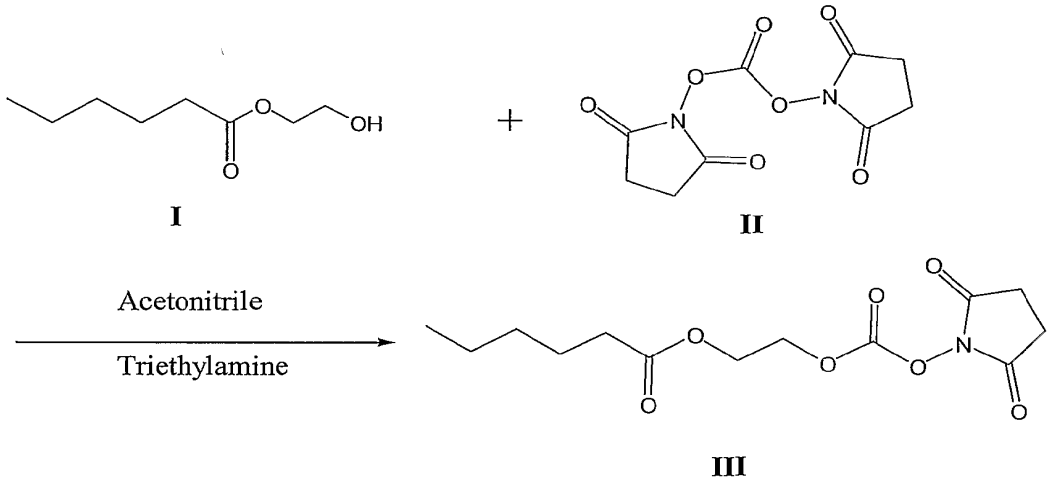
### 9.5 Synthesis of PEG Modifying Moiety (carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-methoxy-ethyl ester)

[221] Monodispersed branched MPEG1 **I** (200 mg, 2.63 mmol) was dissolved in acetonitrile (20 mL) and disuccinimidyl carbonate (DSC, **II**, 1.00 g , 3.94 mmol) was added. Then triethylamine (0.399 g , 3.94 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (20 mL), washed ethyl acetate (2 x 50 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the solid **III** (0.346 g, 60% yield). ESI MS: *m/e* 218.09 $(M+H)^+$.



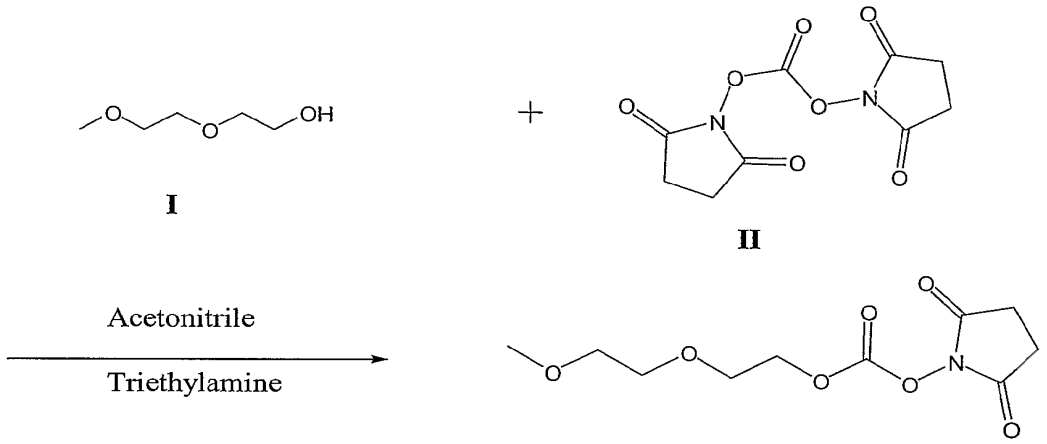

### 9.6 Synthesis of Hydrolysable MicroPAGylated Modifying Moiety (hexanoic acid 2-(2,5-dioxo-pyrrolidin-1-yloxycarbonyloxy)-ethyl ester)

[222] Monodispersed branched C6-PEG1 **I** (100 mg, 0.625 mmol) was dissolved in acetonitrile (10 mL) and disuccinimidyl carbonate (DSC, **II**, 0.240 g , 0.936 mmol) was added. Then triethylamine (0.095 g , 0.936 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (10 mL), washed ethyl acetate (2 x 20 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded an off-white solid **III** (0.146 g, 78% yield). ESI MS: *m/e* 302.29 $(M+H)^+$.



### 9.7 Synthesis of Linear mPEG Modifying Moiety (carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-(2-methoxy-ethoxy)-ethyl ester)

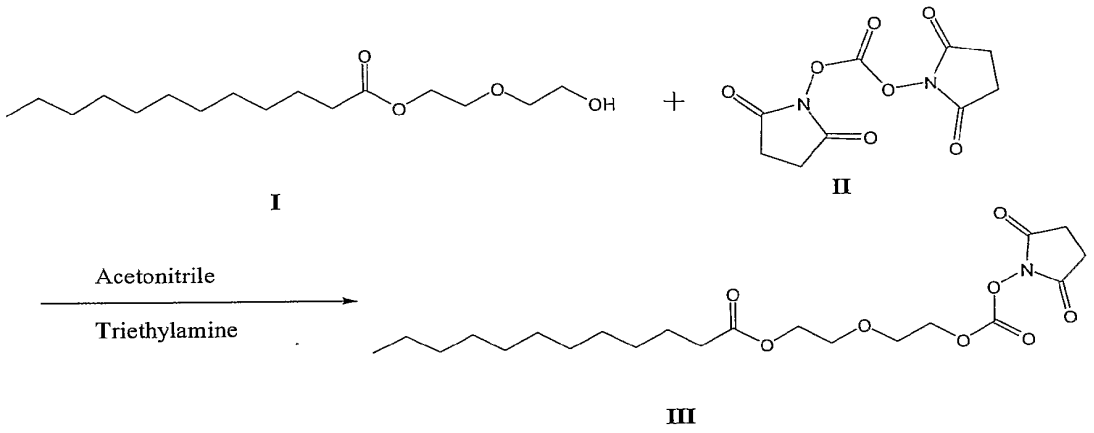
[223] Monodispersed branched MPEG2 **I** (470 mg, 3.91 mmol) was dissolved in acetonitrile (20 mL) and disuccinimidyl carbonate (DSC, **II**, 1.50 g , 5.87 mmol) was added. Then triethylamine (0.594 g , 5.87 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (20 mL), washed ethyl acetate (2 x 50 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the solid **III** (0..632 g, 62% yield). ESI MS: *m/e* 262.23 $(M+H)^+$.




**I** **II** **III**

### 9.8 Synthesis of Hydrolysable MicroPAGylated Modifying Moiety (dodecanoic acid 2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonyloxy)-ethoxy]-ethyl ester)

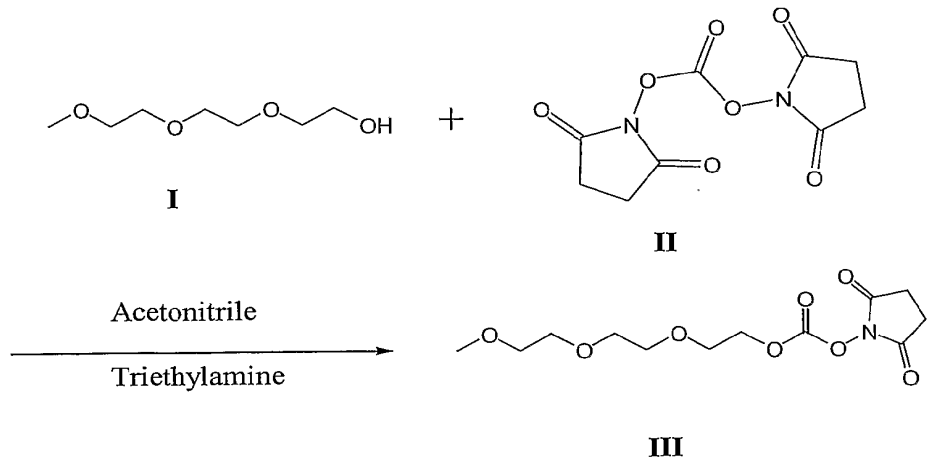
[224] Monodispersed branched C12-PEG2 **I** (200 mg, 0.69 mmol) was dissolved in acetonitrile (10 mL) and disuccinimidyl carbonate (DSC, **II**, 0.265 g , 1.035 mmol) was added. Then triethylamine (0.104 g , 1.035 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (10 mL), washed ethyl acetate (2 x 20 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the oil **III** (0.247 g, 83% yield). ESI MS: *m/e* 430.50$(M+H)^+$.




I II III

## 9.9 Synthesis Linear PEG Modifying Moiety (carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl ester)

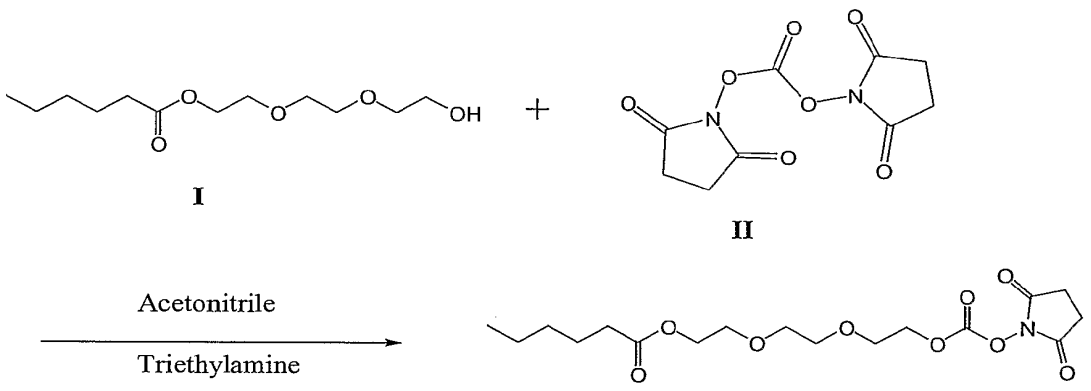
[225] Monodispersed branched MPEG3 **I** (200 mg, 1.21 mmol) was dissolved in acetonitrile (20 mL) and disuccinimidyl carbonate (DSC, **II**, 0.468 g, 1.82 mmol) was added. Then triethylamine (0.184g , 1.82 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (20 mL), washed ethyl acetate (2 x 50 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the solid **III** (0.206 g, 55% yield). ESI MS: *m/e* 306.11 $(M+H)^+$.




I II III

### 9.10 Synthesis of Hydrolysable MicroPAGylated Modifying Moiety (hexanoic acid 2-{2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonyloxy)-ethoxy]-ethoxy}-ethyl ester)

[226] Monodispersed branched C6-PEG3 **I** (200 mg, 0.80 mmol) was dissolved in acetonitrile (20 mL) and disuccinimidyl carbonate (DSC, **II**, 0.309 g , 1.209 mmol) was added. Then triethylamine (0.122 g , 1.209 mmol) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (10 mL), washed ethyl acetate (2 x 20 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the oil **III** (0.203 g, 64% yield). ESI MS: *m/e* 390.40 $(M+H)^+$.




**I** **II** **III**

### 9.11 Synthesis of Benzyl Elimination Hydrolysable Oligomer (6-{2-[2-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-hexanoic acid 4-(4-nitro-phenoxycarbonyloxymethyl)-phenyl ester)

[227] Potassium *tert*-butoxide (3.64 g, 32.4 mmol) was dissolved in 250 mL THF. $MPEG_6$ alcohol (9.58 g, 32.3 mmol) in 10 mL THF was added. The solution was stirred for two hours The mesylate (7.0 g, 29.4 mmol) prepared from commercially available ethyl 6-hydroxy-hexanoate was dissolved in 15 mL THF and added to the PEG solution. The reaction was stirred at room temperature overnight. The reaction was quenched with 25 mL MeOH and filtered through a short pad of Celite. The filtrate was concentrated in vacuo and the residue purified by flash chromatography (EtOAc/2 % MeOH) to give 3.19 g (25 %) of **I**. ESI MS: *m/e* 461.07 $(M+Na)^+$.

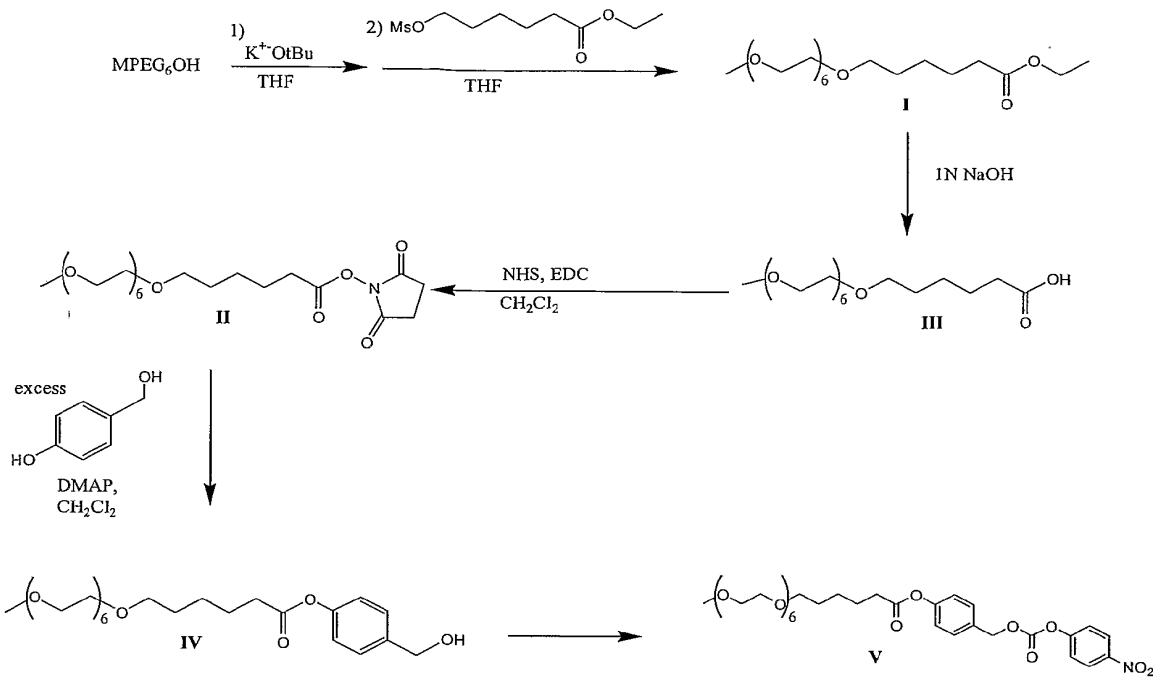
[228] To hydrolyze the ethyl ester, 1.1 g (2.51 mmol) of **I** was treated with 35 mL 1 N NaOH. After six hours, the initially cloudy mixture had become a clear, yellow-colored solution. The mixture was saturated

with NaCl and acidified with concentrated HCl until the pH was 2. The solution was extracted with 100 mL $CH_2Cl_2$. The organics were dried over $Na_2SO_4$, filtered and concentrated in vacuo to afford 0.80 g (78 %) of the carboxylic acid **II**. ESI MS: *m/e* 411.07 $(M+H)^+$, 433.10 $(M+Na)^+$.

[229] Carboxylic acid **III** (0.80 g, 1.95 mmol) was dissolved in 16 mL $CH_2Cl_2$ and placed under $N_2$. To the solution, 0.486 g (2.5 mmol) EDC and 0.288 g (2.5 mmol) N-hydroxysuccinimide (NHS) were added. After five hours, another 0.2 g EDC and 0.12 g NHS were added to drive reaction to completion. When TLC indicated that no unreacted carboxylic acid remained, the mixture was diluted with 60 mL $CH_2Cl_2$ and washed with cold 1 N HCl (1 x 100 mL), cold water (2 x 100 mL) and brine (3 x 100 mL). The organics were dried over $Na_2SO_4$, filtered and concentrated in vacuo to yield 0.71 g (71 %) of **III**. ESI MS: *m/e* 508.17 $(M+H)^+$, 530.07 $(M+Na)^+$.

[230] In 120 mL dry $CH_2Cl_2$, 4-hydroxybenzyl alcohol (2.93 g, 3.6 mmol) and 2.98 g (24.4 mmol) DMAP were dissolved. Compound **III** (1.2 g, (2.37 mmol) was dissolved in another 40 mL $CH_2Cl_2$ and added. The reaction was stirred at room temperature overnight. The mixture was washed with 1 N HCl (2 x 200 mL) and brine (2 x 200 mL). The organics were dried over $Na_2SO_4$, filtered and evaporated to dryness. The residue was purified *via* flash chromatography (silica, EtOAc/10 % MeOH) to give 0.701 g (58 %) of oligomer **IV**. ESI MS: 539.10 *m/e* $(M+Na)^+$.

[231] The oligomer **IV** (0.562 g, 1.09 mmol) was dissolved in 15 mL dry $CH_2Cl_2$. To this solution was added 0.23 mL (1.64 mmol) TEA and 0.329 g (1.64 mmol) $_p$-nitro-phenylchloroformate. The reaction was stirred overnight at room temperature. The mixture was then diluted with a further 15 mL $CH_2Cl_2$ and washed with 15 mL 1 N HCl followed by 15 mL water. The organics were dried over $MgSO_4$, filtered and concentrated to dryness. The crude product was purified via flash chromatography (silica, gradient elution: 3/1 EtOAc/hexanes – EtOAc) to give 504 mg (74 %) of the activated oligomer. ESI MS: *m/e* 682.72 $(M+H)^+$, 704.72 $(M+Na)^+$.




### 9.12 Synthesis of Aryl Carbamate Hydrolysable Modifying Moiety (carbonic acid 4-(6-{2-[2-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-hexyloxy)-phenyl ester 4-nitro-phenyl ester)

[232] $MPEG_6$ alcohol (10.0 g, 33.7 mmol) was dissolved in 40 mL dry $CH_2Cl_2$ and the resulting solution was cooled to 0 °C in an ice bath. TEA (5.64 mL, 40.5 mmol) was added and then 3.13 mL (40.5 mmol) methanesulfonyl chloride was added drop wise. The reaction was stirred for thirty minutes at 0 °C and then removed from the ice bath, allowed to come to room temperature and stirred overnight. The reaction mixture was diluted with more $CH_2Cl_2$ and washed with saturated $NaHCO_3$ and water. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford 12.4 g (98 %) of $MPEG_6$ mesylate, **I**.

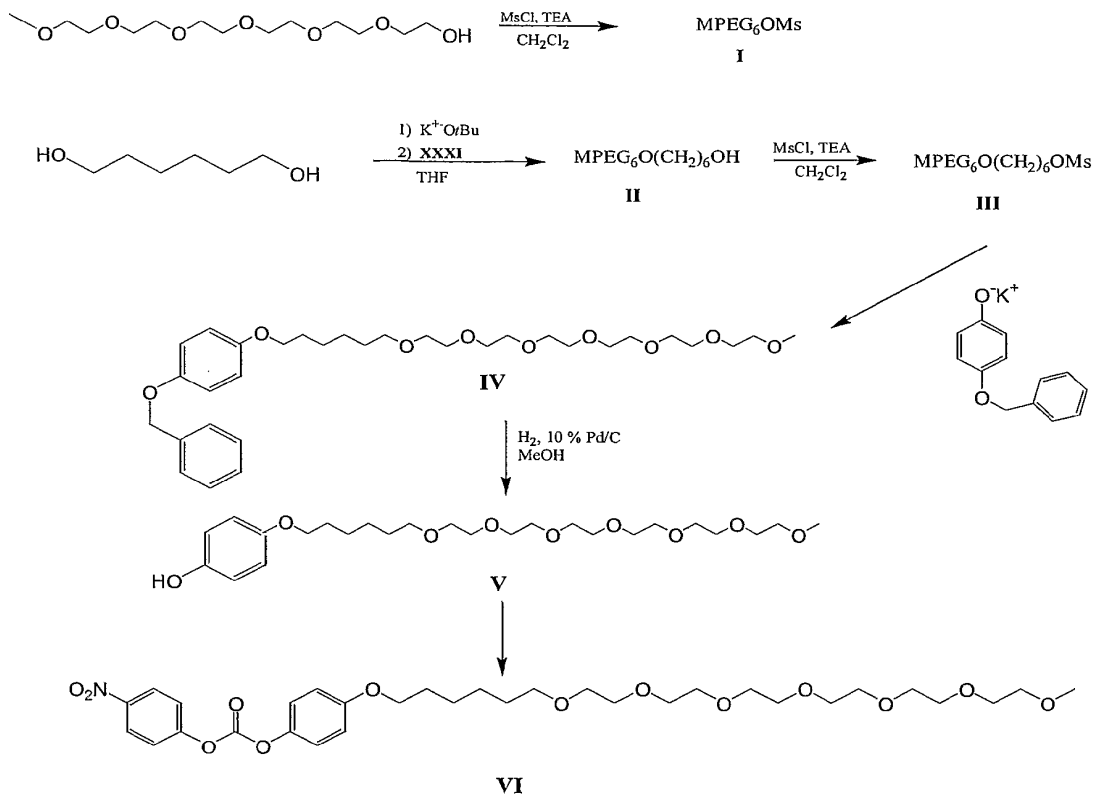
[233] A solution of 1,6-hexanediol was prepared from 6.311 g of the diol (53.41 mmol) and 180 mL of dry THF. The solution was cooled to 0 °C and placed under a $N_2$ atmosphere. Potassium *tert*-butoxide (5.996 g, 53.41 mmol) was added to the solution and the resulting mixture was stirred for one hour. **I** (10.0 g, 26.7 mmol) in 30 mL THF was added to the mixture. All was stirred for a further 30 minutes at 0 °C, then allowed to warm to room temperature and stirred overnight. The reaction mixture was filtered through Celite. The Celite was rinsed with $CH_2Cl_2$ and the combined filtrate was concentrated in *vacuo*. The residue was redissolved in $CH_2Cl_2$ and washed with water. The organics were dried over $Na_2SO_4$, filtered and evaporated to dryness. Purification by flash chromatography (silica, $CHCl_3$/10 % MeOH). Some material was further purified by preparatory TLC (EtOAc/10 % MeOH). Combined yield was 3.923 g (37 %) of **II**.

[234] **II** (3.923 g, 9.89 mmol) was dissolved in 16 mL dry $CH_2Cl_2$ and the resulting solution was cooled to 0 °C and placed under $N_2$. Triethylamine (1.65 mL, 11.9 mmol) was added and then 0.92 mL (11.9 mmol) methanesulfonyl chloride was added dropwise. The reaction was stirred at 0 °C for a further thirty minutes and then allowed to come to room temperature and stirred overnight. The reaction mixture was diluted with more $CH_2Cl_2$ and washed with saturated $NaHCO_3$ and water. The organics were dried over $Mg_2SO_4$, filtered and concentrated *in vacuo* to provide 4.25 g (91 %) of mesylate **III**.

[235] In a flask containing 50 mL dry THF, 5.001g (24.97 mmol) of 4-benzyloxyphenol was dissolved. Potassium *tert*-butoxide (1.202 g, 9.989 mmol) was added and the resulting mixture was stirred for one hour at room temperature under an inert atmosphere. A solution of 3.950 g (8.324 mmol) of **III** in 20 mL THF was added. After a further 18 hours, the entire mixture was quenched with 10 mL MeOH and filtered through a short pad of Celite. The filtrate was concentrated in vacuo and the residue purified by flash chromatography (silica, EtOAc/MeOH 20:1) to provide 1.584 g (33 %) of compound **IV**. ESI MS: *m/e* 579.16 $(M+H)^+$, 601.14 $(M+Na)^+$.

[236] Compound **IV** (0.683 g, 1.18 mmol) was dissolved in 20 mL MeOH. To this solution was added a slurry of 136 mg of 5 % Pd/C in MeOH. The entire mixture was placed under $H_2$ and stirred until TLC confirmed that all of the starting material had been consumed. The mixture was then filtered through Celite and the filtrate was evaporated to dryness to yield 412 mg (71 %) of **V**. ESI MS: *m/e* 511.09 $(M+Na)^+$.

[237] The oligomer **V** (0.605 g, 1.09 mmol) was dissolved in 15 mL dry $CH_2Cl_2$. To this solution was added 0.23 mL (1.64 mmol) TEA and 0.329 g (1.64 mmol) $_p$-nitro-phenylchloroformate. The reaction was stirred overnight at room temperature. The mixture was then diluted with a further 15 mL $CH_2Cl_2$ and washed with 15 mL 1 N HCl followed by 15 mL water. The organics were dried over $MgSO_4$, filtered and concentrated to dryness. The crude product was purified via flash chromatography (silica, gradient elution: 3/1 EtOAc/hexanes – EtOAc) to give 491 mg (75 %) of the activated oligomer. ESI MS: *m/e* 654.71 $(M+H)^+$, 675.71 $(M+Na)^+$.

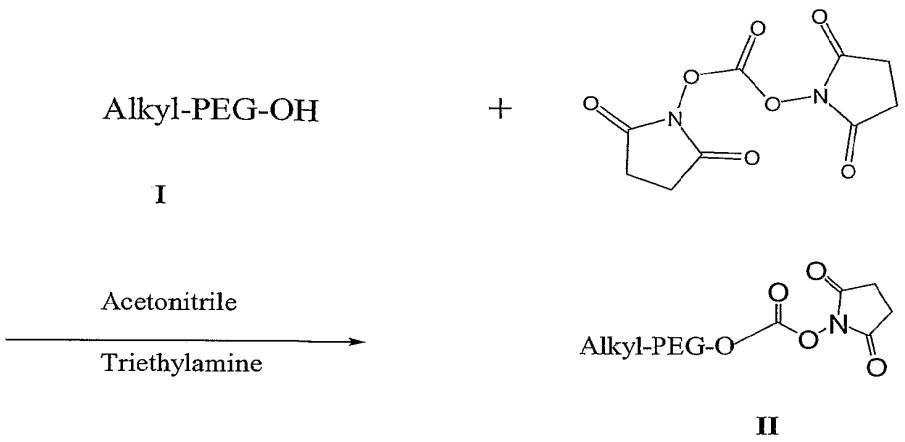



### 9.13 Methods For Activating Oligomeric Moieties

[238] The present example describes methods by which a oligomeric moiety of the present invention may be activated.

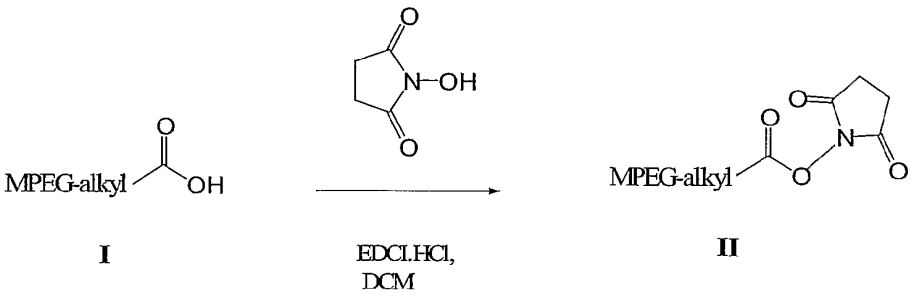
#### 9.13.1 Method I - Activation Using DSC

[239] Alkyl-PEG-OH, **I** (0.4 mmol, 1 eq.) was dissolved in acetonitrile (5 mL) and disuccinimidyl carbonate (DSC, 0.6 mmol, 1.5 eq.) was added. Then triethylamine (1.2 mmol, 1.5 eq.) was added dropwise and after 10 min the reaction mixture became clear. Reaction was stirred overnight at RT. After stirring for ~16h, the crude reaction was evaporated to dryness and then dissolved in sat. $NaHCO_3$ (10 mL), washed ethyl acetate (2 x 20 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, EtOAc/MeOH, 10:1) afforded the activated oligomer **II**.

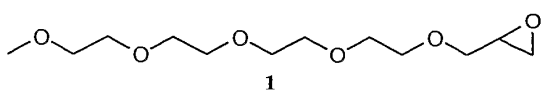



### 9.13.2 METHOD II: Activation Using NHS

[240] MPEG-alkyl-acid **I** (0.544 mmol, 1.0 eq.) was dissolved in 15 ml of anhydrous methylene chloride and then solution of N-hydroxysuccinimide (0.816 mmol, 1.5 eq.) and 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide. HCl (EDCI·HCl, 0.816 mmol, 1.5 eq.) in anhydrous methylene chloride added. Reaction was stirred for several hours, then washed with 1N HCl, water, dried over $MgSO_4$, filtered and concentrated. Crude material was purified by flash chromatography (silica, gradient elution: 2-5 % methanol in $CHCl_3$), to afford activated MPEG-alkyl-acid **II**.

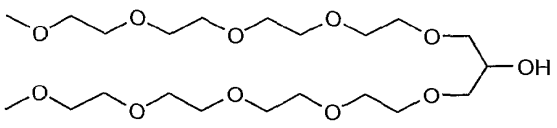



### 9.14 Synthesis of Modifying Moiety with Branched PEG (6-[2-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-1-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxymethyl)-ethoxycarbonylamino]-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester)



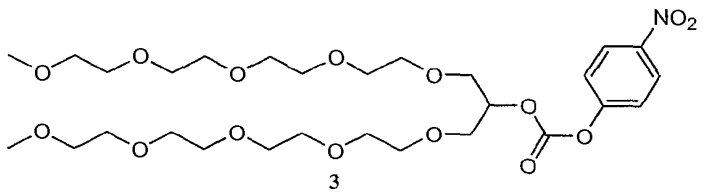
1

[241] **1**. Tetraethylene glycol monomethyl ether (14.0 g, 67 mmol) was dissolved in tetrahydrofuran (90 mL) and NaH (1.77 g, 74 mmol) was added portion wise and reaction was stirred for 2 h. Then epichlorohydrin (26.3 mL, 0.34 mol) was added dropwise and the reaction was stirred at RT for 48 h. The crude reaction mixture was filtered through Celite and washed $CH_2Cl_2$ (250 mL). The filtrate was washed $H_2O$ (2 x 250 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate) afforded **1** a clear oil (10.15 g, 57% yield).



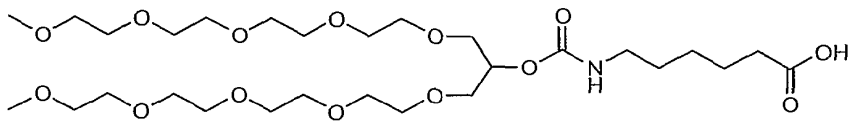

**2**

[242] **2**. Tetraethylene glycol monomethyl ether (7.96g, 0.038 mol) and **1** (10.1, 0.038 mol) were dissolved in $CH_2Cl_2$ (100 mL) and $BF_3{\cdot}OEt_2$ (0.48 mL, 0.0038 mol) was added. The reaction was stirred overnight at RT. Crude reaction was diluted with $CH_2Cl_2$ (200 mL), washed sat. $NaHCO_3$ (300 mL), $H_2O$ (300 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 10:1) afforded **2** a clear oil (4.5 g, 25% yield).




**3**

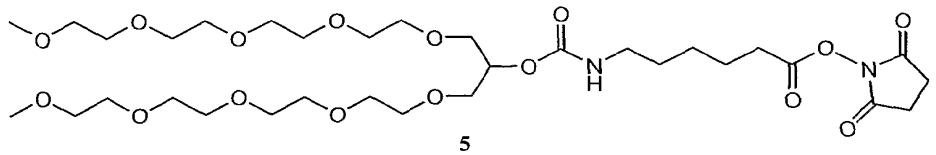
[243] **3**. 4-Nitrochloroformate (2.87 g, 14.3 mmol) and **2** (4.5 g, 9.5 mmol) were dissolved in $CH_2Cl_2$ (45 mL). After stirring for 10 min, TEA (2.1 mL, 15 mmol) was added and reaction stirred overnight at RT. Crude reaction was diluted with $CH_2Cl_2$ (130 mL), washed 1M HCl (175 mL), $H_2O$ (175 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 15:1) afforded **3** a yellowish oil (2.38 g, 40% yield).




**4**

[244] **4**. 6-Aminocaproic acid (0.126 g, 0.96 mmol) and $K_2CO_3$ (0.221 g, 1.6 mmol) were dissolved in $H_2O$ (DI, 5 mL). Then **3** (0.5 g, 0.8 mmol) was dissolved in THF (0.7 mL) and added dropwise. The

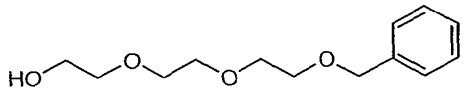
reaction was stirred overnight at RT. Crude reaction was diluted with $H_2O$ (20 mL), acidified to pH ~ 1 with HCl, washed $CH_2Cl_2$ (2 x 25 mL), organic layers dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, $CHCl_3$ / MeOH, 15:1) afforded **4** a clear oil (0.428 g, 85% yield)



5

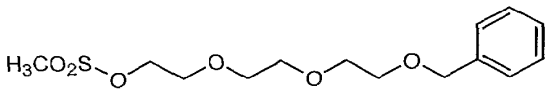
[245] **5**. Activated using **Method II:** **4** (0.40 g, 0.64 mmol), N-hydroxysuccinimide (0.088 g, 0.77 mmol), EDCI (0.160 g, 0.83 mmol), and $CH_2Cl_2$ (5 mL). Column chromatography (silica, ethyl acetate / MeOH, 10:1) afforded **5** a clear oil (0.320 g, 69% yield).

## 9.15 Synthesis of Linear PEG-Alkyl Modifying Moiety (Carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-{2-[2-(2-{2-[2-(2-hexyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl ester)



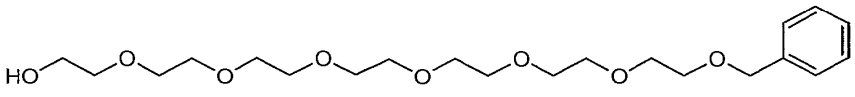
1

[246] **1**. Triethylene glycol (30 g, 0.2 mol) was dissolved in a solution of NaOH (8 g in 8 mL of $H_2O$) and stirred for 10 min. Then benzyl chloride (7 mL, 0.062 mol) was added and the reaction mixture was heated to 100 °C and stirred overnight. The crude reaction was diluted with sat NaCl (500 mL), washed $CH_2Cl_2$ (2 x 400 mL), organic layers dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate to ethyl acetate / MeOH, 10:1) afforded **1** a yellowish oil (9.87 g, 67% yield).



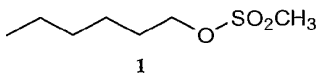
2

[247] **2**. To a solution of **1** (9.87 g, 0.041 mol) in $CH_2Cl_2$ (50 mL) was added TEA (7.1 mL, 0.054 mol). The solution was then cooled to 0 °C in an ice bath and then methanesulphonyl chloride (3.9 mL, 0.049 mol) dissolved in $CH_2Cl_2$ (10 mL) was added dropwise. The reaction was stirred at 0 °C for 0.5 h and then at RT for 4 hours. The crude reaction was filtered through Celite, washed $CH_2Cl_2$ (100 mL), filtrate washed with sat $NaHCO_3$ (150 mL), $H_2O$ (150 mL), dried $MgSO_4$, and evaporated to dryness to afford **2** a yellow oil (11.06 g, 85% yield).



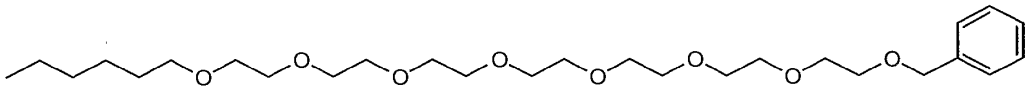
3

[248] **3**. Tetraethylene glycol (7.32 g, 0.038 mol) was dissolved in tetrahydrofuran (140 mL) and NaH was added portion wise over 0.5 h and the reaction was stirred for an additional 1 h. Then **2** (6.0 g, 0.019 mol) was dissolved in $CH_2Cl_2$ (20 mL) and added dropwise and the reaction was stirred overnight at RT. Crude reaction was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved in $CH_2Cl_2$ (150 ml), washed $H_2O$ (150 mL), sat. $NaHCO_3$ (150 mL), $H_2O$ (150 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 10:1) afforded **3** yellowish oil (3.83 g, 49% yield).



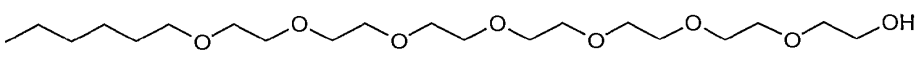
1

[249] **4**. Prepared in the same manner as **2**: hexanol (6.2 mL, 0.05 mol), methanesulphonyl chloride (4.6 mL, 0.058 mol), TEA (8.6 mL, 0.065 mol), and $CH_2Cl_2$ (60 mL) afforded **4** a yellow oil (7.8 g, 86% yield).



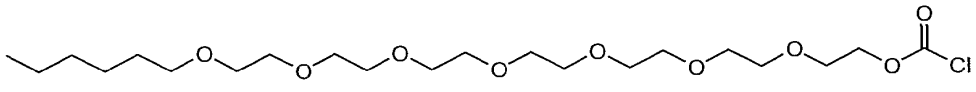
5

[250] **5**. To a solution of **3** (5.45 g, 0.13 mol) in tetrahydrofuran (160 mL) was added potassium tert-butoxide (1.60 g, 0.0144 mol) and the reaction was stirred for 1.5 h. Then **4** (2.59 g, 0.0144 mol) dissolved in tetrahydrofuran (20 mL) was added dropwise and the reaction was stirred overnight. The crude reaction was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved in ethyl acetate (150 mL), washed $H_2O$ (2 x 150 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate) afforded **5** a yellowish oil (2.40 g, 36% yield).



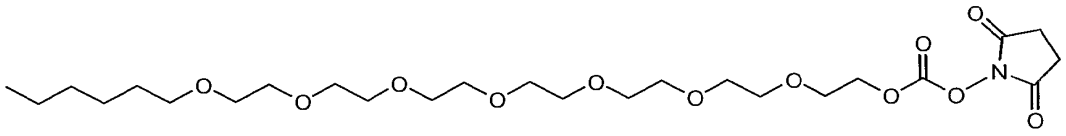
6

[251] **6**. To a solution of **5** (2.4 g, 4.8 mmol) in ethyl acetate (16 mL) was added palladium on activated carbon 10 wt% (1.0 g) and the reaction vessel sealed with a septum. A balloon containing $H_2$ was then inserted in the septum via needle and the reaction was stirred overnight at RT. Crude reaction mixture was filtered through Celite, washed ethyl acetate, and evaporated to dryness to afford **6** a clear oil (1.61 g, 82% yield).



**7**

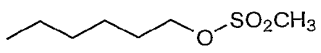
[252] **7**. A phosgene solution (15 mL of a 20% phosgene in toluene) was cooled to -10 °C and **6** (1.60 g, 3.9 mmol) dissolved in toluene (5 mL) was added dropwise. The reaction was stirred at -10 °C for 0.5 h and then 4 h at RT. The phosgene and toluene was then distilled off and the resultant oil was dried under vacuum to afford **7** a yellowish oil.



**8**

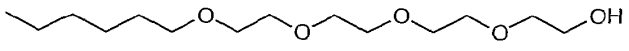
[253] **8**. Activated using **Method II:** **7** (1.65 g, 0.79 mmol), N-hydroxysuccinimide (0.437 g, 3.8 mmol), TEA (2.7 mL, 3.8 mmol), and $CH_2Cl_2$ (10 mL). Column chromatography (silica, ethyl acetate / MeOH, 15:1) afforded **8** a clear oil (1.06 g, 57% yield).

## 9.16 Synthesis Branched Alkyl-PEG-Alkyl (6-[2-(2-{2-[2-(2-Heptyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-1-(2-{2-[2-(2-heptyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxymethyl)-ethoxycarbonylamino]-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester)



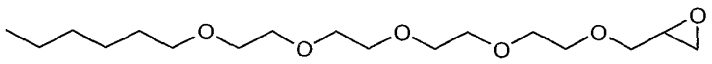
**1**

[254] **1**. Prepared in the same manner as shown in Example 9.15: hexanol (18 mL, 0.15 mol), methanesulphonyl chloride (12.3 mL, 0.16 mol), TEA (25 mL, 0.18 mol), and $CH_2Cl_2$ (180 mL) afforded **1** a yellow oil (23.1 g, 85% yield).



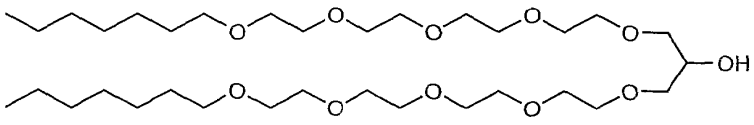
2

[255] **2**. Tetraethylene glycol (50.5 g, 0.26 mol) was dissolved in tetrahydrofuran (350 mL) and potassium *tert*-butoxide (29.2 g, 0.26 mol) was added portion wise over 0.5 h. The reaction was stirred an additional 1h and then **1** (23.0 g, 0.13 mol) dissolved in THF (50 mL) was added. The reaction was stirred overnight at RT. The crude reaction was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved in $CH_2Cl_2$ (300 mL), washed $H_2O$ (2 x 300 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate) afforded **2** a clear oil (18.51 g, 51% yield).



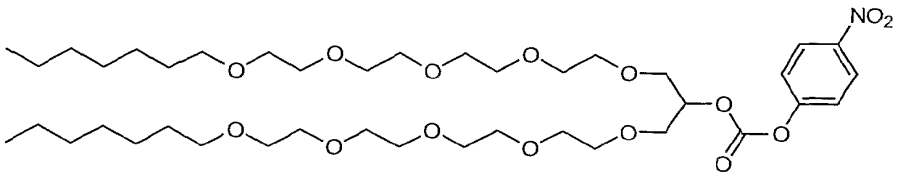
3

[256] **3**. To a solution of **2** (10.0 g, 36 mmol) in tetrahydrofuran (60 mL) was added NaH (0.95 g, 40 mmol) portion wise and reaction was stirred for 0.5 h. Then epichlorohydrin (14.1 mL, 0.34 mol) was added dropwise and the reaction was stirred at RT for 48 h. The crude reaction mixture was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved $CH_2Cl_2$ (200 mL), washed sat. NaCl (200 mL), sat. $NaHCO_3$ (200 mL), $H_2O$ (200 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / hexanes, 10:1) afforded **3** a clear oil (5.46 g, 45% yield).



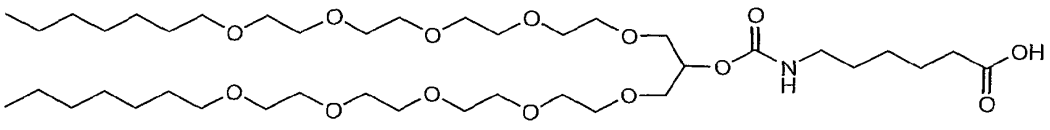
4

[257] **4**. To a solution of **2** (4.54 g, 16 mmol) and **3** (5.46, 16 mmol) in $CH_2Cl_2$ (50 mL) was added $BF_3.OEt_2$ (0.48 mL, 0.0038 mol). The reaction was stirred overnight at RT. Crude reaction was diluted with $CH_2Cl_2$ (50 mL), washed sat. $NaHCO_3$ (100 mL), $H_2O$ (100 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate to ethyl acetate / MeOH, 10:1) afforded **4** a clear oil (2.40 g, 24% yield).



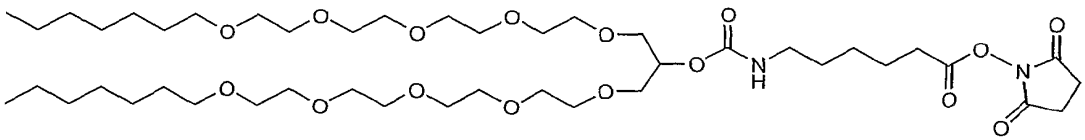
5

[258] **5**. 4-nitrochloroformate (1.18 g, 5.8 mmol) and **4** (2.4 g, 3.9 mmol) were dissolved in $CH_2Cl_2$ (25 mL). After stirring for 10 min, TEA (0.89 mL, 6.4 mmol) was added and reaction stirred overnight at RT. Crude reaction was diluted with $CH_2Cl_2$ (75 mL), washed 1M HCl (100 mL), $H_2O$ (100 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate) afforded **5** a yellowish oil (1.04 g, 34% yield).



6

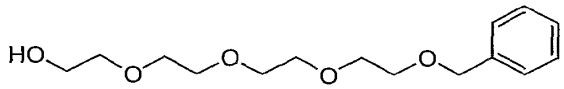
[259] **6**. 6-Aminocaproic acid (0.157 g, 1.2 mmol) and $K_2CO_3$ (0.276 g, 2.0 mmol) were dissolved in $H_2O$ (DI, 8 mL). Then **5** (0.80 g, 1.0 mmol) was dissolved in THF (1.0 mL) and added dropwise. Oil droplets formed when 3 was added and ethanol (2 mL) was added and the reaction was stirred overnight at RT. Crude reaction was diluted with $H_2O$ (30 mL), acidified to pH ~ 1 with HCl, washed $CH_2Cl_2$ (2 x 35 mL), organic layers dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 20:1) afforded **6** a clear oil (0.720 g, 46% yield)



7

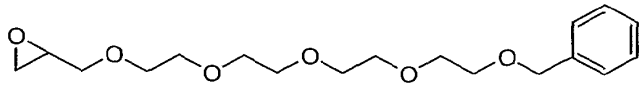
[260] 7. Activated using **Method II: 6** (0.356 g, 0.46 mmol), N-hydroxysuccinimide (0.063 g, 0.55 mmol), EDCI (0.115 g, 0.6 mmol), and $CH_2Cl_2$ (3 mL). Column chromatography (silica, ethyl acetate) afforded 7 a clear oil (0.180 g, 45% yield).

### 9.17 Hexanoic acid 2-[2-(2-{2-[2-[6-(2,5-dioxo-pyrrolidin-1-yloxycarbonyloxy)-hexylcarbamoyloxy]-3-(2-{2-[2-(2-hexanoyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-propoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester



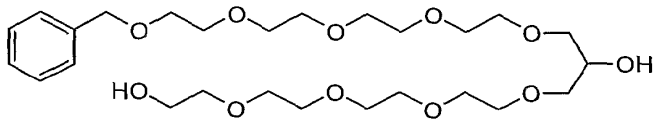
1

[261] **1.** Prepared in the same manner as shown in example 9.15: Tetraethylene glycol (58.27 g, 0.3 mol), NaOH solution (12 g in 12 mL of H2O), benzyl chloride (10.6 mL, 0.092 mol). Column chromatography (silica, ethyl acetate) afforded **1** a yellowish oil (16.8 g, 64% yield).



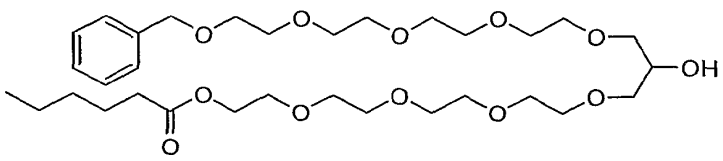
2

[262] **2.** Prepared in the same manner as shown in example 9.14: **1** (9.6 g, 34 mmol), NaH (0.898 g, 37 mmol), tetrahydrofuran (50 mL), epichlorohydrin (13.2 mL, 0.17 mol). Column chromatography (silica, ethyl acetate) afforded **2** a clear oil (6.78 g, 58% yield).



3

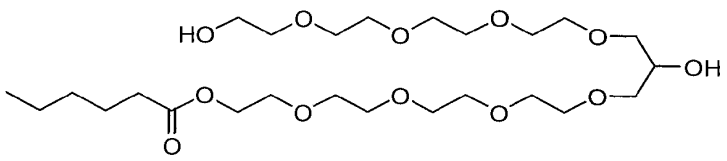
[263] **3.** Prepared in the same manner as shown in example 9.14: Tetraethylene glycol (4.85 g, 20 mmol), **2** (6.78 g, 25 mmol), $BF_3{\cdot}OEt_2$ (0.25 mL, 2.0 mmol), $CH_2Cl_2$ (75 mL). Column chromatography (silica, $CHCl_3$ / MeOH, 20:1) afforded **3** a clear oil (2.85 g, 27% yield).



4

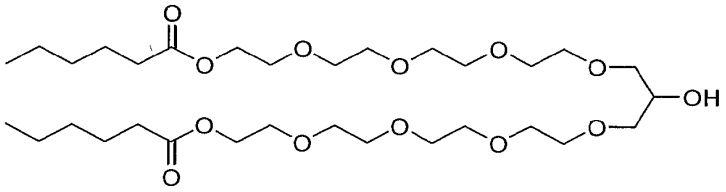
[264] **4.** To a solution of **3** (2.80 g, 5.2 mmol) in $CH_2Cl_2$ (20 mL), was added TEA (0.8 mL, 5.7 mmol) and the solution was cooled to 0 °C. Then hexanoyl chloride (0.73 mL, 5.2 mmol) was added dropwise. The reaction was stirred at 0 °C for 0.5 h, then RT overnight. Crude reaction was diluted with $CH_2Cl_2$ (80 mL), washed $H_2O$ (100 mL), sat. $NaHCO_3$ (100 mL), $H_2O$ (100 mL), dried $MgSO_4$, and evaporated to

dryness. Column chromatography (silica, ethyl acetate / MeOH, 15:1) to afford **4** a yellowish oil (1.95 g, 59% yield).



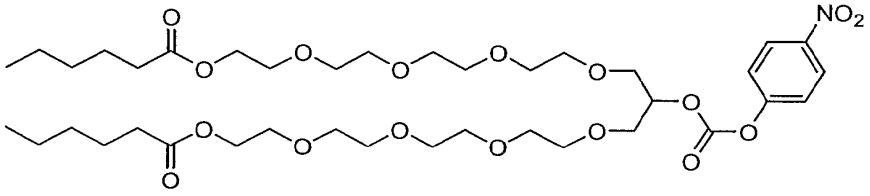
**5**

[265] **5**. To a solution of **4** (1.94 g, 3.1 mmol) in ethyl acetate (20 mL) was added palladium (1.60 g, 5 wt% on activated carbon). The reaction was sealed and stirred under H2 overnight. Crude reaction mixture was filtered through Celite, washed ethyl acetate and evaporated to dryness to afford **6** a clear oil (1.09 g, 65% yield).



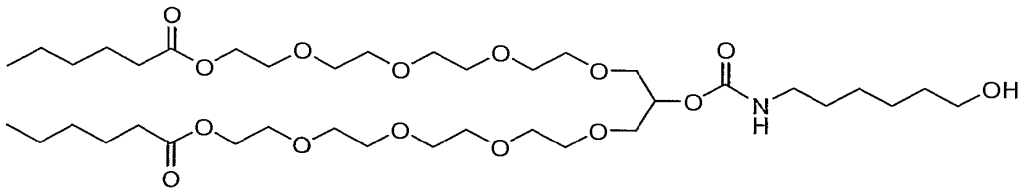
**6**

[266] **6**. To a solution of **5** (1.09 g, 2.0 mmol) in $CH_2Cl_2$ (14 mL), was added TEA (0.31 mL, 2.2 mmol) and the solution was cooled to 0 °C. Then hexanoyl chloride (0.28 mL, 2.0 mmol) was added dropwise. The reaction was stirred at 0 °C for 0.5 h, then RT overnight. Crude reaction was diluted with $CH_2Cl_2$ (86 mL), washed $H_2O$ (100 mL), sat. $NaHCO_3$ (100 mL), $H_2O$ (100 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 15:1) to afford **6** a yellowish oil (0.698 g, 55% yield).



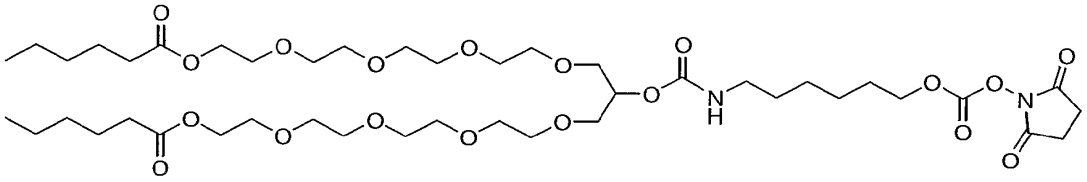
**7**

[267] **7**. Prepared in the same manner as shown in example 9.14: 4-Nitrochloroformate (0.332 g, 1.65 mmol), **6** (0.698 g, 1.1 mmol), TEA (0.28 mL, 2.0 mmol), $CH_2Cl_2$ (7 mL). Column chromatography (silica, ethyl acetate) afforded **7** a yellowish oil (0.688 g, 78% yield).



**8**

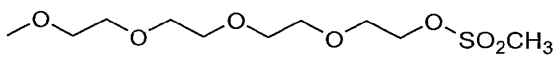
[268] **8**. Prepared in the same manner as shown in example 9.14: 6-amino-1-hexanol (0.142 g, 1.2 mmol), **7** (0.488 g, 0.6 mmol), TEA (0.12 mL, 0.9 mmol), $CH_2Cl_2$ (5 mL). Column chromatography (silica, ethyl acetate / MeOH, 15:1) afford **8** a clear oil (0.30 g, 65% yield).



**9**

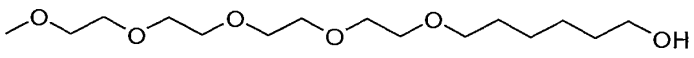
[269] **9**. Activated using **Method I**: N,N'-Disuccinimidyl carbonate (0.118 g, 0.46 mmol), **8** (0.30 g, 0.38 mmol), TEA (80 μL, 0.57 mmol), acetonitrile (4 mL). Washings afforded **9** a clear oil (0.344 g, 90% yield).

### 9.18 6-{2-(2-{2-[2-(2-Hexyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-1-[6-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-hexyloxymethyl]-ethoxycarbonylamino}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester



[270] **1**

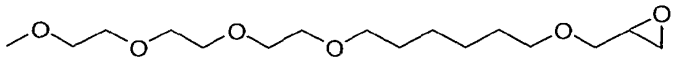
[271] **1**. Prepared in the same manner as shown in example 9.15: Tetraethylene glycol monomethyl ether (25 g, 0.12 mol), TEA (19.5 mL, 0.14 mol), methanesulphonyl chloride (10.0 mL, 0.13 mol), $CH_2Cl_2$ (100 mL) afforded **1** a yellow oil (31.35 g, 91% yield).



**2**

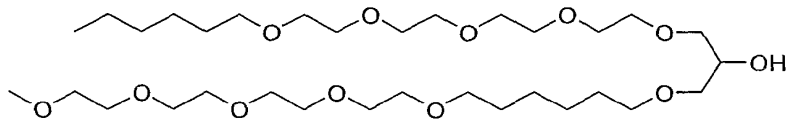
[272] **2**. 1,6-hexane diol (7.93 g, 0.084 mol) was dissolved in tetrahydrofuran (200 mL). Potassium *t*-butoxide (10.37 g, 0.092 mol) was added portionwise over 0.5 h and stirred an additional 1 h. Then **1** (12.0 g, 0.042 mol) dissolved in tetrahydrofuran (40 mL) added added dropwise via addition funnel and the reaction was stirred overnight at RT. Crude reaction was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved in $CH_2Cl_2$ (300 mL), washed $H_2O$ (2 x 300 mL),

dried $MgSO_4$, evaporated to dryness. Column chromatography (silica, ethyl acetate) afforded **2** a clear oil (5.09 g, 39% yield).



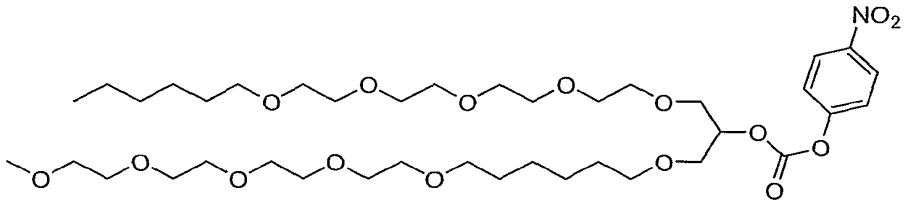
**3**

[273] **3**. To a solution of **2** (5.0 g, 16 mmol) in tetrahydrofuran (35 mL) was added NaH (0.422 g, 17.6 mmol) portion wise and reaction was stirred for 2 h. Then epichlorohydrin (6.3 mL, 8.0 mmol) was added dropwise and the reaction was stirred at RT for 48 h. The crude reaction mixture was filtered through Celite and washed $CH_2Cl_2$. The resultant oil was dissolved in $CH_2Cl_2$ (125 mL), washed $H_2O$ (125 mL), sat. $NaHCO_3$ (125 mL), $H_2O$ (125 mL), dried $MgSO_4$, and evaporated to dryness Column chromatography (silica, ethyl acetate / MeOH, 20:1) afforded **3** a clear oil (2.00 g, 34% yield).



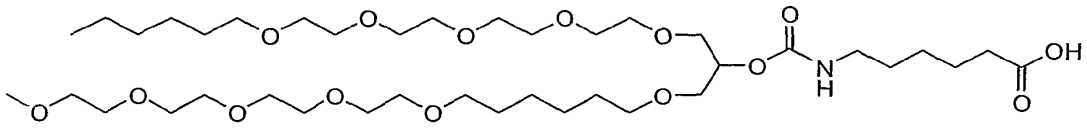
**4**

[274] **4**. Prepared in the same manner as shown in example 9.14: **2** (1.34 g, 4.8 mmol), **3** (1.75 g, 4.8 mmol), $BF_3 \cdot OEt_2$ (0.06 mL, 0.48 mmol), $CH_2Cl_2$ (30 mL). Column chromatography (silica, ethyl acetate / MeOH, 20:1) afforded **4** a clear oil (1.05 g, 34% yield).



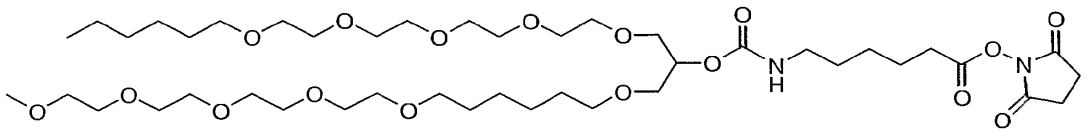
**5**

[275] **5**. Prepared in the same manner as shown in example 9.14: 4-Nitrochloroformate (0.464 g, 2.3 mmol), **4** (1.0 g, 9.5 mmol), TEA (2.1 mL, 1.55 mmol), $CH_2Cl_2$ (10 mL). Column chromatography (silica, ethyl acetate to ethyl acetate / MeOH, 20:1) afforded **5** a yellowish oil (0.663 g, 53% yield).



**6**

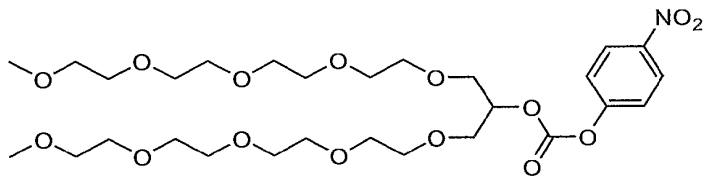
[276] **6**. Prepared in the same manner as shown in example 9.14: 6-Aminocaproic acid (0.054 g, 0.41 mmol), **5** (0.277 g, 0.34 mmol), $K_2CO_3$ (0.094 g, 0.068 mmol), $H_2O$ (DI, 5 mL) afforded **6** a clear oil (0.268 g, 96% yield).



**7**

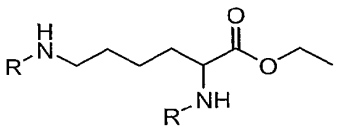
[277] **7**. Activated using **Method II: 6** (0.268 g, 0.34 mmol), N-hydroxysuccinimide (0.047 g, 0.41 mmol), EDCI (0.084 g, 0.44 mmol), and $CH_2Cl_2$ (4 mL). Column chromatography (silica, ethyl acetate / MeOH, 15:1) afforded **7** a clear oil (0.178 g, 58% yield).

### 9.19 Synthesis of multi-branched oligomer

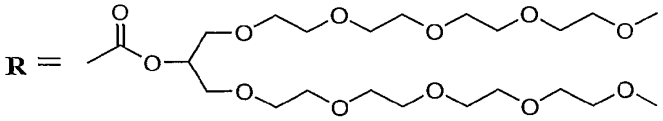


**1**

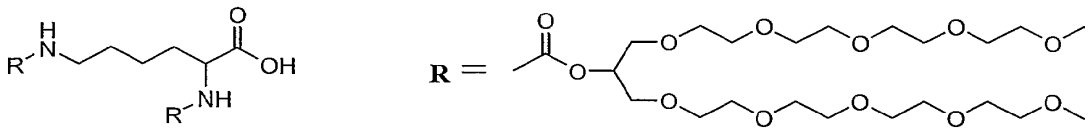
[278] **1**. As prepared in Example 9.14.



**2**

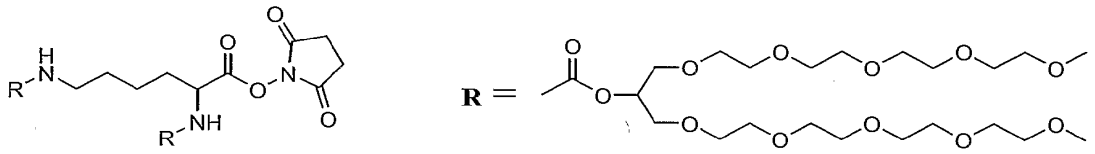


[279] **2**. L-Lysine ethyl ester dihydrochloride (0.32 g, 1.3 mmol) and **1** (1.71 g, 2.7 mmol) were dissolved in DMF (30 mL) and TEA (0.90 mL, 6.5 mmol) was added. The reaction mixture was stirred overnight at room temperature. Crude reaction was evaporated to dryness, dissolve $CH_2Cl_2$ (30 mL), washed 1M HCl (30 mL), $H_2O$ (30mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (Silica, $CHCl_3$ / MeOH, 25:1) afforded 2 a clear oil (1.01 g, 66% yield).



3

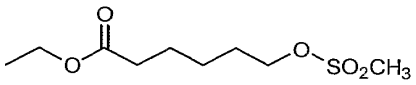
[280] 3. A solution of **2** (1.0 g, 0.85 mmol) in 1M NaOH (10mL) was stirred for 6H at RT. Crude reaction was diluted with sat. NaCl (50 mL), acidified to pH ~ 2, washed $CH_2Cl_2$ (2 x 50 mL), dried $MgSO_4$, and evaporated to dryness to afford **3** a clear oil (0.724 g, 74% yield).



4

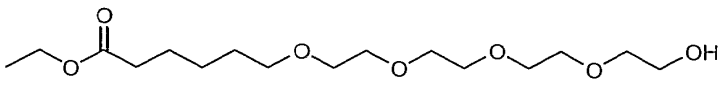
[281] 4. Activated using **Method II:** **3** (0.070 g, 0.61 mmol), N-hydroxysuccinimide (0.091 g, 0.79 mmol), EDCI (0.182 g, 0.95 mmol), and $CH_2Cl_2$ (7 mL). Column chromatography (silica, $CHCl_3$ / MeOH, 20:1) afforded **4** a clear oil (0.480 g, 63% yield).

## 9.20 Synthesis of Sugar-PEG-Alkyl Modifying Moiety 2,2-Dimethyl-propionic acid 4,5-bis-(2,2-dimethyl-propionyloxy)-6-(2,2-dimethyl-propionyloxymethyl)-3-{6-[2-(2-{2-[2-(2,5-dioxo-pyrrolidin-1-yloxycarbonyloxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-hexanoylamino}-tetrahydro-pyran-2-yl ester)



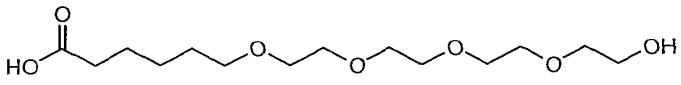
1

[282] 1. Prepared in the same manner as shown in Example 9.15: Ethyl 6-hydroxyhexanoate (8.0 g, 0.05 mol), methanesulphonyl chloride (4.6 mL, 0.06 mol), TEA (10 mL, 0.072 mol), and $CH_2Cl_2$ (32 mL) afforded **1** a yellow oil (11.15 g, 93% yield).



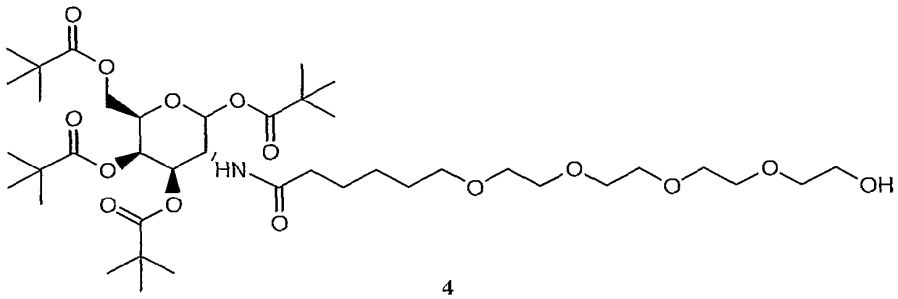
2

[283] **2**. Tetraethylene glycol (19.1 g, 0.098 mol) was dissolved in tetrahydrofuran (190 mL) and NaH (1.69 g, 0.071 mol) was added portion wise over 0.5 h. The reaction was stirred an additional 1h and then **1** (23.0 g, 0.13 mol) dissolved in tetrahydrofuran (10 mL) was added. The reaction was stirred overnight at RT. The crude reaction was filtered through Celite, washed $CH_2Cl_2$, and evaporated to dryness. The resultant oil was dissolved in $CH_2Cl_2$ (200 mL), washed sat. NaCl (200 mL), $H_2O$ (200 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH, 25:1) afforded **2** a clear oil (1.60 g, 10% yield).



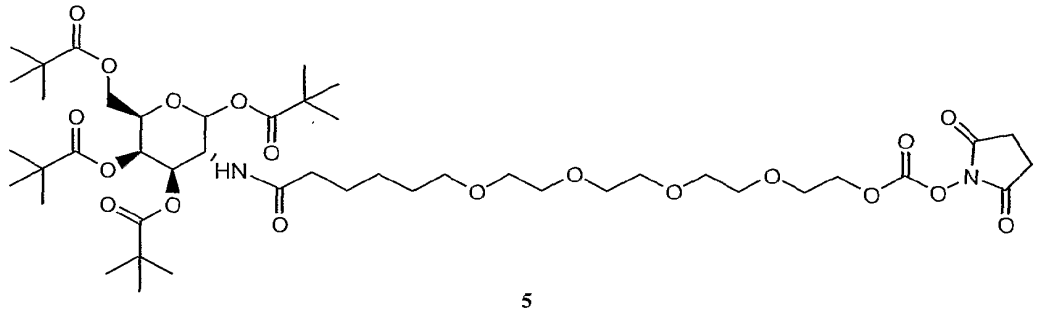
**3**

[284] **3**. A solution of **2** (1.60 g, 4.7 mmol) in 1 M NaOH (6 mL) was stirred for 2 h at RT. The crude reaction was diluted with sat. NaCl (24 mL), acidified to pH ~ 2, washed $CH_2Cl_2$ (2 x 30 mL), dried $MgSO_4$, and evaporated to dryness to afford **3** a clear oil (1.08 g, 73% yield).



**4**

[285] **4**. 2,3,4,6-*Tetra*-O-pivaloyl-ß-D-galactospyranosylamine (0.836 g, 1.6 mmol) and **3** (0.50 g, 1.6 mmol) were dissolved in $CH_2Cl_2$ (8 mL). Then EDCI (0.368 g, 1.92 mmol) was added and the reaction was stirred overnight at RT. After stirring overnight, reaction was incomplete so EDCI (0.368 g, 1.92 mmol) was added and the reaction was stirred overnight at RT. Crude reaction was diluted with $CH_2Cl_2$ (22 mL), washed 1 M HCl (30mL), $H_2O$ (30 mL), sat. NaCl (30 mL), dried $MgSO_4$, and evaporated to dryness. Column chromatography (silica, ethyl acetate / MeOH) afforded **4** a viscous oil (0.397 g, 31% yield).



5

[286] 5. Activated using **Method I:** **4** (0.397 g, 0.50 mmol), N-hydroxysuccinimide (0.063 g, 0.60 mmol), TEA (0.10 mL, 0.75 mmol), and acetonitrile (4 mL). Column chromatography (silica, ethyl acetate) afforded **5** a viscous oil (0.256 g, 56% yield).

## 9.21 Hydrolyzable, Non-hydrolyzable and Pegylated Natriuretic Conjugates

[287] The present example is provided to demonstrate the utility of the present invention for providing natriuretic compound conjugates that have been modified to include virtually all classes of oligomeric moieties, particularly non-hydrolyzed oligomers, microPAGylated oligomers, and hydrolyzable oligomers.

[288] The present hBNP conjugates were synthesized utilizing various oligomers conjugated at different positions on the peptide. The conjugates having the best combination of traits (agonist activity at the receptor, resistance to proteolysis, and oral bioavailability) have become the lead candidates for more extensive *in vivo* testing.

[289] The native hBNP was obtained from a contract peptide synthesis company. The amphiphilic oligomers that were used in the conjugation came from a supply of oligomers and from oligomers designed and synthesized specifically for conjugation to hBNP. The conjugation followed a three-tiered conjugation strategy as illustrated in Figure 2. Class 1 oligomers were tested first. Because extensive conjugation with Class 1 oligomers lessened activity, tri and tetra conjugates with Class 2 oligomers were investigated. Because Class 2 oligomers were not as efficacious, two pro-drug conjugates (Class 3 oligomers) were evaluated.

[290] A first class of conjugates is non-hydrolysable. For conjugates of this class, the drug substance that is dosed (i.e., the conjugate) is the substance that acts at the receptor. In other words, the oligomer and its attachment to the peptide remain intact from the time of dosing to the time of clearance. These oligomers may generally be comprised of an alkyl portion and a PEG portion. To maximize the effectiveness of the oligomer to make the conjugate orally available and resistant to proteolysis, the lengths of the alkyl and PEG portions can be altered and the order can be switched. The extent of conjugation (e.g. mono-, diconjugate) can also be manipulated. Some oligomers that can provide conjugates falling within this first class as well

as methods for providing such conjugates are described in U.S. Patent No. 5,359,030 to Ekwuribe; U.S. Patent No. 5,438,040 to Ekwuribe; U.S. Patent No. 5,681,811 to Ekwuribe; U.S. Patent No. 6,191,105 to Ekwuribe; U.S. Application No. 09/474,915, filed December 31, 1999; U.S. Application No. 09/459,443, filed December 13, 1999; and U.S. Application No. 09/873,797, filed June 4, 2001, the disclosures of which are incorporated by reference herein in their entireties.

[291] A second class of conjugates are microPAGylated. For conjugates of this class, the alkyl portion of the oligomer is cleaved once the conjugate is in the bloodstream. These conjugates may be particularly useful when conjugation occurs within a region of the natriuretic pepide that is necessary for binding to receptor, NPR-A. In such cases, the first class of oligomers may be beneficial to stability and delivery, but may be detrimental to activity. The second class of conjugates reduces or eliminates that problem. The amphiphilic oligomer remains intact through the digestive tract and enhances absorption in the upper duodenum. Once in circulation, the alkyl portion is cleaved. Thus, a smaller oligomer is attached to the circulating peptide when it reaches the receptor. In some embodiments, the decreased steric hindrance leads to increased activity at the receptor. Some oligomers that can provide conjugates falling within this second class as well as methods for providing such conjugates are described in U.S. Patent No. 6,309,633 to Ekwuribe et al. and U.S. Application No. 10/018,879, filed December 19, 2001, the disclosures of which are incorporated by reference herein in their entireties.

[292] A third class of conjugates is fully hydrolysable. For conjugates of this class, the entire oligomer is cleaved once the conjugate is absorbed. Like the second class, these conjugates may be particularly useful when conjugation occurs within a region that is necessary for binding. However, in the event that the microPAGylated conjugates still do not retain sufficient activity, the third class of conjugates may completely obviate the possibility of the oligomer interfering with receptor binding. In this case, the conjugate remains intact through the digestive tract. Once the conjugate is absorbed, the oligomer is cleaved, which releases the native peptide in circulation.

[293] *Conjugation of hBNP.* The carboxyl group of the amphiphilic oligomer ($C_6PEG_7$) is activated with N-hydroxy succinimide, a common activating group in peptide chemistry. Once activated, the oligomers are attached to the peptide either in aqueous or DMSO solution. hBNP has four sites for conjugation: three Lys residues and the N-terminus. By varying the stoichiometry of the reaction, the extent of conjugation (mono-, di-, etc.) can be controlled. Product distribution can be altered by varying the reaction conditions. As preferred sites for conjugation are discovered through the activity assays, preferential synthesis of the desired conjugates can be obtained by varying the stoichiometry and the reaction conditions.

[294] *Choice of PEG-alkyl Oligomers.* By varying the relative length of the alkane (hydrophobic) and PEG (hydrophilic) components, the amphiphilicity and solution structure of the conjugate can be improved.

The PEG portion is very flexible in solution and may play an important role in resistance to enzymes. The alkyl portion may enhance absorption in the gut and/or enable interaction with cell membranes. The latter feature may be particularly important when the target is a membrane-bound protein on the cell surface, such as NPR-A. Thus, the choice of the oligomer may determine the effectiveness of the conjugate in terms of enzyme stability and oral bioavailability.

[295] *Purification of hBNP Conjugates.* The reaction mixtures are purified on a preparative HPLC column (C-18) with a solvent gradient system made of isopropanol/water (0.1% trifluoroacetic acid). The solvent is evaporated and lyophilized to give dry products. Purity of the conjugates is determined by reversed-phase HPLC and mass spectrometry.

### 9.21.1 Class 1 Oligomers: Non-hydrolyzable

[296] Over thirty conjugates that utilized non-hydrolyzable oligomers (Class 1) were synthesized. For conjugates of this class, the drug substance that is dosed is the substance that acts as the receptor. In other words, the oligomer and its attachment to the peptide remain intact from the time of dosing to the time of clearance. Peptide mapping experiments revealed the sites on hBNP to which the oligomers were attached. By changing the amount of oligomer added to the reaction, product distribution could be skewed. The predominant monoconjugate that formed was conjugated at $Lys^3$; the predominant diconjugate had the oligomers attached at the $Lys^3$ and $Ly^{s4}$. By varying the reaction conditions, the triconjugate and or tetraconjugate could be formed as the exclusive product. The triconjugate featured oligomer attachment at $Lys^3$, $Lys^{14}$, and $Lys^{27}$. The tetra conjugate added a fourth attachment at the N-terminus. Initially all the available mono, di, tri, and tetra conjugates were isolated for testing activity in vitro. Based on the activity data, the $Lys^3$ monoconjugates when using Class 1 oligomers were focused.

### 9.21.2 Class 2 Oligomers: Micropegylated

[297] Eight conjugates that utilized micropegylation (Class 2) were synthesized based on the theory that, because Lys14 and Lys27 are in (or proximal to) the binding portion of BNP, micropeg conjugation of these sites would enable the peptide to be more fully conjugated and still retain activity. The amphiphilic oligomer remains intact through the digestive tract and enhances absorption in the upper duodenum. Once in circulation, the alkyl portion is cleaved. Thus, a smaller oligomer is attached to the circulating peptide when it reaches the receptor. Tri- and tetra- conjugates of this class were synthesized both before and after cleavage of the alkyl group. Even after the alkyl groups were cleaved, small PEG units attached to BNP at three or four sites were detrimental to activity (data shown in the next section), though these conjugates retained a therapeutically significant degree of activity.

### 9.21.3 Class 3 Oligomers: Hydrolyzable Oligomers

[298] Eight conjugates that utilized fully hydrolyzable oligomers (Class 3) were synthesized. For conjugates of this class, the conjugate remains intact through the digestive tract. Once the conjugate is absorbed, the oligomer is cleaved, releasing the native peptide in circulation. Like the second class, these conjugates are useful when conjugation occurs within a region that is necessary for binding. However, in situations where the micropegylated conjugates still do not retain activity, the third class of conjugates completely obviates the possibility of the oligomer interfering with receptor binding. Mono, di, tri, and tetra conjugates were made from this class of oligomers. Tri and tetra conjugates were less stable. Two conjugates were tested.

[299] The reaction mixtures were purified on a preparative HPLC column (C18) with a solvent gradient system made of isopropanol/water (0.1% trifluoroacetic acid). The solvent was evaporated and lyophilized to provide the conjugates was dry powders. Purities of the conjugates were determined by reverse-phase HPLC and mass spectrometry.

[300] Native BNP was examined in the assay to provide a measure of activity for the native, wild-type hBNP peptide. The native hBNP peptide used was the 1-32 amino acid sequence, SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH, (SEQ ID NO: ) in which $C^{10}$ and $C^{26}$ are joined by a disulfide bond to form a bond. The results and structures of twenty-nine of the constructs are provided in Table 1. The BNP conjugates were assessed for EC50 and Emax, and these values were compared to those obtained under the same experimental conditions for the native peptide. These data as compared to native BNP (1-32 aa) without an oligomeric moiety are provided in Table 1. The results point to a preference for the monoconjugate BNP that included a Class 1 modifying moiety Lys3 (BNP-002), and the monoconjugate BNP that include a Class 2 modifying moiety at Lys 14 or Lys 27.

The mono-1, mono-2, mono-3 and mono-4 are the monoconjugates of BNP and labeled as in the order they elute on HPLC. In the following Table, the mono-1 BNP is the BNP peptide conjugate that that includes the indicated modifying moiety (oligomer structure) at the Lys-3 BNP residue. The mono-2 and mono-3 co-elute on HPLC and its a mixture of Lys-14 and Lys-27. The diconjugates are generally obtained as a mixtures that elute closely together on HPLC. The major diconujates are Lys3/Lys14 and Lys3/Lys27. The predominant triconjugate is conjugated at Lys3, Lys14, and Lys27. The product identified as "mono-4" includes the modifying moiety (oligomer) at the N-terminus of the BNP peptide. The "mono-1" includes the modifying moiety conjugated at Lys3 of the BNP peptide. The "mono-2" product includes the modifying moiety (oligomer) conjugated at Lys14 of the BNP peptide, or at Lys 27 of the BNP peptide. For results see the table set forth in Figures 6a-d.

## 9.22 Natriuretic Peptide Candidates – Urodilatin, Dendroaspis Natriuretic Peptide (DNP), and Canine Natriuretic Peptide

[301] It is anticipated that the present conjugation technology may be used with many different natriuretic peptides and analogs of these peptides to construct any number of different bioactive natriuretic peptide conjugate embodiments with retained pharmacological activity, enhanced cell-membrane permeability, and/or protease resistance. In addition to the hBNP described in several of the examples here, these candidate peptides include by way of a partial list, peptides, peptide fragments and whole peptides, and multi-constructs peptides prepared and/or isolated from the following assembly of bioequivalent peptides/proteins. It is within the scope of the present invention to include these constructs and conservative substituted constructs thereof in the preparation of the embodiments, the present invention, as well as in pharmaceutical preparations containing these constructs in a conjugated from with at least one modifying moiety as defined herein in the treatment of congestive heart failure. These peptides posses a structure amenable to modifying conjugation moiety.

[302] 1. Urodilatin (hANP with four additional residues at the N-terminus)

[303] TAPRSLRRSSCFGGRMDRIGAQSGLGCNSF**X**$^1$Y (SEQ ID NO: )

[304] The amino acid T defines a modifying moiety conjugation site. In the above sequence, $X^1$ is lysine or an amino acid other than arginine. Where $X^1$ is lysine, a second modifying moiety conjugation site is provided.

[305] 2. Canine natriuretic peptide (Canine NP)

[306] Canine BNP offers natural advantages for manufacturing of conjugates. No conjugation sites exist in the loop region. Conjugation sites are present in the N- and C- terminal tails. These features would enable conjugation without substantial loss of activity. It should also lead to a smaller distribution of products, resulting in higher yield and easier purification.

[307] SP**X**$^1$MMH**X**$^2$GGCFGRRLDRIGSLSGLGCNVLR**X**$^3$Y (SEQ. ID. NO: )

[308] The amino acid sites of $X_1$, $X_2$, and $X_3$ present modifying moiety conjugation sites. In this neutral peptide, all 3 sites of the peptide are available for conjugation with a modifying moiety. The loop region is identified at amino acid 10 (C) to amino acid 26 (C). It is envisioned that any 2 or all 3 of the amino acids at position 3, 14, or 27 may be substituted with a residue other than Lys, such as Arg.

[309] 3. Dendroaspis natriuretic peptide (DNP)

[310] EV**X**$^1$YDPCFGH **X**$^2$IDRINHVSN LGCPSLRDPRPNAPSTSA (SEQ ID NO. )

[311] The amino acid site of the $X_1$ and $X_2$ are modifying moiety conjugation sites. In this example, both $X_1$ and $X^2$ are the amino acid Lys. In some embodiments, $X^1$ is Arg or $X^2$ is Arg. The N terminus is also a conjugation site. Preferably, where $X^1$ is lysine, $X^2$ is arginine (or other than lysine). Optionally, the peptide may include a further conjugation site at the N-terminus.

[312] 4. C-type natriuretic peptide (CNP)

[313] GLSK$^1$GCFGLK$^2$LDRIGSMSGLGC (SEQ ID NO.: )

[314] The amino acid site of the $K^1$ and $K^2$ are modifying moiety conjugation sites. In this example, both $K^1$ and $K^2$ are the amino acid Lys. However, analogs of the peptide may include an Arg (R) in place of Lys at either or both of these positions in the peptide. Optionally, the peptide may include a further conjugation site at the N-terminus.

[315] 5. ANP (human)(rat)(porcine)

[316] SLRRSSCFGGR**X**DRIGAQSGLGCNSFRY (SEQ ID NO.: )

[317] In this example, X is Met(M) or Ile(I), and wherein a modifying moiety conjugation site is at the N-terminus, or R is changed to K to provide a modifying moiety site.

### 9.23 Agonist Activity at the Human Natriuretic Peptide Receptor A (NPR-A)

[318] The vasorelaxant, natriuretic, and diuretic properties of BNP are ascribed to a secondary messenger, cyclic GMP (cGMP). The production of cGMP is accomplished by guanylate cyclase, an enzyme that is activated when BNP binds to the natriuretic peptide receptor A (NPR-A) on the surface of endothelial cells. The ability of the conjugates with either non-hydrolyzable (Class 1) or micropegylated (Class 2) oligomers to stimulate the production of cGMP in human aortic endothelial cells (HAEC) expressing the natriuretic peptide receptor-A (NPR-A) was evaluated. For the micropeglyated group, the conjugates were tested with and without the alkyl portion attached. The conjugates with fully hydrolyzable oligomers (Class 3) were not evaluated in this assay because the compound that is ultimately released in circulation is the native peptide.

[319] Tri- and tetra-conjugates utilizing non-hydrolyzable (Class 1) oligomers were less active. Therefore, tri- and tetra-conjugates utilizing micropegylated (Class 2) oligomers were prepared and tested. The in vitro data generated from these Class 2 oligomers is presented in **Table 2**.

[320] Table 2: In vitro activity of hBNP conjugates utilizing Class 2 oligomers.

| hBNP or hBNP Conjugate | Extent of Conjugation | Average $EC_{50}$ (nM) | Average $E_{max}$ (%) |
|---|---|---|---|

| Native hBNP | None | 236 (+/-) 120 | 100 |
|---|---|---|---|
| BN-007 | Tri | >10,000 | <20 |
| BN-008 | Tetra | >10,000 | <20 |
| BN-010 | Tetra | >10,000 | <20 |
| BN-013 | Tetra | >10,000 | <20 |
| BN-014 | Tri | >10,000 | 26.5 |
| BN-015 | Tetra | >10,000 | <20 |
| BN-016 | Tetra | >10,000 | 24.6 |
| BN-018 | Tetra | >10,000 | <20 |

[321] **Figure 3** shows the activity curves for various Lys-3 conjugates utilizing Class 1 oligomers. The four conjugates in Table 2 demonstrates an average $E_{max}$ and an average $EC_{50}$ closest to those the activity obtained with native forms of the BNP peptide **(Table 3)** and were thus evaluated further in other assays.

[322] Table 3: *In vitro* activity of hBNP conjugates.

| Compound | Average $EC_{50}$ (nM) | Average $E_{max}$ (%) | n |
|---|---|---|---|
| Native hBNP | 236 (+/-) 120 | 100 | 25 |
| BN-002 | 387 (+/-) 171 | 102 | 5 |
| BN-021 | 355 (+/-) 140 | 90 | 5 |
| BN-022 | 364 (+/-) 99 | 79 | 5 |
| BN-024 | 296 (+/-) 172 | 87 | 6 |

[323] Primary HAEC were purchased from Clonetics for cGMP screening. Cells were plated into 12 well plates the day before the experiment. On the day of the experiment, cells were pre-incubated for 10 min at 37 °C with 0.5 mM IBMX to inhibit phosphodiesterases. Test compounds were added to the cells for an additional 60 min at 37 °C and the incubation was stopped by lysing cells to measure cGMP. An ELISA-based cGMP kit was used to measure cGMP production (CatchPoint-cyclic GMP Fluorescent Assay Kit, catalog #R8074, Molecular Devices Corp, Sunnyvale, CA). This kit measures cGMP via a competitive immunoassay in 96-well format. Cell lysates were added to the coated microplate followed by the addition of an anti-cGMP antibody and a horseradish peroxidase (HRP)-cGMP conjugate. Plates were incubated for two hours at room temperature, followed by four washes. A substrate solution was added and the fluorescent intensity of each well was quantitated. The fluorescent signal intensity decreased with increasing levels of cGMP. Native hBNP was be tested in each experiment as a positive control.

### 9.24 BNP Conjugates and Increased Resistance to Proteases

[324] The natriuretic compound that were active *in vitro* are being tested for their stability in the presence of various proteases, such as trypsin and chymotrypsin. The stability of these compounds conjugated to proteases can be determined by the half-lives of the compound conjugates in the presence of

trypsin and chymotrypsin. Thus, several conjugates evaluated in these assays had a longer half-life than did native hBNP. For example, see Figure 4.

[325] Conjugates were incubated with the enzyme for 2 to 120 minutes at 37 °C. Digestions were stopped by adding a 1:1 1% trifluoroacetic acid (TFA): isopropanol quenching solution. Digestion of the hBNP conjugates were compared to the digestion of native hBNP in each experiment. The amount of parent compound remaining in each sample was quantitated by HPLC analysis.

### 9.25 BNP Conjugates and Oral Bioavailability

[326] The conjugates that were active *in vitro* were tested for their oral bioavailabily in rats. The conjugates were administered to the gastrointestinal tract by oral gavage and the presence of hBNP conjugates in the bloodstream was assayed using available radioimmunoassay procedures. The antibodies for detection of hBNP are specific; cross reactivity with rat BNP is less than 1%. Consequently, cross reactivity and interference by endogenous rat BNP was not an issue.

[327] Adult, male rats weighing approximately 250 g were used for determining oral bioavailability of hBNP and hBNP conjugates. Rats were fasted overnight and tap water was provided ad libitum (except for a period of no water for 2 hours pre-dosing until 1 hour post dosing).

[328] Prior to dosing, rats were weighed and distributed throughout the dosing groups by body weight so that each dosing group weighed approximately the same. Five rats were used per time point. Conjugates were administered in a liquid fatty acid formulation at a dose of 2.5 mg/kg. Blood samples were taken at 5, 15, 30, and 60 min after dosing. Central venous blood for all dosing experiments was collected and centrifuged. Plasma samples were frozen at –80 °C for analysis .

[329] The plasma concentrations of hBNP conjugates were measured by a commercial immunoradiometric assay (IRMA) specific for the quantitative determination of human BNP in plasma (SHIONORIA™ BNP, Catalog # 127024, Shionogi & Co., Ltd, Osaka, Japan). Blood was drawn from the dosed rats into EDTA coated plastic polyethylene telepthalate (PET) blood collection tubes and centrifuged at 1600 – 2000 x g for 5 minutes in a refrigerated (2 – 8 °C) centrifuge. Samples were stored in plastic tubes at -80 °C in non-frost free freezers until analysis. 500 µL of sample were used for the IRMA. 100 µL of the sample was added to a tube with 200 µL of $^{125}$I-BNP reagent and one anti-BNP antibody coated bead. Each tube was vortexed and incubated without shaking, for 18 to 22 hours at 2 to 8 °C. The tubes were then aspirated and washed with 2.0 mL of washing solution (buffer solution + 0.05% $NaN_3$) and then reaspirated. The wash process was repeated and the contents of the tube aspirated. The remaining radioactivity in each tube was counted by a gamma counter. The radioactivity was directly proportional to the concentration of hBNP or hBNP conjugates in the sample. In order to accurately quantify samples of hBNP conjugates and

allow for differences of antibody recognition between hBNP conjugates and the native molecule, concentration was determined from a standard curve obtained for the appropriate hBNP conjugate.

[330] The four conjugates that were dosed in rats were all detectable in circulation five minutes after dosing (Figure 5). These four conjugates were BNP-002, BN-021, BN-022, and BN-024.

### 9.26 Preparation of a Diconjugate, a Monoconjugate and a Triconjugate polymer Modifications on the Peptide Structure

[331] The present example is provided to demonstrate the utility for the present invention in the creation of multi-conjugate forms of the bioactive peptide of choice. By way of example, the present description will describe a monoconjugate, a diconjugate and a triconjugate form of the human natriuretic peptide, hBNP.

[332] Protocol for conjugating to hBNP:

[333] The oligomers would be attached via the same procedure used for conjugation to hBNP. One difference will be more of the activated oligomer may be added (1 – 10 equivalents; preferably 3-5 equivalents).

[334] Lysines are in the tails of the sequence. Multiple conjugation sites would presumably afford greater stability in the presence of proteases. The lack of conjugation sites within the loop is advantageous for binding at the NPR-A binding motif.

### 9.27 Synthesis of an hBNP amphiphilic polymer conjugate

[335] By using amphiphilic oligomers of different size and chemical composition, the absorption and partitioning properties of a peptide conjugate, such as hBNP conjugate, can be altered. Conjugate screening is used to determine which of the conjugates retain the activity of the native peptide and show enhanced resistance to enzymes. The conjugates that have a desirable combination of traits (e.g., agonist activity at the receptor, resistance to proteolysis, and oral bioavailability) may become lead candidates for more extensive *in vivo* testing.

#### 9.27.1 General Procedure for Conjugation to BNP

[336] Monoconjugate hBNP use sites Lys 3 or Lys 14, or Lys 27, or at the N-terminus of the peptide.

[337] **Method I:** Preparation of monoconjugates

h-BNP (1 equiv) was dissolved in DMSO (1 ml / 35 mg of h-BNP). The activated oligomer (1.1 equiv) was dissolved in a minimal amount of THF and added to the solution of h-BNP in DMSO. The reaction was monitored by HPLC. Samples for HPLC monitoring were prepared by taking 50 μL of the reaction and

diluting it in 500 μL of $H_2O$ containing 0.1% TFA. Reactions were carried out for 45 min. If reactions were not immediately purified they were frozen until purification could be performed.

[338] **Method II:** Preparation of multiple conjugates

h-BNP (1 equiv) was dissolved in DMSO (1 ml / 35 mg of h-BNP). Once h-BNP was dissolved, TEA (120 equiv) was added and the solution stirred for 5 min. Then the activated oligomer (2.2 equiv for diconjugate, 4 equiv for triconjugate, 5 equiv for tetraconjugate) was dissolved in a minimal amount of THF and added to the solution of h-BNP in DMSO. The reaction was monitored by HPLC. Samples for HPLC monitoring were prepared by taking 50 μL of the reaction and diluting it in 500 μL of $H_2O$ containing 0.1% TFA. Reactions were carried out for 45 min. If reactions were not immediately purified they were frozen until purification could be performed.

[339] Diconjugate hBNP use sites Lys 3, and Lys 14, or Lys 3 and Lys 27 site on hBNP.

[340] Triconjugate hBNP use sites Lys 3, Lys 14 and Lys 27.

### 9.28 Natriuretic Compound Analogs

[341] The present example is provided to demonstrate the utility of the present invention for providing a variety of forms of bioactive BNP-like peptide and peptide fragments thereof for use in the practice of the present invention. These variant forms, or analogs, are characterized by the presence of one or more mutated amino acids in place of a naturally occurring amino acid from the corresponding native peptide/protein.

[342] 1. Analog of hBNP-loop region alone

[343] CFGR**X**MDRISSSSGLGC- (SEQ ID NO. 1)

[344] wherein X is an amino acid other than Lys, or X is Arg or Gly.

[345] 2. Analog of hBNP-3Arg or an amino acid other than Lys

[346] -SP**R**MVQGSG-CFGR**K**MDRISSSSGLGC-$\mathbf{X^2}$- (SEQ ID NO. )

[347] wherein $X^2$ is 1 to 10 amino acids, preferably 1-6 amino acids in length. In some embodiments, $X^2$ is KVLRRH, KVLRR, KVLR, KVL, KV, K, RVLRRH, RVLRR, RVLR, RVL, RV, or R.

[348] 3. Analog of hBNP–3 mutation sites; 3Arg, 14Arg, 27Arg

[349] SP$\mathbf{X^1}$MVQGSG-CFGR$\mathbf{X^2}$MDRISSSSGLGC-$\mathbf{X^3}$VLRRH (SEQ ID NO. )

[350] wherein $X^1$ is Lys or an amino acid other than Lys, $X^2$ is an amino acid other than Lys, and $X^3$ is Lys or an amino acid other than Lys. In some embodiments, $X^1$, $X^2$, and $X^3$, are independently Arg or Gly. In other embodiments, $X^1$ is Lys, $X^2$ and $X^3$ areindependently Arg or Gly. In a preferred embodiment, at lease one of $X^1$, $X^2$, and $X^3$ is Lys.

[351] 4. Analog of hBNP-14 and 27Arg, and a terminal modification site, $X^1$.

[352] **$X^1$**SPKMVQGSG-CFGR**$X^2$**MDRISSSSGLGC- **$X^3$**VLRRH- (SEQ ID NO. )

[353] Wherein $X^1$ is a C-terminus modification site (Ser); and wherein $X^2$ and $X^3$ are an amino acid other than Lys. In some embodiments $X^2$ and $X^3$ are independently Arg or Gly. In other embodiments, $X^2$ is Arg and $X^3$ is Lys.

[354] 5. Analog of hBNP-14Arg

[355] (All fragments in which one or both tails are shortened up to the loop)

[356] **$X^1$**---CFGR**R**MDRISSSSGLGC - **$X^2$** (SEQ ID NO. 1)

[357] wherein $X^1$ is 1 to 10 amino acids, preferably 1-9 amino acids in length, and wherein $X^2$ is 1 to 10, preferably 1-6 amino acids in length. $X^1$ may comprise SPKMVQGSGC, PKMVQGSGC, KMVQGSGC, MVQGSGC, VQGSGC, QGSGC, GSGC, SGC, GC, C, SPK, SPKM, SPKMV, SPKMVQ, SPKMVQ, KMVQ, KMV, KMVQG, KMVQGS, KMVQGSG, or KMVQGSGC. $X^2$ may comprise KVLRRH, KVLRR, KVLR, KVL, KV, K, RVLRRH, RVLRR, RVLR, RVL, RV, or R.

[358] 6. Analog of hBNP 1-29-3Arg or amino acids other than Lys

[359] SP **$X^1$**MVQGSG-CFGRKMDRISSSSGLGC- KVL (SEQ ID NO. )

[360] wherein $X^1$ is Arg, or amino acid other than Lys

[361] 7. Analog of hBNP 1-26-3Arg or amino acid other than Lys

[362] SP**$X^1$**MVQGSG-CFGRKMDRISSSSGLGC (SEQ ID NO. )

[363] wherein $X^1$ is Arg, Gly, or another amino acid other than Lys.

[364] 8. Analog of hBNP-shortened C-terminal tail Lys 14 Arg, 27Arg, or amino acid other than Lys

[365] **$X^1$** - CFGRRMDRISSSSGLGC-RVLRRH (SEQ ID NO: )

[366] wherein $X^1$ is 1 to 10 amino acids, preferably 1 to 9 amino acids in length. $X^1$ may comprise SPKMVQGSGC, PKMVQGSGC, KMVQGSGC, MVQGSGC, VQGSGC, QGSGC, GSGC, SGC, GC, or C.

[367] 9. Analog hBNP-Lys 14Arg or an amino acid other than Lys

[368] -CFGR $\mathbf{X^1}$MDRI$\mathbf{X^2}$GLGC- (SEQ. ID. NO. ___)

[369] wherein $X^1$ is Arg or an amino acid other than Lys, and $X^2$ is one to four amino acids. In some embodiments, $X^2$ is SSSS, SSS, SS, S, KSSS, KSS, or KS.

[370] 10. Analog hBNP-Arg 30 Lys or other equivalent amino acid of like charge

[371] SP**K**MVQGSGCFGRKMDRISSSSGLGC**K**VR$\mathbf{X_1}$RH (SEQ ID NO. )

[372] wherein $X^1$ is Lys or an amino acid other than Arg.

[373] 11. Analog of hBNP-27Arg or an amino acid other than Lys

[374] SP**K**MVQGSGCFGRKMDRISSSSGLGC $\mathbf{X^1}$VLRRH (SEQ ID NO. )

[375] wherein $X^1$ is Arg or an amino acid other than Lys.

[376] 12. Extension Forms of hBNP

[377] – SPKMVQGSG-CFGRKMDRISSSSGLGC- KVLRRH - $\mathbf{X^2}$ (SEQ ID NO. )

[378] $X^2$ is Lys, Cys, or Lys + $Xaa_n$ where n is 1-100, 1-50 or 1-10, and Xaa is any amino acid, or group of amino acids independently selected, or an unknown amino acid

[379] 13. Deletion mutant analog - hBNP

[380] -CFGR $\mathbf{X^1}$MDRI$\mathbf{X^2}$GLGC – (SEQ ID NO. )

[381] wherein $X^1$ is Arg or an amino acid other than Lys and wherein $X^2$ is 1 to 4 amino acids, such as SSSS, SSS, SS, S, KSSS, KSS, or KS.

[382] 14. hBNP Analog – Receptor Specificity

[383] SP$\mathbf{Z^1}$MVQGSG-CFGR$\mathbf{Z^2}$MDRISSSS$\mathbf{X^1X^2X^3}$C

[384] Wherein $Z^1$ is arginine or an amino acid other than lysine, and wherein $Z^2$ is arginine or an amino acid other than lysine, wherein $X^1$ is Gly Met Leu, Phe, Ile or a conservative substitutions thereof, wherein

$X^2$ is Leu, Trp, Tyr, and Phe or a conservative substitutions thereof, and wherein $X^3$ is Gly, Arg, or a conservative substitution thereof. In another embodiment of this analog, $Z^1$ is lysine and $Z^2$ is arginine or an amino acid other than lysine.

[385] 15. ANP analogs

[386] K CFKGKNDR**X**$^1$ K**X**$^2$ QSGL**X**$^3$ C-NSFKY

[387] Wherein $X^1$ is T, a, R, H, P, E;

[388] Wherein $X^2$ is K, N-methyl, Arg, S, D, or P;

[389] Wherein $X^3$ is Arg, K, Y, F, S, P, Orn, Har, Har, p-amidinophenyl Ala, I, any other amino acid that has a positive charge other than Gly, or Try

## 9.26 Recombinant Production of Native BNP and BNP Pro- Peptide and Pro-Peptide Approach to Manufacturing of BNP Conjugate

[390] An oral route of administration will require a large volume supply of BNP peptide. Due to the high cost and supply volume limitations associated with synthetic means to supply BNP, a recombinant technology will be preferred for preparing the conjugated BNP peptide. A recombinant technology for the supply of peptide for the production of the conjugate is described here.

### 9.26.1 Selection of Recombinant Technology

[391] The goal is to select a high expression recombinant technology that is known to express small proteins (>10,000K) free of glycosylation and have the peptides secreted in soluble form for easy isolation.

[392] An E-coli based expression system (U.S. 5,114,923, Seilhamer et. al. is incorporated herein by reference), is used for production of bulk BNP for the approved drug Natrecor®. Use of the E-coli bacterial system is well known and well utilized in the industry for the past many decades for recombinant production of single chain proteins. The E-coli system is in general a simpler system for laboratory uses. Many new E-coli systems have been developed with high cell density to provide high yield of protein expression. However, in general, there exists a limitation to the use of an E-coli based system because of its tendency to secrete the protein in its insoluble form into an inclusion body and to be improperly folded (improper disulfide bond between cysteine amino acid residues). These limitations often leads to high cost of goods, expensive down stream processing steps must be implemented to isolate the protein from inclusion body, and refolding the improperly folded protein to its natural state.

## 9.27 Construct of Pro-protein (pro-BNP) Sequences

[393] The natriuretic compound may also be a multipeptide having two or more natriuretic compound units in sequence and optionally including a spacer sequence between the natriuretic compound unit, and the construct may also optionally comprise a leader and/or extendor sequence at either or both ends of the natriuretic peptide compound. For example, without limiting the multipeptide, to any particular construct, the multipeptide may have the following structures:

NP-$[NP]_n$;

NP-$[Spacer\text{-}NP]_n$;

Leader-NP-$[NP]_n$;

Leader-NP-$[Spacer\text{-}NP]_n$;

Leader-$[Spacer\text{-}NP]_n$;

Leader-$[Spacer\text{-}NP]_n$-Extension;

Leader-NP-$[Spacer\text{-}NP]_n$-Extension;

[394] where n may, for example be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10; NP is a natriuretic peptide or natriuretic peptide analog:

## 9.29 Pro-BNP

[395] The invention also provides a pro-X-polypeptide, where X is a natriuretic peptide. The Pro-X-peptide for BNP can be designed to carry a leader peptide as the Pro moiety and which can be linked to BNP sequence via an enzymatic cleavage site. A gene sequence can be designed that encodes the expression of peptide as pro-BNP peptide in the selected recombinant technology. The pro-moiety can also be selected to aid more efficient purification from the fermentation scheme. Pro-BNP peptide can be conjugated post-expression with the oligomer and then the pro moiety can be cleaved by a selected enzyme, mobilized or immobilized, to provide the BNP conjugate which can be more easily purified via conventional chromatographic methods in high yield. Specific enzyme cleavage sites will be included between pro moiety and BNP sequence so that the pro moiety can be enzymatically cleaved to yield the BNP sequence.

### 9.29.1 Pro-BNP model synthesis

[396] The pro-BNP construct will be assessed with a synthetic pro-BNP model having a BNP sequence and additional specific amino acids. This synthetic model will be conjugated with oligomer and subjected to cleavage by a specific enzyme to monitor the production of BNP-Oligomer conjugate.

### 9.29.2 Designs of pro-BNP

[397] The leader sequence (promoiety) can include a small peptide with a specific enzyme cleavage sequence based on the synthetic model. Other functional amino acid sequences can also be inserted in the leader/spacer sequence to allow easy purification of the pro-BNP protein. The leader sequence can also serve as the pro-moiety to protect the N-terminus from undesired modification during conjugation and can be cleaved upon specific enzyme treatment. Other features can also be built into the leader peptide sequences to allow ease of isolation as pro-BNP or as pro-BNP oligomer conjugate. The leader peptide can also be attached to the C-terminus of the BNP sequence. The leader peptide can also be designed to allow attachment of known fusion proteins.

### 9.29.3 Pro-BNP expression

[398] Functionally specific leader sequences can be provided at the N-terminus or/and C-terminus of BNP for insertion into the expression gene sequence or expression cassette of the selected recombinant technology. The expression sequence of a known fusion protein (see Gaken et al, 2000, the entire disclosure of which is incorporated herein by reference for its teaching concerning fusion proteins) can also be inserted into the expression gene in one of the constructs. Using established procedures, the successful transformation expressed genes in the cells can be monitored. The positive transgenic isolates or cells can be isolated and grown for evaluation for the expression of the designed proteins. Expressed proteins can be purified and sequenced. The purified pro-BNP constructs can then be evaluated. Selected cell lines can be characterized and selected for selection future use.

### 9.29.4 Construction of pro-pentapeptide BNP-1 with Trypsin and Carboxy peptidase-B cleavage spacers and His tagged Leader peptide

[399] The coding sequence for the full length of pro-pentapeptide BNP-1 can be provided according to the following formula: Leader-NP-[Spacer-NP]$_n$ where:

Spacer is Arg-Arg-Asp-Ala-Glu-Asp-Pro-Arg,
Leader is Glu-Gly-Asp-Arg-Arg,
Extension is $(His)_6$-Glu-Gly-Asp-Arg-Arg;
NP is hBNP.

[400] In this embodiment, the NP or NP conjugate can be released using a Trypsin and Carboxypeptidse B enzyme cocktail.

[401] (a) Plasmid construction

[402] Using the standard molecular biology techniques, a plasmid is constructed for expressing the amino acid sequence of pro-pentapeptide BNP-1 [$(His)_6$-Glu-Gly-Asp-Arg-Arg.)-BNP- Arg-Arg-Asp-Ala-

Glu-Asp- BNP- Arg-Arg-Asp-Ala-Glu-Asp- BNP- Arg-Arg-Asp-Ala-Glu-Asp- BNP- Arg-Arg-Asp-Ala-Glu-Asp- BNP] . The plasmid is Codon optimized for the host cell (e.g., E coli) used.

[403] DNA fragment coding this multipeptide sequence is assembled synthetically, starting from post leader sequence and the cleavage sites are added in the order of 3' of the His tag/5' of BNP sequence. cDNA of the sequence is purified from ployacrylamide gel using standard techniques. The plasmid structure is confirmed by restriction enzyme analysis.

[404] (b) Expression and cell recovery

[405] The E-coli cells expressing pro-pentapeptide BNP-1 are cultured with nutrients sufficient to produce the pro-multipeptide. The $(His)_6$ tag pro-penta BNP is recovered from the cells by cell disruption followed by centrifugation, tangential filtration/untrafiltration , homogenization and solubilization of inclusion bodies.

[406] (c) Isolation of the pro-pentapetide BNP-1 from the soulubilized inclusion via affinity chromatography

[407] A HiTrp chelating ($Ni^{2+}$) HP column (Amersham Bioscience) is prepared and the columns are washed with 10 column volumes of distilled water to remove the storage solution, is charged with metal ion solution ($NiSO_4$, 0.1M) and washed with distilled water to remove unbound. The filtered solution after inclusion body solubilization, is loaded to this column and the column is washed with 10 column volume of biding buffer (20mM phosphate buffer, pH 7.4, containing 0.5M sodium chloride and 20mM imidazole), Using a linear gradient 10 column volumes of elution buffer (20mM sodium phosphate, pH 7.4, containing 0.5M Sodium chloride and 0.5M imidazole) the column is eluted and followed by another 2 column volume of elution buffer at 100%. This procedure purifies the $(His)_6$ tag pentapeptide from other components from the cell recovery sample via chelation to $Ni^{2+}$ affinity of the column. The purity of the pentapeptide is analyzed by RP-HPLC method to be > 30%.

[408] (d) Purification and analysis

[409] The pentapeptide is then further purified via C-18 preparative HPLC to a purity >75%. The purified pentapeptide is analyzed by ES/MS analysis and provided M+1 ion peaks for the expected MW of pro-pentapeptide BNP-1. Micro sequencing of the material is used to confirm the amino acid sequence of the multipeptide.

### 9.29.5 Production of multiple units of Lys-3 BNP conjugate from pro-pentapeptide BNP-1

[410] (a) Conjugation of pentapeptide

[411] Pro-pentapeptide BNP-1 (3.20 x 10-4 mmol) is dissolved in 5 mL of DMSO. To the solution is added 45 uL of triethylamine. The solution is allowed to stir for 5 minutes before a solution of activated PEG7-hexyl oligomer (19.6 x 10-4 mmol) in ethanol is added. After the reaction has progressed such that HPLC analysis indicates that the pro-multipeptide has been consumed (or the concentration of pro-multipeptide is no longer decreasing), the reaction is quenched by addition of 0.5 mL of 50% aqueous acetic acid solution. The reaction mixture is then processed and exchanged into 100 mM Tris-HCl Buffer, pH 7.6. The major composition of this mixture is expected to be conjugated multipeptide at Lys3 of each BNP unit.

[412] (b) Enzyme Cocktail Cleavage of Oligomer-Conjugated Pro-pentapeptide BNP-1 to conjugated BNP units

[413] An aliquot of the Tris-HCl solution of the product mixture from Example 2(a) is analyzed by HPLC to determine the polypeptide concentration therein. A solution of trypsin (TPCK treated; from bovine pancreas) is prepared in 100 mM Tris-HCl Buffer, pH 7.6. A solution of carboxypeptidase B (from porcine pancreas) is prepared in 100 mM Tris-HCl Buffer, pH 7.6.

[414] The crude mixture (1 mol eq.) is then allowed to react with trypsin (1.39 x $10^{-3}$mol eq) and carboxypeptidase B (4.56 x $10^{-4}$ mol eq.). After 30 minutes, the reaction is quenched by addition of 1% trifluoroacetic acid in acetonitrile. The product mixture of the reaction is processed and analyzed by HPLC. Retention time (versus that of reference standards) and mass spectral analysis are used to determine identity. The expected products of the reaction are Lys3 hexyl-PEG7-conjugated BNP, Lys14 hexyl-PEG7-oligomer-conjugated BNP, Lys 27 hexyl-PEG7-oligomer-conjugated BNP, Di hexyl-PEG7- oligomer conjugated BNP, Des Arg-His BNP and Des Arg-His hexyl-PEG7-oligomer conjugated (Lys3 or Lys14 or Lys27) BNP. The major composition of this mixture is Lys3-hexyl-PEG7-conjugated BNP.

### 9.29.6 Purification of Pro-pentapeptide BNP-1 conjugates from crude conjugation mixture

[415] Each major product obtained from the conjugation reaction described in Example 2(b) is isolated using reversed-phase HPLC. A column (1.0 cm. i.d. x 25 cm. length) is packed with a commercially available C18 stationary phase known to be useful for the resolution of polypeptides and proteins, and then is incorporated into an HPLC system. The system is equilibrated with elution buffer that comprises a mixture of 75% mobile phase A (H2O with 0.1% trifluoroacetic acid) and 25% mobile phase B (acetonitrile with 0.1% trifluoroacetic acid). The Tris-HCl solution of the product mixture from Example 21(a) is applied to the column, and the major products are separated and eluted using a gradient elution in which the percentage of the acetonitrile component is increased from 25%-55% over 120 minutes. Fractions are collected and analyzed by HPLC to determine the identity and purity of the product therein. Common fractions of each product are pooled, and the solvent is removed by rotary evaporation. The identity and purity of each product peak are determined by HPLC and mass spectrometry. The expected products consist of 3 multipeptide monoconjugates (conjugated at either Lys3 or Lys 14 or Lys 27 of each unit of BNP), 3

multipeptide diconjugate (conjugated at Lys3&Lys14 or Lys14& Lys27 or Lys27&Lys3), 1 multipeptide triconjuagte (conjugated at Lys3, Lys14 and Lys 27) and 1 multipeptide tetraconjugate (conjugated at N-terminal of leader peptide, Lys3, Lys14 and Lys 27).

### 9.29.7 Preparation of Lys3-Hexyl-PEG7-oligomer conjugated BNP from Enzyme Cocktail Cleavage of Isolated Conjugate of pro-pentapeptide BNP-1

[416] The conjugate, monoconjugated Lys3-hexyl-PEG7-oligomer pro-pentapeptide BNP-1, that is obtained using the procedure described in Example 3 is dissolved in 100 mM Tris-HCl Buffer, pH 7.6, and the resulting solution is analyzed by HPLC to determine the polypeptide concentration therein. A solution of trypsin (TPCK treated; from bovine pancreas) is prepared in 100 mM Tris-HCl Buffer, pH 7.6. A solution of carboxypeptidase B (from porcine pancreas) is prepared in 100 mM Tris-HCl Buffer, pH 7.6. The crude mixture (1 mol eq.) is then allowed to react with trypsin (1.39 x 10-3 mol eq.) and carboxypeptidase B (4.56 x 10-4 mol eq.). After 30 minutes, the reaction is quenched by addition of 1% trifluoroacetic acid in acetonitrile. The products are processed and analyzed by HPLC. Retention time (compared to that of reference standards) and mass spectral analysis are used to determine identity. The expected products of the reaction are respective of each conjugates used. For example, Monoconjugated Lys3- hexyl-PEG7-oligomer-conjugated pro-pentapeptide BNP-1 is to provide Lys3 -hexyl-PEG7-oligomer-conjugated BNP and Des Arg-His Lys3-hexyl-PEG7-oligomer-conjugated BNP.

### 9.29.8 Site selective conjugation at Lys3 of pro-pentapeptide BNP-1 in Borate buffer/ organic solvent

[417] Pro-pentapeptide BNP-1 (0.0195mmol) can be dissolved in 5 mL of 50mM boric acid. The solution can brought to pH 9.3 with 4N sodium hydroxide solution and added to 1mL ethanol and adjusted to pH 10.4-10.9 with sodium hydroxide. To the above stirred solution can added a solution of activated methylheptaethylene glycol ((PEG7)-hexyl oligomer) (7.5x 0.0195 mmol) in 1 mL ethanol. The course of the conjugation (acylation) reaction is monitored by HPLC and the pH is maintained at pH 10.5 using 4N sodium hydroxide. When reaction appeared to be complete after 20 minutes, it is quenched by addition of 4N hydrochloric acid to pH 6.8. The reaction mixture is then processed and exchanged into 100 mM Tris-HCl Buffer, pH 7.6 . The HPLC profile of the product mixture showed >40% conjugation at Lys3 of each unit of the BNP unit of pro-pentapeptide BNP-1

### 9.29.9 Site selective conjugation at Lys3 of native hBNP in Borate buffer/ organic solvent

[418] Pro-pentapeptide BNP-1 (0.0195mmol) is dissolved in 5 mL of 50mM boric acid. The solution is brought to pH 9.3 with 4N sodium hydroxide solution and added to 1mL ethanol and adjusted to pH 10.4-10.9 with sodium hydroxide. To the above stirred solution was added a solution of activated methylheptaethylene glycol ((PEG7)-hexyl oligomer) (1.6x 0.0195 mmol) in 1 mL ethanol. The course of the conjugation (acylation) reaction is monitored by HPLC and the pH is maintained at pH 10.5 using 4N

sodium hydroxide. When reaction appeared to be complete after 20 minutes, it is quenched by addition of 4N hydrochloric acid to pH 6.8. The reaction mixture is then processed and exchanged into 100 mM Tris-HCl Buffer, pH 7.6. The HPLC profile of the product mixture is expected to show >40% conjugation at $Lys^3$ of the BNP molecule.

### 9.30 Preparative Chromatography

[419] Reversed phase HPLC analysis and purification was performed for BN-054 on a Waters Prep-LC system with Waters 2487 Dual λ Absorbance detector. Reaction mixture containing DMSO and water (16 ml) was further diluted with 0.1.%TFA water to make final volume of 100 mL. Five injections, 20 mL each were run on to a preparative column. Column specifications: Symmetry Prep C18 7μm column, 19 x 300 mm. The elution gradient was shown below where A was 0.1% TFA in water (v/v) and B was 0.1% TFA in acetonitrile (v/v). Detection was set at 220 nm.

| | Time | Flow ( in mL) | %A | %B |
|---|---|---|---|---|
| 1 | 0.00 | 8.00 | 90.0 | 10.0 |
| 2 | 10.00 | 8.00 | 90.0 | 10.0 |
| 3 | 70.00 | 8.00 | 60.0 | 40.0 |
| 4 | 71.0 | 8.00 | 90.0 | 10.0 |
| 5 | 80.0 | 8.00 | 90.0 | 10.0 |
| 6 | 85.0 | 0.00 | 90.00 | 10.0 |

[420] Fractions were collected and analyzed for the presence of BN-54 using analytical chromatography on Waters 2690/5 system with 996 Diode array detector and column heater. The conjugates were analyzed Phenomenex Luna C18(2) analytical column, (5□m particle size, 300 Å pore size, 150 x 2 mm) at a flow rate of 1.0 mL/min and a column temperature of 60 °C. The elution gradient was 0-80% B in 23 min where A was 0.1% TFA in water (v/v) and B was 0.1% TFA in acetonitrile (v/v). Detection was at 220 nm.

[421] Fractions containing BN-54 were collected, rotavaped and freeze dried to get BN-54 as white powder. The protein content and purity of BN-54 was determined and summarized as follows.

[422] The following table summarizes the protein content for different lots of Nobex-BN054:

| BN Conjugate | Lot # | Protein Content |
|---|---|---|

| | | (%) |
|---|---|---|
| Nobex-BN054 | MM-332-109-NM041101 | 74.8 |
| Nobex-BN054 | MM-332-149-NM050110 | 73.4 |
| Nobex-BN054 | MM-332-179-NM050214 | 74.4 |

[423] Representative chromatograms for the different lots of BN054, including the associated BNP standards, are included as Figures 8, 9 amd 10. This approach to purification is readily adapted for use with other conjugates of the invention.

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U.S Patent No. 6,586,396 – Seilhamer, et. al.
U.S. Patent No. 6,525,022 – Lowe, et. al.
U.S. Patent No. 6,028,055 – Lowe, et al.
U.S. Patent No 5,114,923 - Seilhamer et. al.
PCTUS0217567

# Claims

What is claimed is:

1. A natriuretic compound conjugate comprising:

a biologically active natriuretic compound comprising:

a natriuretic molecule NPR-A binding site; and

at least one modifying moiety conjugation site; and

at least one modifying moiety attached to said modifying moiety conjugation site;

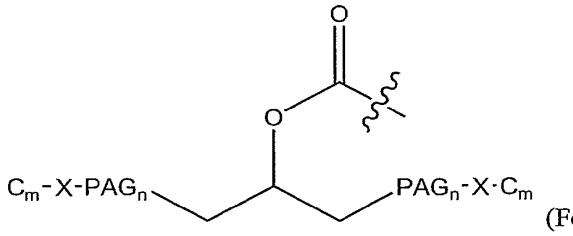
wherein said natriuretic compound conjugate exhibits one or more advantages selected from the group consisting of increased resistance to enzymatic degradation relative to a corresponding unconjugated natriuretic compound, increased circulating half life, increased bioavailability, and prolonged duration of effect.

2. The natriuretic compound conjugate of claim 1 wherein the natriuretic compound comprises a peptide or a biologically active peptide segment of brain natriuretic peptide, atrial natriuretic peptide, C-type natriuretic peptide, or dendroaspis natriuretic peptide.

3. The natriuretic compound conjugate of claim 1 wherein the natriuretic compound comprises a native hBNP sequence having one or more mutations selected from the group consisting of Lys3Arg, Lys14Arg, Arg30Lys, Lys27Arg, and Arg31Lys.

4. The natriuretic compound conjugate of claim 1 wherein the natriuretic compound comprises a native hBNP sequence, having one or more insertions, deletions or extensions.

5. The natriuretic compound conjugate of claim 1 wherein the modifying moiety has a formula:




(Formula I)

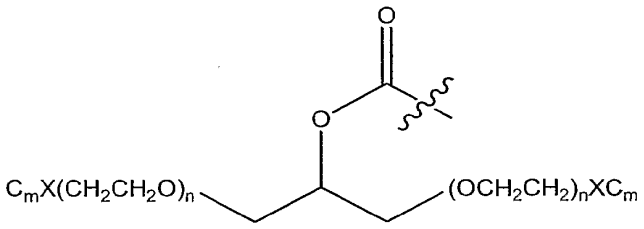
wherein

each C is independently selected and is an alkyl moiety having m carbons and m is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and

each PAG is independently selected and is a polyalkylene glycol moiety having n subunits and n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25;

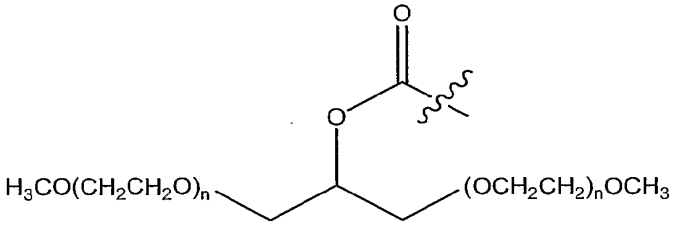
each X is independently selected and is a linking moiety.

6. The natriuretic compound conjugate of claim 5, wherein the modifying moiety has a formula:




(Formula 1A).

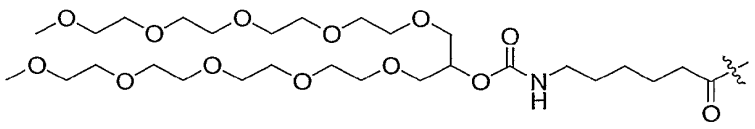
7. The natriuretic compound conjugate of claim 5, wherein the modifying moiety has a formula:



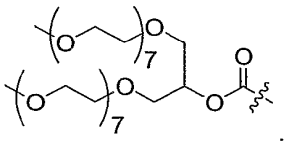

(Formula 1B).

8. A method of treating a condition characterized by an excessive level of extracellular fluid, the method comprising administering to a subject in need thereof a pharmaceutically acceptable amount of a natriuretic compound conjugate of claim 5.

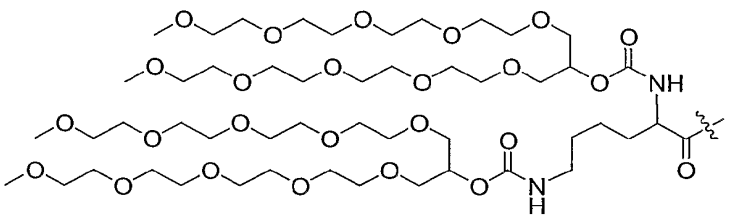
9. The natriuretic compound conjugate of claim 1, wherein the modifying moiety has a formula:



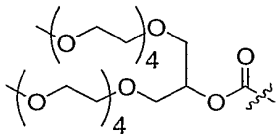
10. The natriuretic compound conjugate of claim 5, wherein the modifying moiety has a formula:



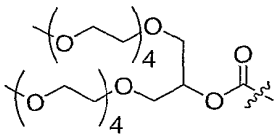
11. The natriuretic compound conjugate of claim 1, wherein the modifying moiety has a formula:



12. The natriuretic compound conjugate of claim 5, wherein the modifying moiety has a formula:



13. The natriuretic compound conjugate of claim 5, wherein the modifying moiety has a formula:



,

and the natriuretic compound is hBNP, and the modifying moiety is coupled at Lys3, and the natriuretic compound conjugate is a monoconjugate.

14. The natriuretic compound conjugate of claim 9 wherein the natriuretic compound is hBNP

15. The natriuretic compound conjugate of claim 10 wherein the natriuretic compound is hBNP.

16. The natriuretic compound conjugate of claim 11 wherein the natriuretic compound is hBNP.

17. The natriuretic compound conjugate of claim 12 wherein the natriuretic compound is hBNP.

18. The natriuretic compound conjugate of claim 9 wherein the natriuretic compound is hBNP, and the modifying moiety is coupled at Lys3, and the natriuretic compound conjugate is a monoconjugate.

19. The natriuretic compound conjugate of claim 10 wherein the natriuretic compound is hBNP, and the modifying moiety is coupled at Lys3, and the natriuretic compound conjugate is a monoconjugate.

20. The natriuretic compound conjugate of claim 11 wherein the natriuretic compound is hBNP, and the modifying moiety is coupled at Lys3, and the natriuretic compound conjugate is a monoconjugate.

21. The natriuretic compound conjugate of claim 12 wherein the natriuretic compound is hBNP, and the modifying moiety is coupled at Lys3, and the natriuretic compound conjugate is a monoconjugate.

22. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 9 and a pharmaceutically acceptable carrier.

23. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 10 and a pharmaceutically acceptable carrier.

24. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 11 and a pharmaceutically acceptable carrier.

25. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 12 and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 14 and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 15 and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 16 and a pharmaceutically acceptable carrier.

29. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 17 and a pharmaceutically acceptable carrier.

30. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 18 and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 19 and a pharmaceutically acceptable carrier.

32. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 20 and a pharmaceutically acceptable carrier.

33. A pharmaceutical composition comprising the natriuretic compound conjugate of claim 21 and a pharmaceutically acceptable carrier.

34. A method of making the natriuretic compound conjugate of claim 9, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

35. A method of making the natriuretic compound conjugate of claim 10, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

36. A method of making the natriuretic compound conjugate of claim 11, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

37. A method of making the natriuretic compound conjugate of claim 12, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

38. A method of making the natriuretic compound conjugate of claim 14, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

39. A method of making the natriuretic compound conjugate of claim 15, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

40. A method of making the natriuretic compound conjugate of claim 16, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

41. A method of making the natriuretic compound conjugate of claim 17, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

42. A method of making the natriuretic compound conjugate of claim 18, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

43. A method of making the natriuretic compound conjugate of claim 19, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

44. A method of making the natriuretic compound conjugate of claim 20, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

45. A method of making the natriuretic compound conjugate of claim 21, the method comprising chemically coupling the modifying moiety to the natriuretic compound.

46. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 9.

47. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 10.

48. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 11.

49. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 12.

50. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 14.

51. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 15.

52. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 16.

53. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 17.

54. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 18.

55. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 19.

56. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 20.

57. A pharmaceutically pure composition of the natriuretic compound conjugate of claim 21.

58. The natriuretic compound conjugate of claim 5 wherein m is 1, X is -O-, n is 4, and C is $-CH_3$.

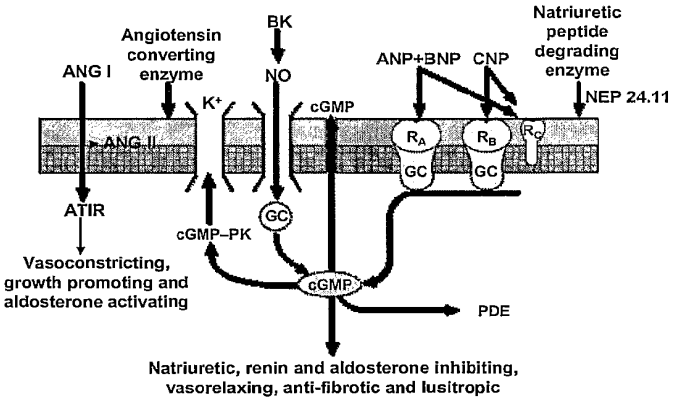

BK
Angiotensin converting enzyme
ANG I
NO
ANP+BNP
CNP
Natriuretic peptide degrading enzyme
NEP 24.11
K+
cGMP
ANG II
$R_A$
$R_B$
$R_C$
GC
GC
ATIR
GC
cGMP–PK
Vasoconstricting, growth promoting and aldosterone activating
cGMP
PDE
Natriuretic, renin and aldosterone inhibiting, vasorelaxing, anti-fibrotic and lusitropic

Figure 1

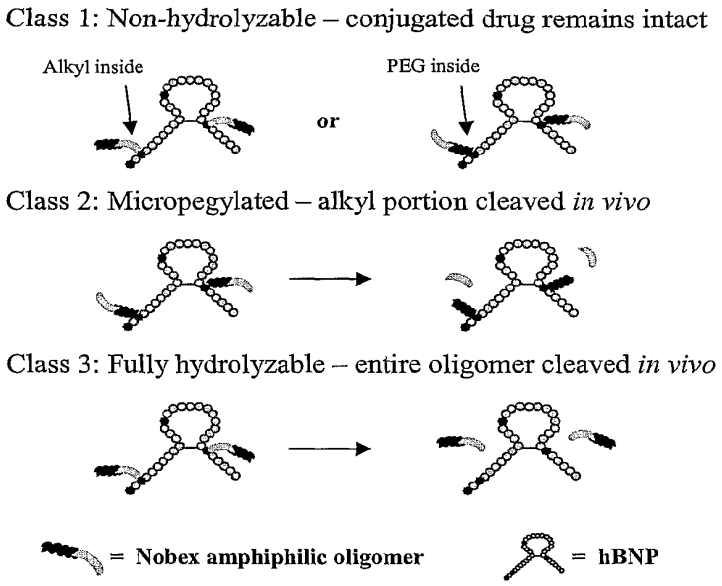

Class 1: Non-hydrolyzable – conjugated drug remains intact
Alkyl inside
PEG inside
or
Class 2: Micropegylated – alkyl portion cleaved *in vivo*
Class 3: Fully hydrolyzable – entire oligomer cleaved *in vivo*
= Nobex amphiphilic oligomer
= hBNP

Figure 2

**Figure 3:**

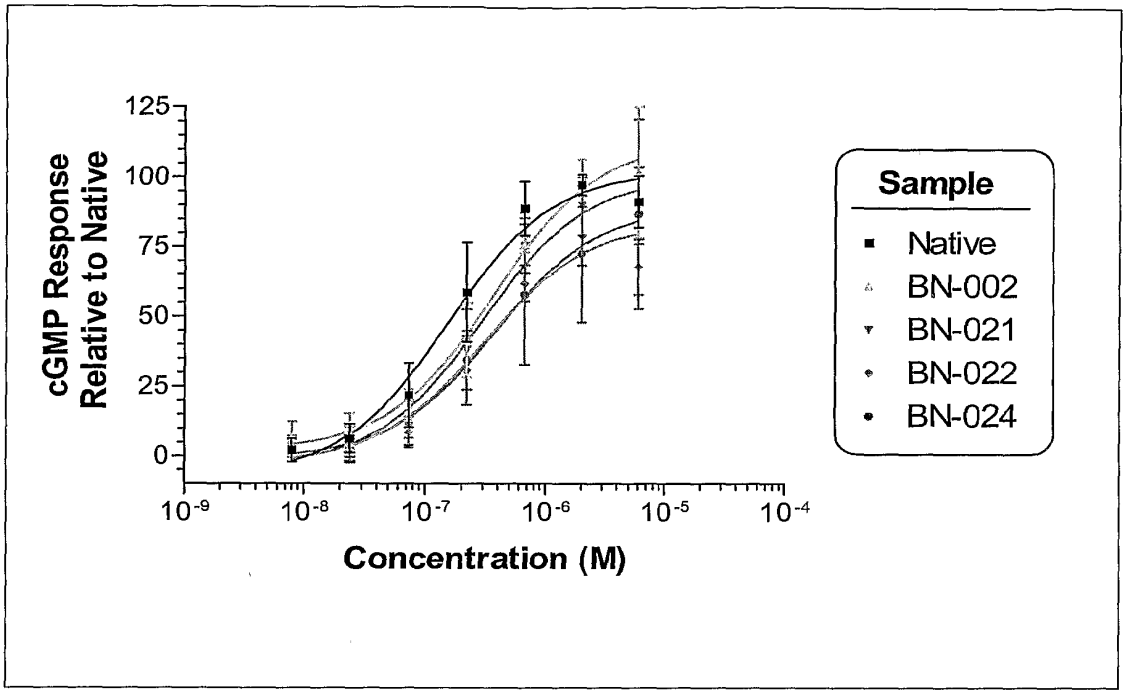

cGMP Response Relative to Native
125
100
75
50
25
0
$10^{-9}$
$10^{-8}$
$10^{-7}$
$10^{-6}$
$10^{-5}$
$10^{-4}$
Concentration (M)
Sample
Native
BN-002
BN-021
BN-022
BN-024

**Figure 4:**

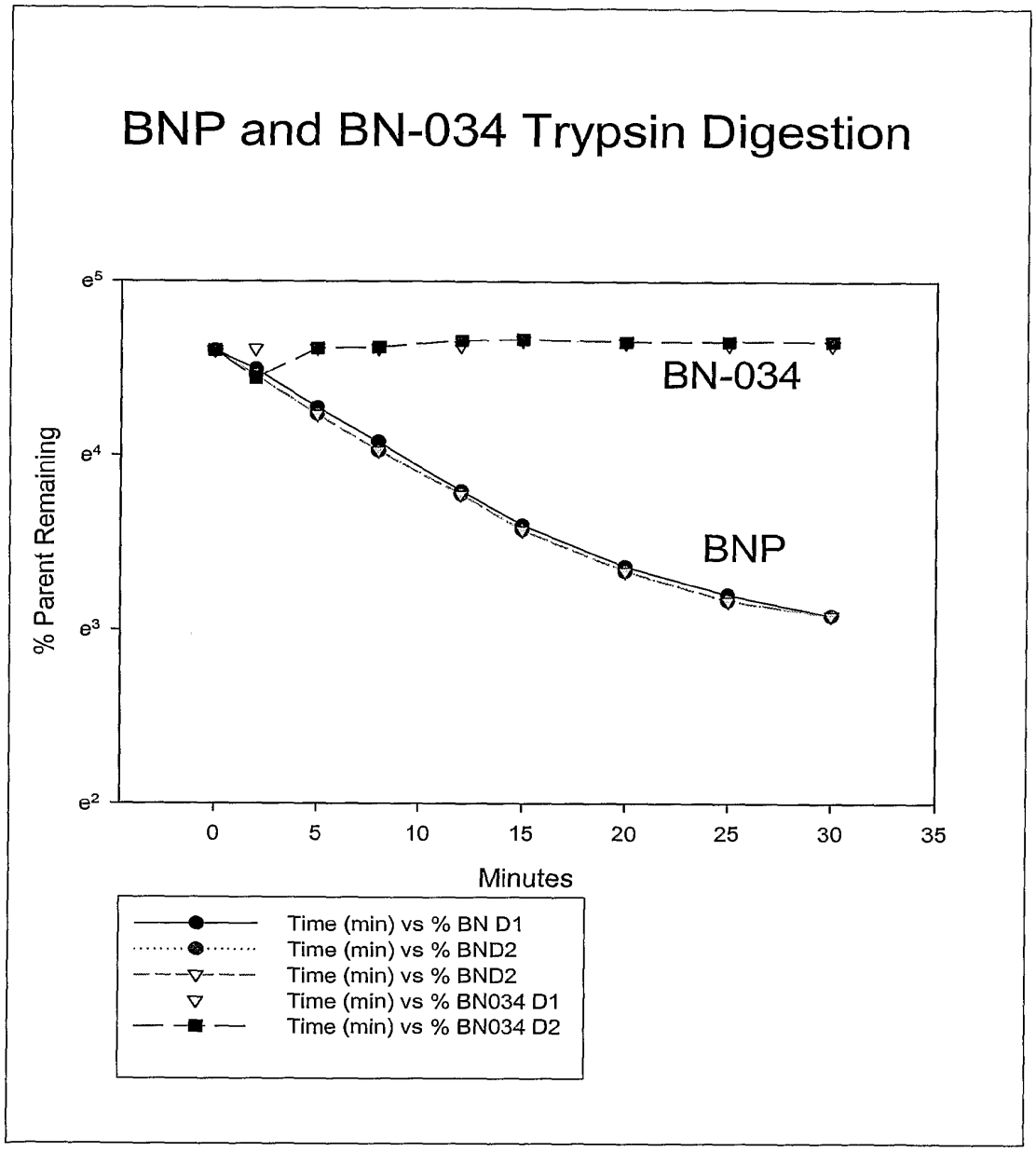
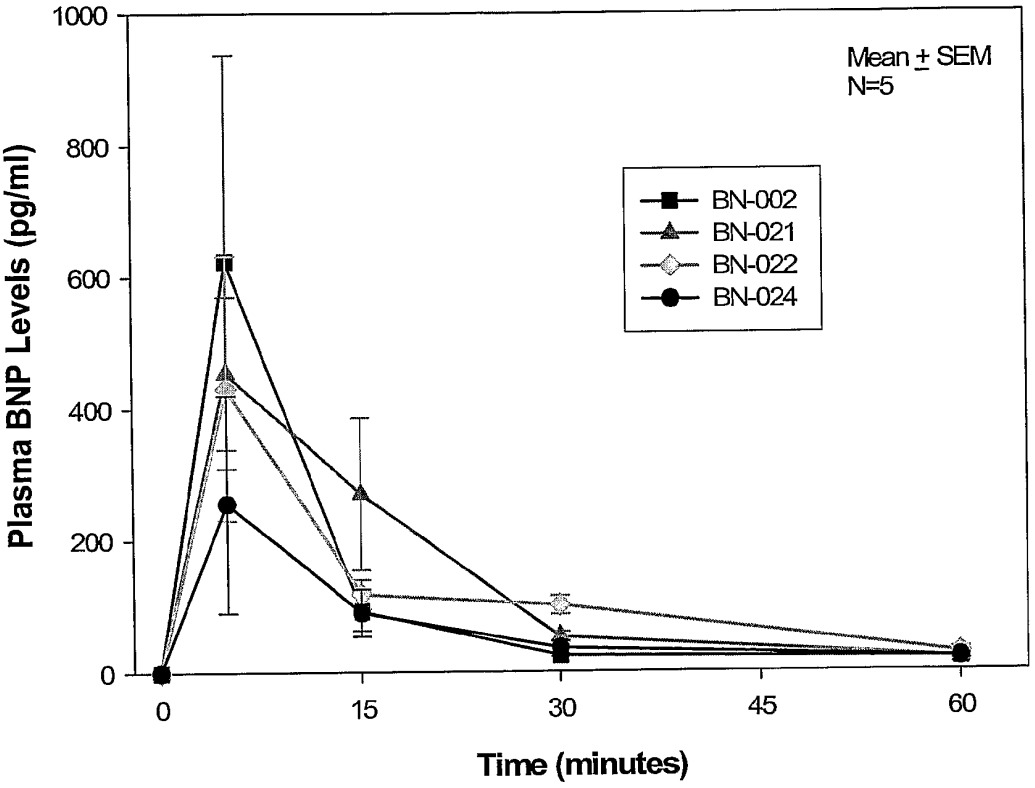

BNP and BN-034 Trypsin Digestion
% Parent Remaining
e5
e4
e3
e2
BN-034
BNP
0
5
10
15
20
25
30
35
Minutes
Time (min) vs % BN D1
Time (min) vs % BND2
Time (min) vs % BND2
Time (min) vs % BN034 D1
Time (min) vs % BN034 D2

**Figure 5:**

Figure 5: Plasma levels of hBNP conjugates at various times after oral dosing.



## NOBEX Corporation

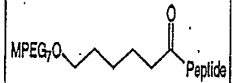
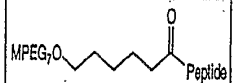
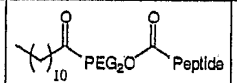
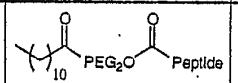
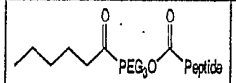
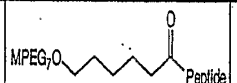
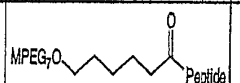
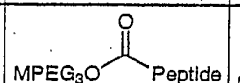
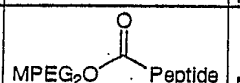
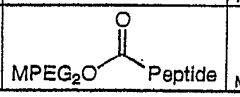
| NOBEX Number | Oligomer Structure | #of Oligomers | Average EC50 | Standard Deviation | Average Emax | Standard Deviation | Hydrolyzable | n= |
|---|---|---|---|---|---|---|---|---|
| Native BNP Lot UCB 050703 | N/A | N/A | 2.35E-07 | 1.1E-07 | 100.0 | 0.0 | N/A | 44 |
| BN-002 | | Mono-1 | 4.44E-07 | 3.0E-08 | 101.0 | 6.8 | No | 2 |
| BN-002 Scaled Up | | Mono-1 | 3.48E-07 | 2.29E-07 | 112.6 | 30.3 | No | 3 |
| BN-003 |   | Tri | >1.00E-05 | N/A | <20.0 | N/A | No | 4 |
| BN-004 |   | Tetra | >1.00E-05 | N/A | <20.0 | N/A | No | 2 |
| BN-007 |   | Tri | >1.00E-05 | N/A | <20.0 | N/A | Micropegylated | 2 |
| BN-008 |   | Tetra | >1.00E-05 | N/A | <20.0 | N/A | Micropegylated | 2 |
| BN-010 |   | Tetra | >1.00E-05 | N/A | <20.0 | N/A | Micropegylated | 3 |
| BN-011 |   | Mono-2 | 1.11E-06 | 3.3E-07 | 71.2 | 21.2 | No | 2 |
| BN-012 |   | DI | 1.71E-06 | 2.5E-07 | 60.8 | 19.2 | No | 2 |
| BN-013 |   | Tetra MPEG 3 of BN-010 | >1.00E-05 | N/A | <20.0 | N/A | MPEG 3 | 3 |
| BN-014 |   | Tri MPEG 2 of BN-007 | >1.00E-05 | N/A | 26.5 | 7.4 | MPEG 2 | 2 |
| BN-015 |   | Tetra MPEG 2 of BN-008 | >1.00E-05 | N/A | <20.0 | N/A | MPEG 2 | 2 |

FIG. 6A

## NOBEX Corporation

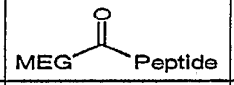
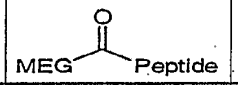
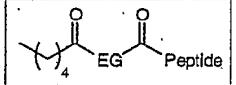
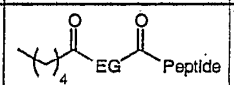
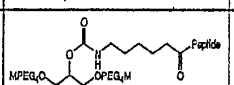
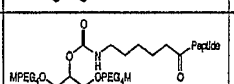
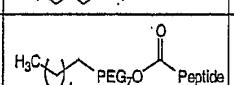
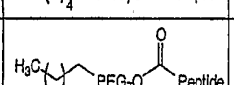
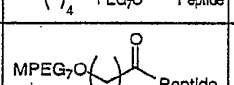
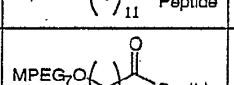
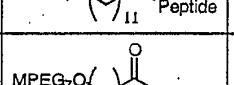
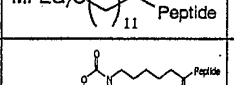
| NOBEX Number | Oligomer Structure | #of Oligomers | Average EC50 | Standard Deviation | Average Emax | Standard Deviation | Hydrolyzable | n= |
|---|---|---|---|---|---|---|---|---|
| BN-016 |  | Tetra | >1.00E-05 | N/A | 24.6 | 6.5 | MEG | 2 |
| BN-017 |  | Penta | >1.00E-05 | N/A | <20.0 | N/A | MEG | 1 |
| BN-018 |  | Tetra | >1.00E-05 | N/A | <20.0 | N/A | No | 2 |
| BN-019 |  | Penta | >1.00E-05 | N/A | <20.0 | N/A | No | 1 |
| BN-021 |  | Mono-1 | 3.69E-07 | 2.2E-07 | 91.8 | 1.9 | No | 2 |
| BN-021 Scaled Up |  | Mono-1 | 3.45E-07 | 1.2E-07 | 90.1 | 0.0 | No | 3 |
| BN-022 |  | Mono-1 | 2.81E-07 | 8.1E-08 | 75.7 | 9.2 | No | 2 |
| BN-022 Scaled Up |  | Mono-1 | 4.18E-07 | 7.03E-08 | 81.2 | 12.8 | No | 3 |
| BN-024 |  | Mono-1 | 3.21E-07 | 1.8E-07 | 106.2 | 42.7 | No | 2 |
| BN-024 Scaled Up |  | Mono-1 | 3.14E-07 | 2.26E-07 | 57.8 | 12.2 | No | 3 |
| BN-025 |  | Di-1 | >1.00E-05 | N/A | 43.5 | 3.0 | No | 2 |
| BN-028 |  | Mono-1 | >1.00E-05 | N/A | 28.3 | 2.4 | No | 2 |

FIG. 6B

## NOBEX Corporation

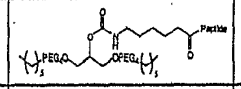
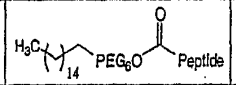
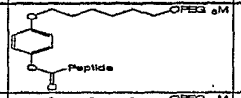
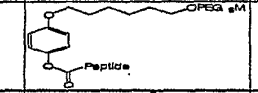
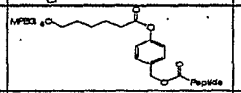
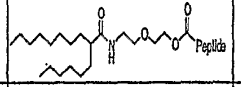
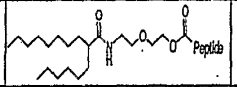
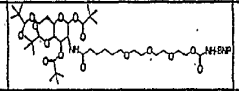
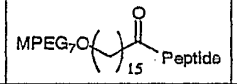
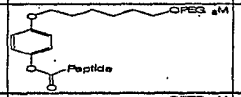
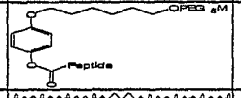
| NOBEX Number | Oligomer Structure | #of Oligomers | Average EC50 | Standard Deviation | Average Emax | Standard Deviation | Hydrolyzable | n= |
|---|---|---|---|---|---|---|---|---|
| BN-029 |  | Mono-2 | >1.00E-05 | N/A | 34.0 | 13.3 | No | 2 |
| BN-030 |  | Mono-2 | 1.26E-07 | 4.7E-08 | 53.6 | 5.6 | No | 2 |
| BN-033 |  | Mono-1/Mono-2 mixture | 1.38E-07 | #DIV/0! | 81.1 | #DIV/0! | Yes | 1 |
| BN-034 |  | Di-mixture | 2.75E-07 | 3.3E-07 | 23.9 | 8.4 | Yes | 2 |
| BN-038 |  | Di-mixture | 2.24E-06 | 2.1E-06 | 62.3 | 31.0 | Yes | 2 |
| BN-041 |  | Mono-1 | 3.58E-07 | 1.8E-07 | 30.5 | 6.9 | No | 2 |
| BN-042 |  | Mono-4 | 1.46E-07 | 9.5E-08 | 37.9 | 12.2 | No | 3 |
| BN-046 |  | Mono-1/Mono-2 Mixture | 2.45E-07 | 1.2E-07 | 60.5 | 23.6 | No | 3 |
| BN-047 |  | Mon0-1/Mono-2 Mixture | 8.15E-07 | 5.5E-07 | 40.2 | 6.6 | No | 4 |
| BN-048 |  | Mono-1 | 6.31E-07 | 6.6E-09 | 98.3 | 9.3 | Yes | 2 |
| BN-049 |  | Mono-2 | 1.25E-06 | 1.5E-08 | 75.2 | 11.7 | Yes | 2 |
| BN-050 | | Mono-1 | 1.71E-07 | 5.2E-08 | 94.4 | 22.9 | No | 3 |

FIG. 6C

NOBEX Corporation

| NOBEX Number | Oligomer Structure | #of Oligomers | Average EC50 | Standard Deviation | Average Emax | Standard Deviation | Hydrolyzable | n= |
|---|---|---|---|---|---|---|---|---|
| BN-050 (scale-up) | | Mono-1 | 4.33E-07 | 1.5E-07 | 104.6 | 23.2 | No | 2 |
| BN-051 | | Mono-1 | 5.56E-07 | 2.4E-07 | 106.2 | 16.6 | No | 3 |
| BN-052 | | Mono-1/ Mono-2 Mixture | 2.12E-07 | 1.0E-08 | 60.1 | 7.4 | No | 2 |
| BN-053 |  | Mono-1 | 2.65E-07 | 7.39E-08 | 99.0 | 3.6 | No | 3 |

FIG. 6D

**Chromatograph showing preparative run for BN-54**

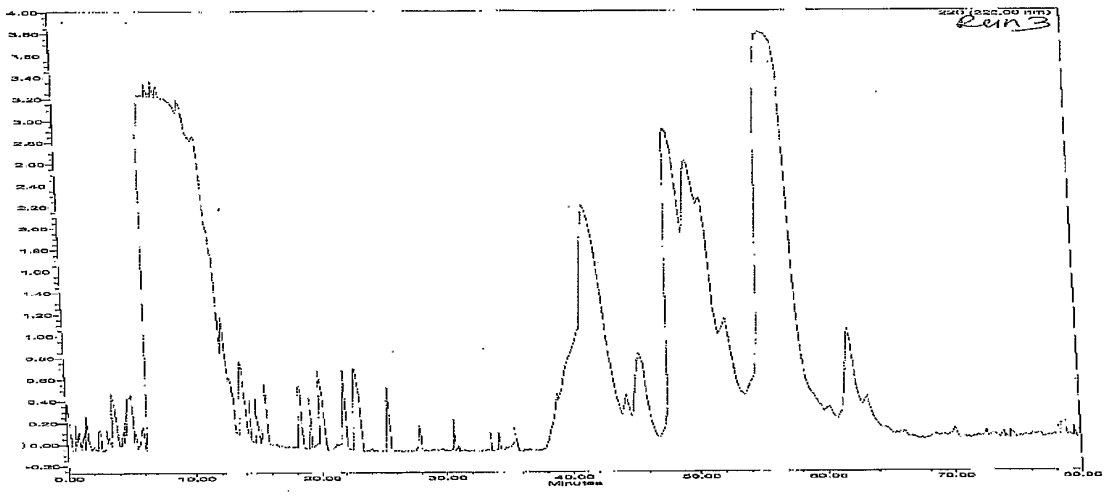



**Figure 7**

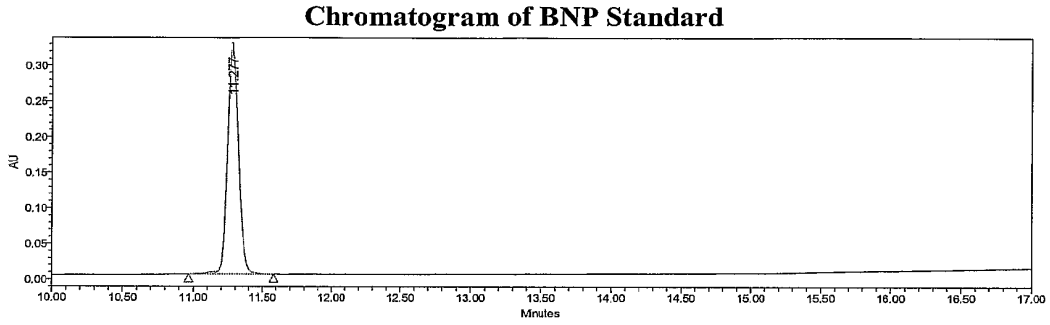

Chromatogram of BNP Standard
11.277
AU
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10.00
10.50
11.00
11.50
12.00
12.50
13.00
13.50
14.00
14.50
15.00
15.50
16.00
16.50
17.00
Minutes

**Figure 8**

**Chromatogram of Nobex-BN054**
**Protein Content 74.8%**

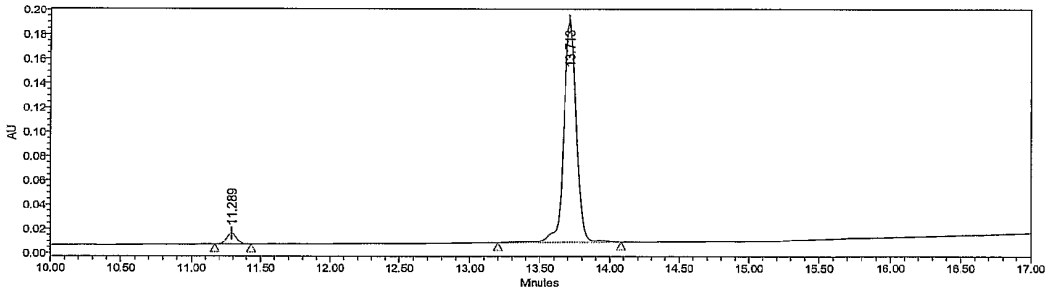



**Figure 9**

**Chromatogram of Nobex-BN054**
**Protein Content 73.4%**

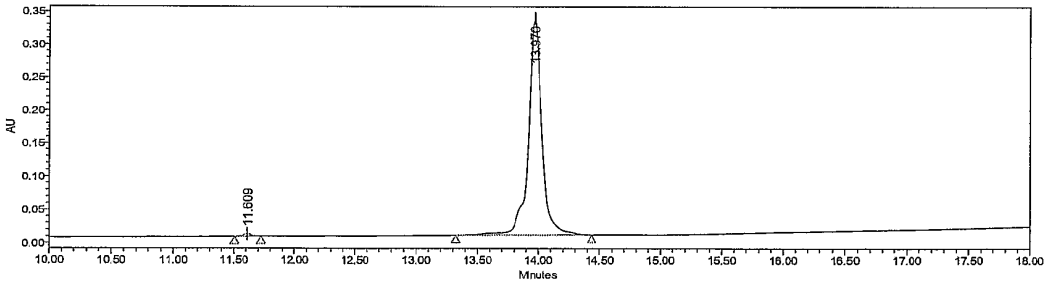



**Figure 10**

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Miscellaneous:** | | | | |
| RCE- 1ST REQUEST | 1801 | 1 | 1300 | 1300 |
| | | **Total in USD ($)** | | **1300** |

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 37427167 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 10-OCT-2019 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 17:51:49 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $1300 |
| RAM confirmation Number | E201900H53257771 |
| Deposit Account | 132855 |
| Authorized User | Katherine Neville |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:<br>37 CFR 1.17 (Patent application and reexamination processing fees) | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Request for Continued Examination (RCE) | Request_for_Continued_Examination_Transmittal.PDF | 27065<br>5ac21b52f6a2ee43ba24ba8c09c55065bc74c8e0 | no | 1 |
| **Warnings:** | | | | | |
| This is not a USPTO supplied RCE SB30 form. | | | | | |
| **Information:** | | | | | |
| 2 | | 44556C1-R2_second_supp_response.PDF | 79399<br>22a19c3fa50683323055ef248bca9867822f1c00 | yes | 23 |

**Multipart Description/PDF files in .zip description**

| Document Description | Start | End |
|---|---|---|
| Amendment Submitted/Entered with Filing of CPA/RCE | 1 | 1 |
| Claims | 2 | 22 |
| Applicant Arguments/Remarks Made in an Amendment | 23 | 23 |

| | | | | | |
|---|---|---|---|---|---|
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Transmittal Letter | Information_Disclosure_Statement.PDF | 17336<br>83c85450c60ede24a472a9a8078478dc9206e891 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Information Disclosure Statement (IDS) Form (SB08) | IDS_Citation_by_Applicant_3_References.PDF | 36219<br>5397e6cbeb8d2911ee977aa7047c2e9381030e96 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| 5 | Foreign Reference | WO2010033217A1.PDF | 5490290<br>d2fd8c3201d44a42dd592539b6658798933<br>3fb6a | no | 112 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Foreign Reference | WO2009095479A2.PDF | 3168815<br>c726ef92f126639d5ea2334a0130311735cd<br>c138 | no | 97 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 7 | Non Patent Literature | Samson504.PDF | 1929210<br>589e3dc7896d7a02591c306e2682c7f34d1<br>0d96b | no | 6 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 8 | Foreign Reference | WO2008154226A1.PDF | 4023065<br>9aa2f89fc6cf7d018cca8b40f4686301a2e73<br>c3b | no | 76 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 9 | Foreign Reference | WO2005087797A1.PDF | 9551703<br>894b94fbc7f6f7362929ff82d5d7d0043ec84<br>8ef | no | 171 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 10 | Foreign Reference | WO2005116655A2.PDF | 5755057<br>fd635a6f727ca0f8118bb0bcc51ca970940e<br>d4ca | no | 120 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 11 | Non Patent Literature | Frankel575.PDF | 4028434<br>d3848b151234400c912b5d16d8e2228aad<br>28d7dc | no | 7 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| 12 | Non Patent Literature | Dyadyk.PDF | 323566<br>e2da63b66450610ef4c33f416e5fe81a3766797c | no | 3 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 13 | Fee Worksheet (SB06) | fee-info.pdf | 30201<br>a59e4a8565035613145 9df015e05a02cf53888d2 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 34460360 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 10/23/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| CAMPELL, BRUCE R | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 10/23/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

| ***Advisory Action Before the Filing of an Appeal Brief*** | **Application No.** 15/646,822 | **Applicant(s)** Wendt et al. | |
|---|---|---|---|
| | **Examiner** BRUCE R CAMPELL | **Art Unit** 3991 | **AIA (FITF) Status** No |

***--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --***

THE REPLY FILED 26 September 2019 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☑ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☐ The period for reply expires ____ months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☑ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection.The current period for reply expires 6 months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   *Examiner Note*: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANTS FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37CFR 41.37(a).

AMENDMENTS

3. ☐ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: _____ (See 37CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☑ Applicants reply has overcome the following rejection(s): The terminal disclaimer has overcome the double patenting rejection.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☐ For purposes of appeal, the proposed amendment(s):(a)☐ will not be entered, or (b)☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☑ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: The claims are not in the correct format for reissue applications..
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☑ Other: The RCE, amendment and IDS filed 10/10/2019 have been entered. An Office action will be issued in due course..

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed:_____.
    Claim(s) objected to:15-30.
    Claim(s) rejected:_____.
    Claim(s) withdrawn from consideration:_____.

| /BRUCE R CAMPELL/ /SHARON TURNER/<br>Patent Reexamination Specialists, CRU 3991 | /JEAN C WITZ/<br>Supervisory Patent Reexamination Specialist, CRU 3991 |
|---|---|

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 11/26/2019

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| CAMPELL, BRUCE R | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 11/26/2019 | ELECTRONIC |

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The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mflores@bmrn.com
mgbdocket@marshallip.com

| *Office Action Summary* | Application No. 15/646,822 | Applicant(s) Wendt et al. | |
|---|---|---|---|
| | Examiner BRUCE R CAMPELL | Art Unit 3991 | AIA (FITF) Status No |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 10 October 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 15-30 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 10/10/2019.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

### *Notice of Pre-AIA or AIA Status*

The present application is being examined under the pre-AIA first to invent provisions.

### *Reissue Applications*

For reissue applications filed before September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the law and rules in effect on September 15, 2012. Where specifically designated, these are "pre-AIA" provisions.

For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.

### *Continued Examination Under 37 CFR 1.114*

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on October 10, 2019 has been entered.

### *Claim Rejections - 35 USC § 102*

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim(s) 15-30 are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by Wendt, U.S. Patent 8,377,884.

The applied reference has a common assignee and inventors with the instant application. Based upon the earlier effective U.S. filing date of the reference, it constitutes prior art under pre-AIA 35 U.S.C. 102(e). This rejection under pre-AIA 35 U.S.C. 102(e) might be overcome either by a showing under 37 CFR 1.132 that any invention disclosed but not claimed in the reference was derived from the inventor or joint inventors (i.e., the inventive entity) of this application and is thus not the invention "by another," or if the same invention is not being claimed, by an appropriate showing under 37 CFR 1.131(a).

Wendt discloses variants of C type natriuretic peptide (CNP) useful for treating achondroplasia. CNP22 can be extended at the N-terminus by 1-40 amino acids derived from NPPC or CNP53 (col. 8, lines 59-65). These variants can be conjugated to PEG at the N-terminus (col. 9, lines 8-14). Thus Wendt anticipates the pegylated forms of SEQ ID Nos. 60 and 146-174 (claims 15-20). With regard to claim 21, Wendt teaches that CNP stimulates cGMP production (col. 2, lines 23-26), and directly demonstrates this with CNP22, CNP27, CNP37 and CNP53 (Figs. 4-6, 9-12). With regard to claim 22, CNP reverses growth arrest induced by activated FGFR3 (Fig. 7). With regard to claims 23-30, Wendt teaches that the CNP variants are useful for treating skeletal dysplasias, including achondroplasia (col. 61, lines 37-59).

### *Claim Rejections - 35 USC § 103*

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to

consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 15-30 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over U.S. patent 6,743,425 ("Nakao '425") taken with U.S. 2007/0197434 ("Nakao '434"), Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang.

State of the Art Regarding CNP

Potter teaches the structure of C type natriuretic peptide (CNP). CNP is synthesized as a 126 amino acid pre-pro-hormone, which is cleaved by furin to release a 53 amino acid polypeptide (CNP53) having an N-terminal aspartate residue. CNP53 can be further cleaved by an unidentified peptidase to yield CNP22. See Fig. 1. CNP binds to two receptors, NPR-A and NPR-B, which are transmembrane guanylyl cyclases (p. 51). Loss of NPR-B function is associated with dwarfism in both humans and mice (pp. 52-53). CNP stimulates bone growth by increasing proliferation of hypertrophic chondrocytes, and dwarfism in mice caused by constitutively active FGF3 receptor can be partially reversed by transgenic expression of CNP (p. 62). Potter suggests using CNP to treat dwarfism (p. 63).

Yeung compared the effects of CNP22 and CNP53 on cGMP production in cultured mouse astrocytes. The two forms of CNP were found to have very similar binding characteristics and effects on cGMP production (see Figs. 1, 3, 4), which did not appear to be caused by conversion of CNP53 to CNP22. It was concluded that CNP53 is biologically active itself, i.e. it is not simply a precursor to CNP22 (p. 105).

Totsune teaches that the methionine residue found in both CNP53 and CNP22 is susceptible to oxidation (p. 39, Fig. 3; also see Potter Fig. 1).

State of the Art Regarding Recombinant Protein Production

Eun reviews protein synthesis and turnover. Eun teaches that a protein's stability and susceptibility to degradation are affected by the amino acid residues at the N-terminal end. Methionine and glycine have a stabilizing effect, while aspartate is destabilizing. See pp. 13-14.

Szoka teaches that small peptides can be produced as fusion proteins in bacteria (p. 11) and discloses a method for cleaving fusion proteins to release the peptide of interest. An expression vector is designed so that the N-terminal of the peptide of interest is joined to the C-terminal of a bacterial protein, linked by an aspartate-proline dipeptide (Fig. 3). Acid treatment cleaves the dipeptide, releasing the peptide of interest with a proline residue added at the N-terminus (pp. 16-17). When bovine growth hormone was produced by this method, presence of the N-terminal protein did not prevent the hormone from binding its receptor (Fig. 7).

Huang teaches, "It is well known that hSA, the most abundant protein in plasma, is a good carrier protein with a substantial circulatory half-life (up to 19 days in the human blood) and is widely distributed in vivo. A number of proteins have been fused to albumin to improve their circulating half-lives and stability characteristics" (p. 2, citations omitted).

Suggestion to Make and Use the Claimed Inventions

Nakao '425 discloses a method for treating achondroplasia caused by constitutive activation of FGFR3 by administering a compound that activates guanylyl cyclase B, such as CNP22 or CNP53 (col. 2, line 48 – col. 3, line 20; col. 8, lines 1-48; claim 5). Nakao also discloses transgenic mice overexpressing FGFR3 which can be used to test potential therapeutics (col. 14, lines 44-46). Nakao '425 does not teach a method in which the administered compound is a CNP analog (i.e. not CNP22 or CNP53) conjugated to a synthetic polymer.

Nakao '434 discloses another use for CNP peptides, treatment of rheumatoid arthritis. Nakao '434 teaches that CNP22 or CNP53 could be modified by addition, deletion or substitution of amino acid residues to yield biologically active analogs (¶¶ 29, 35, 47, 102-115).

Golembo discloses a method for treating skeletal dysplasias by administering natriuretic peptides. The administered peptide can be CNP or a CNP variant (p. 5, lines 26-28; p. 7, lines 14-17). Golembo teaches that deletion of the 5 N-terminal residues from CNP22 (to yield what the instant application calls CNP17) yields a peptide as active as CNP22, and further teaches substitution of the methionine residue, which is a

neutral endopeptidase cleavage site (p. 7, line 29 – p. 8, line 15; pp. 21-24). Golembo teaches that CNP has a short half life in vivo (p. 3, line 31) but can be conjugated to an agent to prolong its half life in circulation (p. 6, line 20).

James discloses natriuretic peptides and analogs conjugated to PEG (¶¶ 4, 49, 50, claim 1). Natriuretic peptide analogs can include an N-terminal "tail" comprising all or part of the native natriuretic peptide sequence (¶ 70). James teaches that the use of pegylated proteins as therapeutics is well established and provides advantages such as increased half life in the bloodstream (¶¶ 13, 16).

It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the method of Nakao '425 by administering a CNP analog.

Many of the claimed peptides are truncated forms of CNP53 (SEQ ID Nos. 60, 145—178). Yeung established that CNP22 and CNP53 are equally effective, and Golembo found that CNP17 is also as effective as CNP22, leading to the conclusion that the CNP17 ring structure is all that is required to activate the NPR-B receptor. Accordingly, Nakao '434 and Golembo both teach that CNP53 or CNP22 analogs with a longer or shorter "tail" attached to the CNP17 ring can be used. There was a reasonable expectation that truncated forms of CNP53 (CNP52, CNP51, etc.) would be active because the longest tail (CNP53) does not interfere with receptor binding, as taught by Yeung. Furthermore, a truncated form of CNP53 should still be cleaved to CNP22 by an endopeptidase, as taught by Potter. It would also have been obvious to include an N-terminal "tail" as an attachment site for HSA or PEG, as taught by James, to avoid steric interference with the CNP17 ring which is involved with receptor binding.

Some of the claimed peptides are truncated forms of CNP53 which have been further modified.

The substitution of the methionine with a different amino acid (SEQ ID Nos. 180-184) is suggested by Totsune, which teaches that the methionine residue is susceptible to oxidation, and Golembo, which teaches that this residue is susceptible to cleavage by endopeptidase.

With regard to SEQ ID Nos. 75, 179, 181, 190, 191, 193 and 194, it would have been obvious to append a glycine or methionine to the N-terminus of any peptide because these residues have a stabilizing effect, as taught by Eun. It was especially obvious to do so for CNP53 because the N-terminal aspartate of CNP53 would have a destabilizing effect.

If the CNP analog were produced by the conventional method of expression as part of a fusion protein (taught by Szoka), an N-terminal proline would remain after cleavage of the fusion protein, as in SEQ ID Nos. 145, 185, 186, 188 and 189.

With regard to SEQ ID Nos. 144, 183, 188 and 193, it was obvious to provide a CNP analog as a fusion with HSA since this was known to increase the half life of circulating proteins, as taught by Huang.

Regarding all the claimed peptides, pegylation is an art recognized method for prolonging the half life of proteins in the bloodstream, and James discloses pegylation of "virtually any natriuretic peptide." James also teaches that undesired conjugation of PEG can be prevented by substituting lysine residues with arginine (¶ 65), a modification found in SEQ ID Nos. 36, 184, 189 and 194.

With regard to the peptides' properties which are recited in the claim preambles, James Fig. 2 discloses pegylated natriuretic peptides in which the PEG can be fully hydrolyzed to release the peptide, as recited in claims 15 and 18. Stimulation of cGMP production (claim 21) and overcoming constitutive activation of FGFR3 (claim 22) were known properties of CNP, and treating skeletal dysplasia including achondroplasia (claim 23) had already been suggested (Potter, Yeung, Golembo, Nakao '425).

Thus the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Claims 15-30 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over U.S. patent 6,743,425 ("Nakao '425") taken with U.S. 2007/0197434 ("Nakao '434"), Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang, and further in view of Wendt.

Nakao '425, Nakao '434, Golembo, James, Potter, Yeung, Totsune, Eun, Szoka and Huang are relied upon as described in the rejection above.

Wendt is relied upon as cited above. Wendt is cited here because it specifically suggests replacing the methionine residue (residue 48 of CNP53 or residue 17 of CNP22) with asparagine (e.g. col. 43, lines 23-25). This is relevant to SEQ ID Nos. 180-184.

Wendt is also cited because the test results showing increased half life of CNP37 and pegylated CNP27 compared to CNP22 (e.g. Figs. 9-12) provide further expectation of success regarding extension of CNP22 by including all or part of the remaining CNP53 sequence, and pegylation of these peptides. This is relevant to SEQ ID Nos. 60 and 145-178.

Thus the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made.

## *Conclusion*

No claim is allowed.

## *Correspondence*

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BRUCE CAMPELL whose telephone number is 571-272-0974. The examiner can normally be reached on Monday - Thursday from 9:00 to 5:00, Central Time. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Timothy Speer, can be reached on 313-446-4825.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov.

Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Bruce Campell/
Patent Reexamination Specialist
Central Reexamination Unit 3991

/Sharon Turner/
Patent Reexamination Specialist
Central Reexamination Unit 3991

/Jean C. Witz/
Supervisory Patent Reexamination Specialist
Central Reexamination Unit 3991

| Notice of References Cited | Application/Control No. 15/646,822 | Applicant(s)/Patent Under Reexamination Wendt et al. | |
|---|---|---|---|
| | Examiner BRUCE R CAMPELL | Art Unit 3991 | Page 1 of 2 |

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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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| *Notice of References Cited* | Application/Control No. 15/646,822 | Applicant(s)/Patent Under Reexamination Wendt et al. | |
|---|---|---|---|
| | Examiner BRUCE R CAMPELL | Art Unit 3991 | Page 2 of 2 |

**U.S. PATENT DOCUMENTS**

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**FOREIGN PATENT DOCUMENTS**

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| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | BRUCE R CAMPELL | 3991 |

| CPC - Searched* | | |
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| Symbol | Date | Examiner |
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| CPC Combination Sets - Searched* | | |
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| Symbol | Date | Examiner |
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| US Classification - Searched* | | | |
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| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Reviewed prosecution history in parent application 13/466,672 | 6/6/2018 | /J.R./ |
| Reviewed prosecution in RE 46,707 E | 6/6/2018 | /J.R./ |
| Google Search Terms: C-type natriuretic peptide bone review " fibroblast growth factor receptor" | 6/12/2018 | /J.R./ |
| Inventor Search | 6/18/2018 | /J.R./ |
| updated Inventor Search | 04/03/2019 | /J.R,/ |
| reviewed 13/466,672 and 14/604,262 | 10/15/2019 | /BC/ |

| Interference Search | | | |
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| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| A61K | 38/2242 | 10/15/2019 | /BC/ |

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | BRUCE R CAMPELL | 3991 |

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.R.C/

PTO/SB/08a (07-09)
Approved for use through 11/30/2020. OMB 0651-0031
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

*(Use as many sheets as necessary)*

Sheet 1 of 2

| *Complete if Known* | |
|---|---|
| Application Number | 15/646,822-Conf. #7727 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

## U. S. PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Document Number<br>Number-Kind Code[2] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear |
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| Examiner Initials* | Cite No.[1] | Foreign Patent Document<br>Country Code[3]-Number[4]-Kind Code[5] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
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| | | | | | | |

| Examiner Signature | /BRUCE R CAMPELL/ | Date Considered | 10/15/2019 |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. 3 Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). 4 For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. 5Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. 6 Applicant is to place a check mark here if English language Translation is attached.

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| Sheet | 2 | of | 2 |
|---|---|---|---|

| *Complete if Known* | |
|---|---|
| **Application Number** | 15/646,822-Conf. #7727 |
| **Filing Date** | July 11, 2017 |
| **First Named Inventor** | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

**NON PATENT LITERATURE DOCUMENTS**

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(71) Applicant *(for all designated States except US)*: **PROCHON BIOTECH LTD.** [IL/IL]; Kiryat Weizmann Science Park, P.O. Box 1482, 76114 Rehovot (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GOLEMBO, Myriam** [IL/IL]; Moshav Netayim 113, 76870 (IL). **YAYON, Avner** [IL/IL]; Moshav Sitria # 104, 76834 (IL).

(74) Agent: **WEBB, Cynthia**; Webb & Associates, P.O. Box 2189, 76121 Rehovot (IL).

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(54) Title: METHOD AND COMPOSITION FOR TREATMENT OF SKELETAL DYSPLASIAS

(57) Abstract: The present invention discloses pharmaceutical compositions for the treatment of skeletal dysplasias, comprising as an active ingredient at least one natriuretic peptide. Unexpectedly, it has been shown that the natriuretic factors may be effective for bone elongation in situations of abnormal bone growth especially for Achondroplasia. The effects of the natriuretic peptide may be further enhanced by prolonging its residence time or action at the target site.

# METHOD AND COMPOSITION FOR TREATMENT OF SKELETAL DYSPLASIAS

## FIELD OF THE INVENTION

The present invention relates to compositions for treatment of skeletal dysplasias, such as achondroplasia, and in particular to pharmaceutical compositions for bone elongation in disorders related to abnormal bone growth comprising natriuretic peptides or natriuretic factors.

## BACKGROUND OF THE INVENTION

Bone Development

Endochondral ossification is a fundamental mechanism for bone formation, whereby cartilage is replaced by bone. Endochondral ossification requires the sequential formation and degradation of cartilaginous structures that serve as molds for the developing bones. The process of endochondral ossification in the cartilaginous growth plate, which is located at both ends of vertebrae and long bones, determines longitudinal bone growth.

During fetal life and until the end of puberty, longitudinal bone growth takes place via endochondral ossification of the growth plate located at the epiphyses (ends) of long bones. The growth plate is divided into several zones of cartilage forming cells, or chondrocytes, with distinct patterns of gene expression. In the Reserve Zone, cells are small and relatively inactive. In the adjacent Proliferative Zone, chondrocytes proliferate, arrange themselves in columns and eventually undergo hypertrophy. In the lower Hypertrophic Region towards the cartilage-bone junction, cells are big and highly active but exhibit no further cell division. The matrix surrounding the hypertrophic cells calcifies and the lowermost cells undergo programmed cell death. Cell death is accompanied by the removal of the cartilaginous matrix and its replacement by bone through the concerted action of recruited bone cells, namely osteoclasts and osteoblasts.

Signaling Pathways in Bone Development

The process of endochondral ossification is the result of the concerted action of several signaling pathways. The signaling pathway triggered by activation of Fibroblast Growth Factor (FGF) receptors have been shown to be involved in several stages of limb and bone development. A number of birth defects are associated with mutations in the

genes encoding FGF receptors (FGFR). For example a mutation in FGFR1 is associated with Pfeiffer syndrome. Certain other mutations in FGFR2 are associated with Crouzon, Pfeiffer, Jackson-Weiss, Apert or Beare-Stevenson syndromes. The clinical manifestation of Apert syndrome (AS) is characterized by both bony and cutaneous fusion of digits of the hands and the feet. Broad thumbs and halluces distinguish Pfeiffer syndrome, while in Crouzon syndrome limbs are normal but a high degree of proptosis is evident. The most prominent malformation syndrome associated with these mutations is craniosynostosis (the premature fusion of the skull bones sutures).

FGFR3 has an inhibitory role in bone elongation as demonstrated by the fact that mice lacking this receptor exhibit a phenotype of skeletal overgrowth. Moreover, mutations at various positions in this receptor result in skeletal dysplasias (SD). Thanatophoric dysplasia is a severe and lethal form, while hypochondroplasia is a milder form than Achondroplasia. Examination of the sequence of FGFR3 in Achondroplasia patients identified a mutation in the transmembrane domain of the receptor (reviewed in Vajo et al. (2000) Endocrine Rev 21:23-39).

Achondroplasia is the most common form of short-limbed dwarfism occurring with a frequency of 1:20,000 live births. Patients show characteristic shortening of proximal long bones (rhizomelia), relative macrocephaly, depressed nasal bridge and lumbar lordosis. Achondroplasia is mainly caused by a Gly380Arg (G380R) mutation in the transmembrane domain of the FGFR3 and is transmitted in an autosomal dominant fashion (Shiang et al. (1994) Cell 78: 335-342 and Rousseau et al. (1994) Nature 371: 252-254). A Gly375Cys (G375C) mutation has also been reported in some Achondroplasia patients. These mutations affect the process of endochondral ossification by inhibiting proliferation and delaying maturation of chondrocytes in the growth plate cartilage of long bones, resulting in decreased elongation.

Other major regulators of bone growth include growth hormone (GH, reviewed in Kelly et al., (2001) Horm Res;55 Suppl 2:14-7); insulin-like growth factor 1 (IGF-1, reviewed in McCarthy and Centrella (2001) Growth Horm IGF Res 11:213-9), glucocorticoids (GC) thyroid hormone (TH, Harvey et al., (2002) Mol Genet Metab 75:17-30) and Vitamin D (van Leeuwen et al, (2001) Steroids 66:375-80).

Each of these molecules exerts its function by binding to specific cell-surface or nuclear receptors of skeletal cells.

Natriuretic Peptides

Natriuretic peptides are known for their role in cardiovascular homeostasis, diuresis, natriuresis and vasodilation. Four isoforms constitute this family: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP). While ANP and BNP are circulating peptides produced by the atria and the ventricle respectively, CNP is hardly found in circulation and is mainly produced in the brain, in vascular endothelial cells and other tissues where it is supposed to work in an autocrine/paracrine manner (Chen and Burnett (1998) J. Cardiovasc. Pharm. 32 Suppl 3:S22-8). DNP is present in human plasma and atrial myocardium (Chen et al (2000) Curr Cardiol 2:198-205) and its sequence disclosed (Schweltz et al (1992) JBC 267:13928-32). CNP from different species have been disclosed in US Patent nos. 5,336,759 (frog); 5,338,759 (chicken); 5,973,134 (rat); 6,020,168 (pig) and 6,034,231 (human).

Natriuretic peptides effect their biological role through two receptors: NPR-A and NPR-B. These receptors have cytoplasmic guanylyl cyclase domains, which are activated upon ligand binding and lead to accumulation of intracellular cGMP. Some of the effects of cGMP are mediated through two known protein kinases: cGMP-dependent protein kinase I and II. The peptides bind the receptors with different affinities: ANP≥BNP>>CNP for NPR-A and CNP> ANP≥ BNP for NPR-B. The tissue distribution of each receptor is different. While NPR-A is expressed in vasculature, kidney and adrenal glands, NPR-B is mainly expressed in the brain.

NPR-C, a third receptor devoid of the kinase and cytoplasmic GC domains is generally considered to be a clearance receptor for removing natriuretic peptides from the circulation, though some other biological functions have been attributed to it (Murthy and Makhlouf (1999) JBC 274:17587-92). This is a widely distributed receptor expressed in almost all the tissues that express a guanylyl cyclase receptor. US Patent no. 5,846,932 discloses potent ANP variants having decreased affinity for the human clearance or C-receptor. These ANP variants exhibit natriuretic, diuretic and vasorelaxant activity but have increased metabolic stability, making them suitable for treating congestive heart failure, acute kidney failure and renal hypertension. Furthermore, WO00/61631 discloses novel pentapeptide antagonists of the NPR-C.

Natriuretic peptides have a short half life *in vivo*. In addition to the clearance receptor, they are further cleared from the circulation by degradation. The peptides are

cleaved at specific sites, by the neutral endopeptidase 24.11 (NEP) which is found in endothelial cells covering the vascular walls. Human BNP is more resistant to this degradation while ANP and CNP are readily degraded by this enzyme. Inhibition of NEP by inhibitors, including the compounds thiorphan or candoxatril (Ohbayashi et al. (1997) Clin. Exp. Pharma. Physiol. 25: 986-91; Brandt et al. (1997) Hyperten. 30: 184-90), increases the concentration of endogenous or administered peptides in the circulation.

CNP, like ANP, BNP and DNP, was shown to exhibit natriuretic and hypotensive actions. Novel CNP-related peptides capable of eliciting a strong cGMP response and suppressing the growth of vascular smooth muscle cells have been disclosed in US Patent no. 5,434,133. Also disclosed are the amino acids responsible for the cGMP producing activities and novel CNP variants capable of inhibiting abnormal growth of smooth muscle cells, for the treatment of, inter alia, restenosis and arteriosclerosis.

Transgenic mice, over-expressing BNP show a skeletal phenotype characterized by overgrowth of the axial and appendicular skeleton (Suda et al. (1998) PNAS 95: 2337-42). Moreover, mice that are null mutants for the clearance receptor, NPR-C, exhibit similar skeletal overgrowth, consistent with a role for the local modulation of natriuretic peptides levels by NPR-C (Matsukawa et al. (1999) PNAS 96: 7403-08). CNP and its specific receptor, NPR-B, have been shown to be expressed in the proliferating zone of the growth plate in fetal mouse tibia while NPR-C has been shown to be expressed in the region of hypertrophic chondrocytes and in osteoblasts (Yamashita et al. (2000) J Biochem 127: 177-9). After the date of the present invention, Chuso et al (Chusho et al. (2001) PNAS 98:4016-21) have disclosed CNP knockout mice which exhibit skeletal phenotypes histologically similar to those seen in Achondroplasia mice. They also reveal the rescue of the CNP knock out skeletal defects by tissue-specific ectopic CNP expression in the growth plate. Moreover, *ex vivo* experiments (fetal bone organ culture) from wild type animals have shown that CNP, more than BNP and ANP, can induce bone elongation (Yasoda et al. (1998) JBC 273: 11695-700, Mericq et al. (2000) Ped Res 47: 189-93).

While much is known about the components of signaling pathways that contribute to the process of endochondral ossification, little is known about the complex interactions between them that coordinate longitudinal bone growth.

## SUMMARY OF THE INVENTION

One object of the present invention is to provide a method for the treatment of skeletal dysplasias. A second object of the present invention is to provide pharmaceutical compositions useful in the treatment of skeletal dysplasias. Yet another object is to provide novel compounds and compositions useful in the methods of the invention.

The method and composition of the invention affect bone elongation, inter alia, by increasing the size of the growth plate of the bone, specifically of limb bones, in skeletal dysplasias such as achondroplasia.

It is an object of the present invention to provide a pharmaceutical composition comprising natriuretic peptides (NP) or functional variants useful in effecting bone elongation and treating skeletal dysplasias.

It is another object of the present invention to provide NP variants with increased stability.

It is yet another object of the present invention to provide a method to enhance NP stabilization in circulation.

It is a further object of the present invention to provide a method of delivering NP or its variants to a target site .

These and other objects are met by the invention disclosed herein.

The term "natriuretic peptides" or "NP" as referred to herein relates to any of the four isoforms, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) or dendroaspis natriuretic peptide (DNP) and to any functional variants thereof. The natriuretic peptides may be of any species but are preferably human.

The invention provides the use of natriuretic peptides (NP), their conjugates, variants and derivatives in effecting bone elongation and treating skeletal dysplasias.

The method of the invention for treating skeletal dysplasias includes the step of administering to a patient an effective amount of an NP. In one currently preferred embodiment of the present invention the natriuretic peptide is CNP. In another currently preferred embodiment the natriuretic peptide is a CNP variant. The method may further include a step of administering to the patient an inhibitor of neutral endopeptidase 24.11 (NEP). Suitable compounds for inhibiting NEP are known in the art, including but not limited to thiorphan or candoxatril. Administration of such an inhibitor of neutral

endopeptidase may be performed either separately or simultaneously with the administration of NP. It may also include administering a clearance receptor (NPR-C) inhibitor either alone or in conjunction with administration of NP.

Hyperactivation of the FGFR pathways has been implicated in several bone skeletal dysplasias. In particular, over stimulation of FGFR3 results in bone growth inhibition. The method of the invention for treating skeletal dysplasias includes administering to a patient a pharmaceutical composition comprising an NP and a receptor kinase inhibitor, in particular a tyrosine kinase inhibitor including, but not limited to, those disclosed in US Patent No. 6,329,375 or 6,344,459.

In another currently preferred embodiment NP is targeted to a desired tissue, specifically the growth plate of the bones. This may be achieved by methods known to one skilled in the art and include, in a nonlimiting manner, a chimeric protein comprising an NP fused to a carrier domain to form a fusion protein. The NP includes all forms of the NPs, CNP and derivatives or variants. A carrier domain includes, for example, a hormone or a ligand for a receptor expressed in the target tissue. The carrier protein may be an active agonist or an inactive targettor moiety or a variant or mutant thereof. According to one currently preferred embodiment CNP or a functional variant is fused to growth hormone. An alternative embodiment comprises conjugating at least one NP to a carrier protein to form an NP-carrier protein conjugate.

Alternatively, NP maybe conjugated to an agent to prolong its half life in circulation or to a peptide that facilitates translocation across a cell membrane.

Administration of NP to a patient can be achieved by any suitable route of administration, including but not limited to injecting NP to the patient, inhalation, or implantation of a depot into the patient. The depot is preferably implanted at the site of the lesion, the lesion being an abnormal bone or a dysplasic bone. The NP may further be administered by an osmotic pump, such as an Alzet pump. The osmotic pump can be implanted subcutaneously, or at any other appropriate site. Preferred sites may be close to the target site of action namely in proximity to the long bones of the limbs, and in particular near the epiphyses.

A further method of administration may be implantation of NP secreting cells. According to one currently preferred embodiment of the invention the NP is CNP. The NP may be natural or a variant or analog. The methods of implanting or transplanting living

cells to provide therapeutically useful substances (cell therapy) is known in the art. In one form of cell therapy, the cells that are implanted have been genetically modified in vitro with exogenous genetic material so as to enable the cells to produce a desired biological substance that is useful as a therapeutic agent. Methods of genetically engineering cells are known to those skilled in the art. Methods for implantation or transplantation of NP secreting cells include encapsulation of the cells in any immunologically inert matrix including gelatin or polymers. According to one currently preferred embodiment the matrix is an alginate-polylysine-alginate (APA) complex encapsulating the cells. These methods of administration and other known methods may be utilized alone or in combination for treating skeletal dysplasia.

The present invention further provides a composition for treatment of skeletal dysplasias, such as achondroplasia. The composition includes an NP or NP variant and any pharmaceutically acceptable diluent or carrier thereof. According to one currently preferred embodiment of the invention, the natriuretic peptide is a BNP. According to one currently more preferred embodiment of the invention the natriuretic peptide is a CNP. According to one currently most preferred embodiment of the invention the natriuretic peptide is a CNP variant. The composition may further include any substance, molecule or vehicle capable of increasing the NP stability in vivo. For example the composition may include an inhibitor of neutral endopeptidase 24.11 (NEP), including but not limited to thiorphan or candoxatril. In another embodiment the composition may include an inhibitor of the natriuretic peptide receptor-C (NPR-C).

The composition may be in any form suitable for being administered to a patient, including but not limited to in dry, liquid or suspension form or injectable, implantable or transplantable form.

Also provided is a composition for treatment of skeletal dysplasias that includes NP secreting cells encapsulated within an immunologically inert matrix. One currently preferred embodiment includes NP secreting cells encapsulated in an alginate-polylysine-alginate (APA) complex and a suitable carrier thereof.

According to one currently preferred embodiment of the present invention natriuretic peptides and variants thereof are provided for the treatment of skeletal dysplasias. In another currently preferred embodiment of the present invention the NP is CNP and novel CNP variants. The peptide variants have been modified by removing the

five N-terminal amino acids of CNP, considered to be a part of the ectocyclic domain and have further been modified by amino acid substitutions. According to one currently more preferred embodiment of the present invention the variant CNP comprises 17 amino acids, from Cysteine5 to the Cysteine22 as depicted in Figure 3B, and is identified herein as SEQ ID NO:2. According to one currently most preferred embodiment of the present invention the variant CNP comprises 17 amino acids and an amino acid substitution at a cleavage site, and is depicted as SEQ ID NO:5

Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys

wherein

Xaa=Leu, Ile, Val; Xbb=Lys, Leu, Met; Xcc=Leu, Ile, Ala, Val;
Xdd=Ser, Ala, Gly, Thr, Asn; Xee=Met, Ala, Lys, Trp; Xff=Gly, Lys, Ala, Leu;
Xgg=Leu, Met.

The variant of SEQ ID NO:10 wherein Met17 has been substituted with various amino acids and exhibits high cGMP activity. A currently preferred embodiment of the present invention provides a CNP variant comprising a Met17 substitution.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in which:

Figure 1 shows the growth rate curves for CNP treated Ach heterozygote femora in the *ex vivo* experiment. Figure 1A shows the growth rate during the first three days of culture. Figure 1B shows the growth rate during days 3-15.

Figure 2 shows growth rates of femora ex vivo. Figure 2A depicts the interpolation of the growth rate curves of Fig. 1. Figure 2B shows the growth rates of CNP or vehicle treated femora derived from wild type, Ach heterozygotes and Ach homozygotes.

Figure 3 shows the amino acid sequence of the NPs and the NEP cleavage sites for CNP. The endocyclic sequences are underlined. The amino acids are represented by either the one-letter code or three-letter codes according to IUPAC conventions. Figure 3A shows the native human CNP 1-22 peptide with the NEP cleavage sites marked with an arrow. Figure 3B shows the amino acid sequence of CNP-5-22. Figure 3C shows the alignment of human ANP, BNP and CNP peptides and the amino acid sequence of the variants of the present invention.

Figure 4 shows a scheme of the growth hormone-CNP constructs for producing GH-CNP fusion protein.

## DETAILED DESCRIPTION OF THE INVENTION

Achondroplasia is characterized, inter alia, by shortening of proximal long bones. In humans the bone growth plate is active until puberty and bone growth is thus achieved until puberty. Thus, treatment aimed at bone elongation, for example, by increasing the size of limb bone growth plate, would be advantageous during this period.

Treatment of skeletal dysplasias such as achondroplasia, includes treating a shortened bone with NP. The bone may be treated by administering to a patient an effective amount of NP. The amount of the active ingredient administered will be determined by the attending physician and is generally proportional to the patient's weight.

According to the present invention it is now disclosed that NP can induce bone elongation in situations of abnormal bone growth such as those typical of skeletal dysplasias.

The role and use of NPs in bone elongation in situations of abnormal bone growth and in the treatment of skeletal dysplasias, such as achondroplasia, is demonstrated in the following examples and experiments.

For convenience certain terms employed in the specification, examples and claims are described here.

The term "natriuretic peptides" or "NP" as referred to herein and in the claims relates to any of the three isoforms, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) , C-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP) and to any functional variants thereof. The NP may be of any species but is preferably human.

The term "FGFR" as used herein denotes a receptor specific for FGF which is necessary for transducing the signal exerted by FGF to the cell interior, typically comprising an extracellular ligand-binding domain, a single transmembrane helix, and a cytoplasmic domain that contains a tyrosine kinase activity.

A "ligand" as used herein is a molecule capable of binding a receptor or a receptor analog. The ligands of the hGh-R (human growth hormone receptor) and FGFRs are the hGH and FGF molecules or variants thereof. The molecule may be chemically synthesized, synthesized by recombinant techniques or may occur in nature.

The terms "variant", "derivative" or "mutant" as used herein interchangeably refer to a polypeptide sequence that possesses some modified structural property of the native sequence. For example, the variant may be truncated at either the amino or carboxy termini (N- or C-termini) or both termini or may have amino acids deleted or substituted. It is contemplated in this invention that a variant may have altered binding to a receptor than the native protein. It may have enhanced or reduced binding which may enhance or depress a biological response. A biological response may be, for example, the stimulation of cell division, differentiation, homeostasis or growth. A biological response may encompass other functional properties of the native protein and would be well known to those practicing the art. Accordingly, the variant may have altered specificity for one or more receptors. The variant may be generated through recombinant DNA technologies, well known to those skilled in the art.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods disclosed in U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,965,188, hereby incorporated by reference.

Pharmacology

The present invention also contemplates pharmaceutical formulations, both for veterinary and for human medical use, which comprise as the active agent one or more peptide(s) of the invention, as well as the use of a peptide of the invention in the manufacture of a medicament for the treatment or prophylaxis of the skeletal conditions variously described herein.

In such pharmaceutical and medicament formulations, the active agent preferably is utilized together with one or more pharmaceutically acceptable carrier(s) therefore and optionally any other therapeutic ingredients. The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof. The active agent is provided in an amount effective to achieve the desired pharmacological effect, as described above, and in a quantity appropriate to achieve the desired daily dose. The pharmaceutical composition of the present invention can be administered either as free forms of the peptides of the present invention or as pharmacologically acceptable acid addition salts thereof.

The dose of the pharmaceutical composition of the present invention may vary with the kind of disease, the age of patient, body weight, the severity of disease, the route of administration, etc.; typically, it can be administered in a daily dose of 0.5-500 microgr/kg.

Apart from other considerations, the fact that the novel active ingredients of the invention are peptides, peptide variants or fusion proteins dictates that the formulation be suitable for delivery of these types of compounds. Clearly, peptides are less suitable for oral administration due to susceptibility to digestion by gastric acids or intestinal enzymes. The peptide analogs of the present invention have been designed to circumvent these problems. The preferred routes of administration of peptides are intra-articular, intravenous, intramuscular, subcutaneous, intradermal, or intrathecal. A more preferred route is by direct injection at or near the site of disorder or disease. Alternatively, they may be administered per orally as microcapsules in which the peptides of the present invention are incorporated as the active ingredient in liposome, polyamide, etc. and which are rendered resistant to degradation in the digestive tract. Another method of administration that can be adopted is to have the drug absorbed through the mucous membrane such as in the rectum, within the nose or eye or beneath the tongue, so that the drug is administered as a suppository, intranasal spray, eye drop or sublingual tablet.

In one currently preferred embodiment of the present invention, cells genetically engineered to express high levels of an NP or an NP variant are provided. Said cells may be implanted at a suitable location, more preferably at or near the intended site of activity, most preferably at or near an affected limb bone. In a currently more preferred embodiment, the cells are encapsulated. The encapsulated cells may be implanted at a suitable location, more preferably at or near the intended site of activity within the body. Implantation is preferably subcutaneous, at a site in close proximity to the growth plate of limb bones.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the peptides or peptide analogs selected from the sequences described herein, or physiologically acceptable salts or prodrugs or fusion proteins thereof, with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

The term "prodrug" refers to an agent, which is converted into an active drug *in vivo*. Prodrugs are often useful because in some instances they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility compared to the parent drug in pharmaceutical compositions.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols. Pharmaceutical compositions may also include one or more additional active ingredients.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, grinding, pulverizing, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries. Proper formulation is dependent upon the route of administration chosen.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants for example polyethylene glycol are generally known in the art.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as

glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration. For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the variants for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the peptide and a suitable powder base such as lactose or starch.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active ingredients in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable natural or synthetic carriers are well known in the art (Pillai et al, (2001) Curr Opin Chem Biol 5:447-51). Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds, to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for reconstitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The compounds of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

The formulations of present invention may be administered topically as a gel, ointment, cream, emulsion or sustained release formulation including a transdermal patch. The pharmaceutical compositions herein described may also comprise suitable solid of gel phase carriers or excipients. Examples of such carriers or excipients include,

but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Administration may be preferred locally by means of a direct injection at or near the site of target or by means of a patch or subcutaneous implant, staples or slow release formulation implanted at or near the target.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of a compound effective to prevent, alleviate or ameliorate symptoms of a disease of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

Toxicity and therapeutic efficacy of the peptides described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the $IC_{50}$ (the concentration which provides 50% inhibition) and the $LD_{50}$ (lethal dose causing death in 50 % of the tested animals) for a subject compound. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (Fingl, et al. (1975) in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Depending on the severity and responsiveness of the condition to be treated, dosing can also be a single administration of a slow release composition, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved. The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, and all other relevant factors.

Utilities

The present invention also relates to methods of treatment of skeletal disorders described above, by administering to a patient in need thereof a therapeutically effective

amount of the compositions of the present invention. The term administration as used herein encompasses oral, parenteral, intravenous, intramuscular, subcutaneous, transdermal, intrathecal, rectal, intralesional and intranasal administration.

The present invention further relates to method for the use of the NP or peptide variants, conjugates or fusions of the present invention to prepare medicaments useful in the treatment of various skeletal disorders such as Achondroplasia and thanatophoric dysplasia.

**Examples**

**Example 1. *Ex Vivo* Bone Culture**

Femora derived from achondroplasia model mice (Ach369 knock-in mice carry the Gly to Cys mutation at position 369, analogous to position 375 in humans or Ach374 knock-in mice that carry the Gly to Arg mutation at position 374, analogous to position 380 in humans) were dissected from P0 wild type, heterozygote and homozygote littermates and cultured in the presence of natriuretic peptides for 15 days.

Protocol for bone culture: Femoral bone cultures were performed by excising the hind limbs of mice. The limbs were carefully cleaned from the surrounding tissue (skin and muscles) and the femora exposed. The femora were removed and further cleared from tissue remains and ligaments. The femora were measured for their initial length, using a binocular with an eyepiece micrometer ruler. The bones were grown in 1 ml of medium (α-MEM supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml), nystatin (12.5 units/ml), BSA (0.2%), β-glycerophosphate (1 mM) and freshly prepared ascorbic acid (50 μg/ml)) with varying concentrations of NPs or CNP variants in a 24 well tissue culture dish. The bones were cultured for 15 days while measurements of bone length and medium replacement were performed every three days. At the end of the experiment, the growth rate of the bones was determined. The growth rate of bones is calculated from the slope of a linear regression fit on the length measurements obtained from day 3 to 15. Units given can be converted to length, 40 units = 1 mm.

Results: *Ex vivo* experiments with fetal bone organ culture from wild type (normal) animals have shown that CNP, more than BNP and much more than ANP, can induce bone elongation. According to the present invention it is now shown that natriuretic peptides can induce longitudinal growth of Achondroplasia-derived bones. CNP increased the total

longitudinal growth of femora derived from Ach374/+, Ach369/Ach369, Ach369/+ and wild type (+/+ or wt) mice compared to vehicle treated animals.

Figs. 1A and 1B depicts the growth rate curves for the CNP treated Ach heterozygote femora in the experiment. Figure 2 shows the interpolation of the rates in Figures 1A and 1B. The growth rate curves shows two stages of growth: an initial stage (days 0-3) in which growth is quick and is most affected by the presence of natriuretic peptides and a second slower linear rate. The maximal growth rate is observed in the initial days of culture. This may be due to the limitations of the culturing conditions, particularly to the depletion of nutrients.

Figure 2B shows the growth rate over 15 days in the homozygote mutant and heterozygote mutant femoras compared to wt femoras. In all cases, the CNP treated femoras increased in size at a rate higher than that of the untreated limbs. The final length if the bone was greater in the CNP treated groups, as well. These results suggest that Achondroplasia derived rudiments have a high capacity to respond to CNP and are able to reach a growth rate similar to that of the wt.

Interestingly, at this stage, femora derived from homozygote Ach animals respond better to CNP than femora derived from heterozygote animals while bones derived from wt animals are very slightly influenced by CNP. This is summarized in Table 1.

Table 1: Growth rate of femora in CNP treated mice

| | Growth rate (days 0-3) | | Growth rate (days 3-15) | |
|---|---|---|---|---|
| CNP | untreated | treated | untreated | treated |
| +/+ wild type (wt) | 9 | 10 | 4.9 | 5.7 |
| Ach/+ heterozygote | 4.4 | 9.1 | 2.1 | 3.4 |
| Ach/Ach homozygote | 2.9 | 7.1 | 1.2 | 2.8 |

**Example 2. Natriuretic peptides lead to an increase in the size of growth plate: Morphological and immunohistological analysis**

Most of the elongation induced by natriuretic peptides observed in bone culture results from epiphyseal growth and not from elongation of the mineralized diaphysis. Morphological analysis of the cellular composition of the growth plate in treated samples revealed that there is an increase in the size of the proliferative region and in the size of the hypertrophic zone. In addition, there is an area of acellular matrix similar to that observed

in the growth plate of FGFR3 knockout mice. Therefore, there seems to be an increase in proliferation which leads to a larger growth plate.

Mouse-specific BNP was synthesized and labeled with biotin. Biotinylated BNP was tested in bone culture on bones derived from Achondroplasia mice and shown to induce bone elongation. Nevertheless, a higher concentration of BNP was needed.

Histological analysis of the distribution of biotinylated BNP in the growth plate shows that it is apparent both in proliferative and in hypertrophic cells. Recent studies have shown the distribution of the NP receptors in the growth plate: NPR-B is expressed in proliferative cells while NPR-C is expressed in hypertrophic cells (Yamashita et al. (2000) J. Biochem 127: 177-179). Therefore, BNP could exert its effect on bone growth either by stimulating the activity of NPR-B or by blocking the clearance of the endogenous peptide, CNP, through NPR-C.

Several signaling pathways contribute to the process of endochondral ossification. The epistatic relationship between the natriuretic peptide and the FGF signaling pathways is not known. The pathways could act in parallel or one could act to induce the other. For example, if the FGF pathway were epistatic (downstream), to the NP pathway, an increase in the activity of NP would not result in a rescue of the FGFR defects. Furthermore, if the pathways acted in parallel it would not be obvious that an increase in NP signaling would be able to overcome the effect of the FGFR mutations.

**Experiment 3. *in vivo* Administration**

1. IP administration of drugs to achondroplasia pups

The fastest growth period for mice is during the first month of life therefore, it is expected that the earlier the experiments are initiated, the greater the influence on growth. The disadvantage of treating such young animals is that there is a limit on volume delivered. Drug delivery cannot be performed intravenously (IV) or by implantation of a continuous delivery device like a pump.

Animals: Heterozygote mice for the achondroplasia mutation, aged P4-P5, weighing approximately 3.5 gr. were randomly separated into groups of 3 animals/group and ear marked. Animals were distributed 6-7 individuals/cage with one foster mother.

Materials and Procedure: Animals were injected daily with drug intraperitoneally (IP) in a volume not exceeding 50µl. This volume can be increased proportionally to weight

increase. Animals are weighed and the tail length is measured on day 0 and every 2 days thereafter. The treatment is continued for 2 weeks. At the end of the administration period animals are sacrificed and skeletal elements are measured and analyzed by histology. CNP is administered at a concentration of $10^{-5}$ M and $10^{-6}$ M (in 1x PBS) during 2 weeks.

2. Administration of drugs to achondroplasia model mice using the Alzet pump

Drug release by osmotic pumps provides a continuous supply and a constant amount of circulating drug. Furthermore, it enables directed release of the drug closer to the target site. Nevertheless, this procedure can only be performed in older mice.

Animals: Heterozygote mice for the achondroplasia mutation, aged P12-P14, weighing approximately 10 gr. were randomly separated into groups of 3 animals/group and ear marked. Animals were distributed 6-7 individuals/cage with one foster mother.

Materials and Procedure: The Alzet pumps used have a total volume of 100µl and a release constant of .25µl/hour over a course of 14 days. Pumps were filled with appropriate drug, calibrated for 4-6 hours, implanted subcutaneously (SC) on the back of anesthetized mice and the contents were directed with a catheter to the femoral artery of one hind limb. Mice were monitored for recovery and returned to mothers. Mice were weighed and measured every 2 days. At the end of the administration period animals were sacrificed and skeletal elements measured and analyzed by histology.

This experiment was performed with a concentration of $10^{-4}$ M of CNP (in 1X PBS) in pump to obtain a concentration of $10^{-7}$ M in the blood stream.

**Example 4: Implantation of alginate encapsulated cells that secrete CNP**

Implantation of cells expressing high levels of NPs is a further method for providing a continuous source of NPs. NIH-3T3 fibroblasts were infected with retrovirus expressing mouse CNP and selected for resistance to neomycin. CNP secretion was tested by assaying the supernatant of the cells using RIA (RadioImmune Assay) specific for CNP (Phoenix Pharmaceuticals) and following manufacturer's instructions. The cells are encapsulated in APA (alginate-polylysine-alginate) complex according to the protocol described by Chang (Chang (1997) Ann N Y Acad Sci 831:461-73) and summarized below.

Materials and Methods:

1.Cell Preparation: Cells are removed from plate with trypsin, resuspended in media, transferred to 50 cc tube, spun down 5-10 minutes 100 rpm, media removed.

2. PBS Wash: Cells are resuspended in PBS, spun 5-10 minutes at 1000 rpm, PBS removed and cells resuspended in 0.5 ml PBS.

3. Alginate: Mix thoroughly with alginate, drawing completely in and then out >10 times, until a homogenous mix with no bubbles is seen.

4. Encapsulate cells: Extrude alginate-cell mix into 40 ml CaCl2 1.1% solution (on ice). Start with 1/10X until spray is invisible, then switch to 1/100X. Watch for accumulation of alginate on end of needle: either suction off or briefly increase extrusion rate to 1/10X. Determine the exact airflow for desired capsule size by checking capsule size / shape with check dishes. Routinely check capsules size / shape throughout process. Approximate settings: Extrusion: 99.9 at 1/100X speed, (brief 1/10X to force out blockages) Airflow of 3-4 yields large capsules (> 600 um), 4-6 regular size capsules (generally 200-600 um), 6-8 small capsules (<200 um) 7-8 very small (50-100 um) and needle: 1-2 mm from end of airflow connector. Transfer capsules and CaCl2 solution to 50 cc conical tube.

5.Capsule washes: Allow capsules to settle. Remove supernatant with suction. Add wash solution, mix gently, and allow 2 minutes for capsules to settle.

6. Capsule implantation: The capsules are implanted intraperitoneally (IP) into P5 mice (Ach369 and wt). The mice are weighed and tail length measured every 2 days for 3 weeks.

**Example 5: Co-administration of CNP with an NEP inhibitor**

The NPs have been shown to have a short half-life in circulation, probably due to the activity of the neutral endopeptidase (NEP). We sought to block the activity of NEP with a specific inhibitor in order to increase the concentration of CNP in the blood .

The experiment is performed so that CNP is administered at $10^{-4}$ M via a pump to obtain a concentration of $10^{-7}$ M in blood stream together with thiorphan (NEP inhibitor) at a concentration of 10 mg/ml in pump (10 μg/ml blood stream) for 2 weeks. Administration is done by using an Alzet pump as described above. The same animals are injected IP with extra CNP daily ($10^{-7}$ M) together with 0.1% BSA. A similar experiment

is performed with biotinylated BNP ($10^{-4}$ M, yielding $10^{-7}$ M in blood stream) injected daily, given in conjunction with thiorphan.

In a similar manner, any combination of NPs with compounds that contribute to NP stability in the circulation can be administered to a patient for efficient bone elongation and/or treatment of skeletal dysplasias. Such combinations may include a mixture of CNP and BNP, NPs in combination with peptidase inhibitors or in combination with NPR-C inhibitors. NP activity may be enhanced in combination with tyrosine kinase inhibitors.

**Example 6: CNP analogs**

CNP is active as a 22 amino acid (22-mer) peptide (CNP 1-22), shown in Figure 3 as SEQ ID NO:1. A shortened version of CNP, the 17 amino acid (17-mer) CNP 5-22 peptide, SEQ ID NO:2, was shown to be active in a cGMP assay. The 17-mer peptide comprises the amino acids of the cyclic domain of CNP and lacks five amino acids of the ectocyclic domain. NEP cleaves CNP at several sites, the primary one being the Cys6-Phe7 bond, as depicted by the thickened arrow in Figure 3A. Peptide analogs were synthesized to obtain a less degradable, i.e. NEP resistant or NRP-C resistant, peptide by incorporating one or more amino acid substitutions. A set of peptides including those with amino acid deletions, insertions and substitutions is shown in Table 2. The 11mer to 15mer peptides were shown to have reduced cGMP activity.

In some peptides, a histidine residue was incorporated between Cys6 and Phe7 (His-CNP) to disrupt the cleavage recognition site. Other peptides were synthetically modified by methylation either on Phe7 only, or both on Phe7 and Leu11, sites of NEP activity. Peptides maybe modified by incorporation of a reporter molecule that allows detection, such as a biotin or fluorescein, at either the N-terminus or C-terminus of the peptide.

**Table 2: Amino Acid Sequence of CNP Variants**

| SEQ ID NO: | Amino Acid Sequence | % relative binding |
|---|---|---|
| **17-mers** | | |
| SEQ ID NO:2 | **C F G L K L D R I G S M S G L G C** | 127.4 |
| SEQ ID NO:6 | C **A** G L K L D R I G S M S G L G C | 43.8 |
| SEQ ID NO:7 | C F G L K L **A** R I G S M S G L G C | 49.1 |
| SEQ ID NO:8 | C F G L K L D **A** I G S M S G L G C | 18.4 |
| SEQ ID NO:9 | C F G L K L D R **A** G S M S G L G C | 11.5 |
| SEQ ID NO:10 | C F G L K L D R I G S **A** S G L G C | 138.1 |
| **15-mers** | | |
| SEQ ID NO:11 | C **H** F G L K L D R I G S M S - - C | 25.9 |
| SEQ ID NO:12 | C **H** F G L K L D R I G S M **A** - - C | 21.7 |
| SEQ ID NO:13 | C **H** F G L K L D R I G A **Q** **S** - - C | 26.5 |
| **14-mers** | | |
| SEQ ID NO:14 | C F G L K L D R I G S M S - - - C | 29.9 |
| SEQ ID NO:15 | C F G L K L D R I G **A** **Q** S - - - C | 20.6 |
| **13-mers** | | |
| SEQ ID NO:16 | C F G L K L D R I G S M - - - - C | 17.3 |
| SEQ ID NO:17 | C F G L K L D R I G **A** M - - - - C | 3.4 |
| SEQ ID NO:18 | C F G L K L D R I G S **Q** - - - - C | 11.8 |
| SEQ ID NO:19 | C F G L K L D R I G **A** **Q** - - - - C | 3.8 |
| SEQ ID NO:20 | C **H** F G L K L D R I G S - - - - C | 11.9 |
| **12-mers** | | |
| SEQ ID NO:21 | C F G L K L D R I G S - - - - - C | 22.7 |
| **11-mers** | | |
| SEQ ID NO:22 | C F G L K L D R I G - - - - - - C | 4.2 |
| SEQ ID NO:23 | C **H** F G L K L D R I - - - - - - C | 3.8 |

A substitution or incorporation of an amino acid is marked in bold and underlined. Amino acid deletions are marked as a dash.

All peptide variants were analyzed for activity using the Biotrak enzyme immunoassay (EIA, Amersham) that measures the amount of secondary messenger, cyclic GMP (cGMP), elicited after activation of the natriuretic peptide receptor by the peptide on C3H10T1/2 cells. Additional 17-mer variants were synthesized and tested with the results of the assay summarized in Table 3. Amino acid substitutions are marked in bold and underlined. The assay was performed for CNP and variants at a concentration of $10^{-6}$ M. The values are given as the percent of relative binding compared to CNP, where CNP 1-22 and CNP 5-22 yield 100% binding.

**Table 3. CNP variants and *in vitro* cGMP values**

| SEQ ID NO: | Amino acid sequence of CNP variants | % relative binding |
|---|---|---|
| SEQ ID NO:2 | C F G L K L D R I G S M S G L G C | 100.000 |
| SEQ ID NO:24 | C **A** G L K L **A** R I G S M S G L G C | -5.848 |
| SEQ ID NO:25 | C **A** G L K L D R I G S **A** S G L G C | 3.933 |
| SEQ ID NO:26 | C F G L K L **A** R I G S **A** S G L G C | 5.755 |
| SEQ ID NO:27 | C **A** G L K L **A** R I G S **A** S G L G C | -10.355 |
| SEQ ID NO:28 | C **I** G L K L D R I G S M S G L G C | 13.899 |
| SEQ ID NO:29 | C **L** G L K L D R I G S M S G L G C | 6.255 |
| SEQ ID NO:30 | C **M** G L K L D R I G S M S G L G C | -1.341 |
| SEQ ID NO:31 | C **W** G L K L D R I G S M S G L G C | -10.834 |
| SEQ ID NO:32 | C **V** G L K L D R I G S M S G L G C | 5.740 |
| SEQ ID NO:33 | C **H** G L K L D R I G S M S G L G C | 5.699 |
| SEQ ID NO:34 | C **T** G L K L D R I G S M S G L G C | -6.903 |
| SEQ ID NO:35 | C F G L K L **E** R I G S M S G L G C | 9.450 |
| SEQ ID NO:36 | C F G L K L **Q** R I G S M S G L G C | -17.095 |
| SEQ ID NO:37 | C F G L K L **N** R I G S M S G L G C | 17.235 |
| SEQ ID NO:38 | C F G L K L **I** R I G S M S G L G C | 7.228 |
| SEQ ID NO:39 | C F G L K L **M** R I G S M S G L G C | 7.784 |
| SEQ ID NO:40 | C F G **A** K L D R I G S M S G L G C | 20.154 |
| SEQ ID NO:41 | C F G **I** K L D R I G S M S G L G C | 47.395 |
| SEQ ID NO:42 | C F G **V** K L D R I G S M S G L G C | 64.212 |
| SEQ ID NO:43 | C F G L **L** L D R I G S M S G L G C | 29.744 |
| SEQ ID NO:44 | C F G L **M** L D R I G S M S G L G C | 5.421 |
| SEQ ID NO:45 | C F G L K **A** D R I G S M S G L G C | 11.259 |
| SEQ ID NO:46 | C F G L K **I** D R I G S M S G L G C | 88.117 |
| SEQ ID NO:47 | C F G L K **V** D R I G S M S G L G C | 66.714 |
| SEQ ID NO:48 | C F G L K L D **H** I G S M S G L G C | 13.482 |
| SEQ ID NO:49 | C F G L K L D **K** I G S M S G L G C | 21.543 |
| SEQ ID NO:50 | C F G L K L D **Q** I G S M S G L G C | 11.953 |
| SEQ ID NO:51 | C F G L K L D R **L** G S M S G L G C | 14.177 |
| SEQ ID NO:52 | C F G L K L D R **V** G S M S G L G C | 27.103 |
| SEQ ID NO:53 | C F G L K L D R **T** G S M S G L G C | -1.945 |
| SEQ ID NO:54 | C F G L K L D R I G **A** M S G L G C | 54.469 |
| SEQ ID NO:55 | C F G L K L D R I G **G** M S G L G C | 27.811 |
| SEQ ID NO:56 | C F G L K L D R I G **T** M S G L G C | 74.080 |
| SEQ ID NO:57 | C F G L K L D R I G **N** M S G L G C | 27.430 |
| SEQ ID NO:58 | C F G L K L D R I G S M S **A** L G C | 53.649 |
| SEQ ID NO:59 | C F G L K L D R I G S M S **L** L G C | 15.289 |
| SEQ ID NO:60 | C F G L K L D R I G S M S **K** L G C | 49.202 |
| SEQ ID NO:61 | C F G L K L D R I G S M S G **Q** G C | -3.474 |
| SEQ ID NO:62 | C F G L K L D R I G S M S G **M** G C | 51.286 |
| SEQ ID NO:63 | C F G L K L D R I G S M S G **A** G C | -1.111 |
| SEQ ID NO:64 | C F G L K L D R I G S M S G **G** G C | -10.145 |
| SEQ ID NO:65 | C F G L K L D R I G S **W** S G L G C | 66.450 |
| SEQ ID NO:66 | C F G L K L D R I G S **H** S G L G C | 66.222 |
| SEQ ID NO:67 | C F G L K L D R I G S **K** S G L G C | 66.910 |
| SEQ ID NO:68 | C F G L K L D R I G S **S** S G L G C | 62.531 |
| SEQ ID NO:69 | C F G L K L D R I G S **G** S G L G C | 62.117 |
| SEQ ID NO:70 | C **H** G L K L D R I G S **A** S G L G C | 3.058 |
| SEQ ID NO:71 | C **T** G L K L D R I G S **A** S G L G C | -9.108 |

The peptide of SEQ ID NO:4 was shown to be as active as the native CNP1-22, SEQ ID NO:3. The modification lies in the removal of the N-terminal amino acids, the ectocyclic part of the peptide. The peptide of SEQ ID NO: 9 contains a substitution of Met17 to Ala. This variant retains high activity perhaps due to the alteration of an NEP cleavage site. Furthermore, the peptide of SEQ ID NO:9 was tested in bone culture and shown to induced elongation of Ach369 femora even at a concentration similar to that of CNP.

The peptide variant of SEQ ID NO: 6 has an amino acid substitution whereby Phe7 is modified to Ala. All tested substitutions of the Phe7, SEQ ID NOs: 24-25, 27-34 and 69-71, resulted in reduced activity.

Another modification includes a gain of activity obtained by the substitution of Met17. Met 17 is a site of NEP cleavage and its substitution by another amino acid including Ala identified as SEQ ID NO:10 increases activity and may increase stability. Other substitutions of Met retaining activity are Trp His, Lys, Ser and Gly identified herein as SEQ IS Nos:65-69. Other examples of variants wherein modifications were directed to the cleavage sites and activity is retained are disclosed as SEQ ID NOs: 45-47 wherein Leu10 is substituted, SEQ ID NOs:54-57 wherein Ser16 is substituted and SEQ ID Nos:58-60 wherein Gly19 is substituted.

Peptide variants exhibiting a high level of activity as determined in the cGMP assay may be useful for treating skeletal disorders. These variants may be useful alone or in combination with other compounds including but not limited to NPs, NEP inhibitors, NPR-C inhibitors or TK inhibitors. The variants with reduced activity may be useful alone or in conjugation with other NPs and may impart stability or synergy.

One skilled in the art will recognize that the variants may be synthesized as peptide mimetics. A peptide mimetic or peptidomimetic, is a molecule that mimics the biological activity of a peptide but is not completely peptidic in nature. Whether completely or partially non-peptide, peptidomimetics provide a spatial arrangement of chemical moieties that closely resembles the three-dimensional arrangement of groups in the peptide on which the peptidomimetic is based. As a result of this similar active-site geometry, the peptidomimetic has effects on biological systems which are similar to the biological activity of the peptide.

**Example 7: *in vivo* assays**

Methods for detecting administered compounds in the blood or tissue of treated mammals are known in the art. The pharmacokinetic properties of the administered compounds are determined using such methods. In animal models, radiolabelled oligonucleotides or peptides can be administered to and their distribution within body fluids and tissues assessed by extraction of the oligonucleotides or peptides followed by autoradiography (Agrawal et al (1991) PNAS 88:7595-99). Other methods include labeling of a peptide with a reporter moiety, including fluorescent or enzyme labels, administration to an animal, extraction of the peptide from body fluids and organs followed by HPLC analysis. Alternatively, immunohistochemical methods are used for detection of the administered peptide in tissue. The present invention contemplates reporter labeled NPs and CNP variants.

**Example 8: GH-CNP fusion construct**

One approach to creating high local concentrations of a therapeutic molecule is to target it to the growth plate (GP) via a GP-specific carrier including but not limited to growth hormone (GH), IGF-1, TH and receptor ligands. GH is an endocrine hormone that is secreted by the pituitary and affects bone elongation. Targeted delivery of a compound into cells comprising a chimeric protein has been disclosed for example in US Patent Application No. 20010025026.

We have synthesized a GH-CNP fusion construct useful as a carrier for CNP on its way into the growth plate. This fusion protein maintains the binding capacity of GH and the activity of CNP. In one aspect a linker has been added between the two peptides which enables independent folding of each part. In another aspect, a linker comprising a cleavage site has been introduced to allow physical separation of the two hormones. Figure 4 shows a scheme of the constructs.

To determine whether GH can enter the growth plate from the circulation, GH was labeled with $^{125}$Iodine and administered to mice IP, one hour after a pulse of unlabelled GH. Blood samples were collected at several time points and analyzed on a protein gel. The samples show strong labeling which increases in the first 2-3 hours, can still be observed at 6 hours and almost disappears at 24hrs.

PCR and Cloning: Mouse Growth Hormone (mGH) and mouse CNP (mCNP) were isolated from a brain cDNA library by method of RT-PCR, using the following oligonucleotide primers (oligos):

Mouse Growth Hormone:

For: 5' TGG CAA TGG CTA CAG ACT CTC GG

Rev: 5' GAA GGC ACA GCT GCT TTC CAC AA

Mouse CNP:

For: 5' ACC CAA GCT TAT GCA CCT CTC CCA GC

Rev: 5' CCA TCG ATC TAA CAT CCC AGA CCG

The generated PCR products were sub-cloned into plasmids and sequenced to verify their identity and fidelity. These plasmids were then used as templates for the generation of the fusion constructs which were generated by a two-step nested PCR approach. The construction is described in detail here, using four different oligonucleotides and three PCR reactions. All other constructs were generated by similar methodology. Construct A harbors sequences encoding:

BamHI - Factor Xa Cleavage site – mCNP – Linker A – mGH - Stop Codon - Hind III.

The first PCR reaction (21 cycles) was performed on mCNP using the forward oligo which harbors sequences to encode a BamHI cloning site, Factor Xa consensus cleavage site as well as the 5' of mouse CNP and the reverse oligo which harbors the 3' component of CNP, as well as sequences to encode linker A. The second PCR reaction (21 cycles) was performed on mGH using forward oligo which harbors sequences to encode a linker A as well as the 5' of mGH and a reverse oligonucleotide which harbors the 3' component of mGH, including a stop codon and a HindIII site for subsequent sub-cloning. The third PCR reaction (21 cycles) was performed by combining the products of the two above PCR reactions, the forward oligo from the first PCR reaction, the reverse oligo from the second PCR reaction. The overlapping linker sequences for the two different template DNAs, allowed the generation of the full-length fusions. The constructs were cloned into an expression vector and used to transfect host cells. The fusion proteins were synthesized and isolated according to methods known in the art. A construct wherein the CNP lies 5' to the mGH was constructed, as well. A scheme of all the constructs that have been made is shown in Figure 4. The legend for Figure 4 is as follows: Linker A is a simple flexible linker encoding six amino acids: Gly Gly Ser Gly Gly Ser while Linker B harbors an MMP9-specific cleavage sequence (Kridel et al. (2001) J Biol Chem 276: 20572-8). B and H are BamHI and HindIII restriction enzyme sites, respectively and Xa is a factor Xa cleavage site.

The activity of the GH and CNP in the fusion protein is assessed by measurement of cGMP accumulation for CNP and activity of GH.

**Example 9: Establishment of assay to assess resistance to NEP degradation**

Endothelial cells express neutral endopeptidase (NEP) in vivo and thus degrade part of the circulating natriuretic peptides. An in vitro assay has been established to determine the stability of the modified peptides in the presence of endopeptidases. Endothelial cell lines have been used but any primary cell or cell line expressing NEP may be useful in the assay. Endothelial cell lines derived from either bovine or human tissue are grown. Cells are overlayed with $10^{-6}$ M CNP or variant, incubated overnight and the medium is assayed for cGMP activity, as described above. The results obtained from the variants are compared to the activity of CNP.

**Example 10: Establishment of assay to assess resistance to NPR-C degradation**

An assay to determine the resistance of the CNP variants to clearance via the NPR-C receptor is being established using astrocytes derived from a NPR-C knockout mouse (Matzukawa et al (1999) PNAS 96:7403-8).

It will be evident to the skilled artisan that administration of NPs according to the principles of the present invention can be performed by any suitable route of administration, utilizing any suitable pharmaceutically acceptable carrier or diluent. Under certain circumstances specific formulations that enable or enhance targeting of the active principle to the bone or growth plate may be utilized such as but not limited to use of the NPs in combination with vehicles such as liposomes, microemulsions, microcapsules, microspheres, and the like. It is also intended antibodies, peptides, hydroxyapatite, glucosamine, collagen especially collagen type X, polyGlu or polyAsp and other molecules having affinity for the growth plate.

It will be appreciated that many improvements may be achieved by stabilization of the NP or otherwise achieving a prolonged half life or improved pharmacokinetic profile. For example, functional variants of NPs having enhanced stability in vivo, such as peptides having altered sequences or configurations, may be administered for treatment of skeletal dysplasias and/or bone elongation.

Further, cells can be engineered to produce and secrete functional variants of NPs having enhanced stability in vivo and these cells may be encapsulated in APA (alginate-polylysine-alginate) complex and implanted intraperitoneally for treatment of skeletal dysplasias and/or bone elongation.

While the present invention has been particularly described, persons skilled in the art will appreciate that many variations and modifications can be made. Therefore, the invention is not to be construed as restricted to the particularly described embodiments, rather the scope, spirit and concept of the invention will be more readily understood by reference to the claims which follow.

**CLAIMS**

1. A method for increasing the size of a bone growth plate in abnormal bone comprising treating the bone with an effective amount of at least one natriuretic peptide, or variants thereof.

2. The method according to claim 1 wherein said at least one natriuretic peptide is a CNP.

3. The method according to claim 1 wherein said at least one natriuretic peptide is a CNP variant according to SEQ ID NO:5
Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys
wherein Xaa=Leu,Ile,Val; Xbb=Lys,Leu,Met; Xcc=Leu,Ile Ala,Val;
Xdd=Ser,Ala,Gly,Thr,Asn; Xee=Met,Ala, Trp, His, Lys, Ser, Gly;
Xff=Gly,Lys,Ala,Leu; Xgg=Leu,Met.

4. The method according to claim 1 wherein said at least one natriuretic peptide is according to any one of SEQ ID NOs:1-4.

5. The method according to claim 1wherein said at least one natriuretic peptide is a BNP.

6. The method according to claim 1 further comprising inhibiting the natriuretic peptide clearance receptor.

7. The method according to claim 1 further comprising an inhibitor of the neutral endopeptidase 24.11.

8. The method according to claim 7 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

9. The method according to claim 7 wherein the step of administering an inhibitor of neutral endopeptidase is performed simultaneously with the step of administering an effective amount of at least one natriuretic peptide.

10. The method according to claim 1 wherein said at least one natriuretic peptide is fused to a carrier protein forming a natriuretic peptide-carrier protein fusion protein.

11. The method according to claim 10 wherein the natriuretic peptide-carrier protein fusion protein comprises a CNP variant fused to growth hormone.

12. The method according to claim 1 wherein the natriuretic peptide is conjugated to a carrier protein forming a natriuretic peptide-carrier protein conjugate.

13. The method according to claim 1 wherein the bone is a limb bone.

14. The method according to claim 13 wherein the limb bone is an achondroplasic bone.

15. A method for elongation of an abnormal bone, comprising treating the bone with an effective amount of at least one natriuretic peptide, or variants thereof.

16. The method according to claim 15 wherein said at least one natriuretic peptide is a CNP.

17. The method according to claim 15 wherein said at least one natriuretic peptide is a CNP variant according to SEQ ID NO:5
Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys
wherein Xaa=Leu,Ile,Val; Xbb=Lys,Leu,Met; Xcc=Leu,Ile Ala,Val;
Xdd=Ser,Ala,Gly,Thr,Asn; Xee=Met,Ala, Trp, His, Lys, Ser, Gly;
Xff=Gly,Lys,Ala,Leu; Xgg=Leu,Met.

18. The method according to claim 15 wherein said at least one natriuretic peptide is according to any one of SEQ ID NOs:1-4.

19. The method according to claim 15 wherein said at least one natriuretic peptide is a BNP.

20. The method according to claim 15 further comprising inhibiting the natriuretic peptide clearance receptor.

21. The method according to claim 15 further comprising an inhibitor of the neutral endopeptidase 24.11.

22. The method according to claim 21 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

23. The method according to claim 21 wherein the step of administering an inhibitor of neutral endopeptidase is performed simultaneously with the step of administering an effective amount of at least one natriuretic peptide.

24. The method according to claim 15 wherein said at least one natriuretic peptide is a natriuretic peptide fused to a carrier protein forming a natriuretic peptide-carrier protein fusion protein.

25. The method according to claim 24 wherein the natriuretic peptide-carrier protein fusion protein comprises a CNP variant fused to growth hormone.

26. The method according to claim 15 wherein said at least one natriuretic peptide is conjugated to a carrier protein forming a natriuretic peptide-carrier protein conjugate.

27. The method according to claim 15 wherein the bone is a limb bone.

28. The method according to claim 15 wherein the limb bone is an achondroplasic bone.

29. Use for the preparation of a medicament for treating skeletal dysplasia of at least one natriuretic peptide, or variants thereof.

30. Use according to claim 29 wherein said at least one natriuretic peptide is a CNP.

31. Use according to claim 29 wherein said at least one natriuretic peptide is a CNP variant according to SEQ ID NO:5
Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys
wherein Xaa=Leu,Ile,Val; Xbb=Lys,Leu,Met; Xcc=Leu,Ile Ala,Val;
Xdd=Ser,Ala,Gly,Thr,Asn; Xee=Met,Ala, Trp, His, Lys, Ser, Gly;
Xff=Gly,Lys,Ala,Leu; Xgg=Leu,Met.

32. Use according to claim 29 wherein said at least one natriuretic peptide is according to any one of SEQ ID NOs:1-4

33. Use according to claim 29 wherein the natriuretic peptide is a BNP.

34. Use according to claim 29 further comprising an inhibitor of the natriuretic peptide clearance receptor.

35. Use according to claim 29 further comprising an inhibitor of the neutral endopeptidase 24.11.

36. Use according to claim 35 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

37. Use according to claim 29 wherein the medicament further comprises an inhibitor of tyrosine kinase.

38. Use according to claim 29 wherein said at least one natriuretic peptide is a natriuretic peptide fused to a carrier protein forming a natriuretic peptide-carrier protein fusion protein.

39. Use according to claim 38 wherein the natriuretic peptide-carrier protein fusion protein comprises a CNP variant fused to growth hormone.

40. Use according to claim 29 wherein said at least one natriuretic peptide is conjugated to a carrier protein forming a natriuretic peptide-carrier protein conjugate.

41. Use according to claim 29 wherein the skeletal dysplasia involves a limb bone.

42. Use according to claim 29 wherein the skeletal dysplasia to be treated comprises Achondroplasia.

43. A pharmaceutical composition for bone elongation or treating skeletal dysplasias comprising at least one natriuretic peptide or variants thereof and a carrier or excipient.

44. The pharmaceutical composition according to claim 43 wherein said at least one natriuretic peptide is a CNP.

45. The pharmaceutical composition according to claim 43 wherein said at least one natriuretic peptide is a CNP variant according to SEQ ID NO:5
Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys
wherein Xaa=Leu,Ile,Val; Xbb=Lys,Leu,Met; Xcc=Leu,Ile Ala,Val;

Xdd=Ser,Ala,Gly,Thr,Asn; Xee=Met,Ala, Trp, His, Lys, Ser, Gly; Xff=Gly,Lys,Ala,Leu; Xgg=Leu,Met.

46. The pharmaceutical composition according to claim 43 wherein said at least one natriuretic peptide is according to any one of SEQ ID NOs:1-4.

47. The pharmaceutical composition according to claim 43 wherein the natriuretic peptide is a BNP.

48. The pharmaceutical composition according to claim 43 further comprising an inhibitor of the natriuretic peptide clearance receptor.

49. The pharmaceutical composition according to claim 43 further comprising an inhibitor of the neutral endopeptidase 24.11.

50. The pharmaceutical composition according to claim 49 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

51. The pharmaceutical composition according to claim 43 further comprising an inhibitor of tyrosine kinase,

52. The pharmaceutical composition according to claim 43 wherein said at least one natriuretic peptide is a natriuretic peptide fused to a carrier protein forming a natriuretic peptide-carrier protein fusion protein.

53. The pharmaceutical composition according to claim 52 wherein the natriuretic peptide-carrier protein fusion protein comprises a CNP variant fused to growth hormone.

54. The pharmaceutical composition according to claim 43 wherein said at least one natriuretic peptide is conjugated to a carrier protein forming a natriuretic peptide-carrier protein conjugate.

55. The pharmaceutical composition according to claim 43 wherein the bone is a limb bone.

56. The pharmaceutical composition according to claim 43 wherein the skeletal dysplasia to be treated comprises achondroplasia.

57. A method for treatment of skeletal dysplasias comprising the step of administering to a patient a therapeutically effective amount of at least one natriuretic peptide.

58. The method according to claim 57 wherein said at least one natriuretic peptide is a CNP.

59. The method according to claim 57 wherein said at least one natriuretic peptide is a CNP variant according to SEQ ID NO:5
Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys
wherein Xaa=Leu,Ile,Val; Xbb=Lys,Leu,Met; Xcc=Leu,Ile Ala,Val;
Xdd=Ser,Ala,Gly,Thr,Asn; Xee=Met,Ala, Trp, His, Lys, Ser, Gly;
Xff=Gly,Lys,Ala,Leu; Xgg=Leu,Met.

60. The method according to claim 57 wherein said at least one natriuretic peptide is according to any one of SEQ ID NOs:1-4

61. The method according to claim 57 wherein said at least one natriuretic peptide is a BNP.

62. The method according to claim 57 further comprising inhibiting the natriuretic peptide clearance receptor.

63. The method according to claim 57 further comprising an inhibitor of neutral endopeptidase 24.11 with an inhibitor.

64. The method according to claim 57 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

65. The method according to claim 57 wherein the step of administering to the patient an inhibitor of neutral endopeptidase is performed simultaneously with the step of administering to a patient an effective amount of at least one natriuretic peptide.

66. The method according to claim 65 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

67. The method according to claim 57 further comprising administering to the patient an inhibitor of tyrosine kinase.

68. The method according to claim 57 wherein said at least one natriuretic peptide is a natriuretic peptide fused to a carrier protein forming a natriuretic peptide-carrier protein fusion protein.

69. The method according to claim 68 wherein the natriuretic peptide-carrier protein fusion protein comprises a CNP variant fused to growth hormone.

70. The method according to claim 57 wherein said at least one natriuretic peptide is conjugated to a carrier protein forming a natriuretic peptide-carrier protein conjugate.

71. The method according to claim 57 wherein the skeletal dysplasia is achondroplasia.

72. A composition for treatment of skeletal dysplasias comprising natriuretic peptide secreting cells.

73. The composition of claim 72 wherein the secreted cells are encapsulated within an inert matrix.

74. The composition of claim 73 comprising natriuretic peptide secreting cells encapsulated within an alginate-polylysine-alginate complex and a carrier thereof.

75. A method for treating skeletal dysplasias comprising transplanting or implanting to a patient in need thereof natriuretic peptide secreting cells.

76. The method of claim 75 wherein the natriuretic peptide secreting cells are transplanted or implanted to the site of the lesion.

77. The method of claim 75 wherein the natriuretic peptide secreting cells are encapsulated within an inert matrix

78. The method of claim 77 wherein the natriuretic peptide secreting cells are encapsulated within an alginate-polylysine-alginate complex.

Figure 1

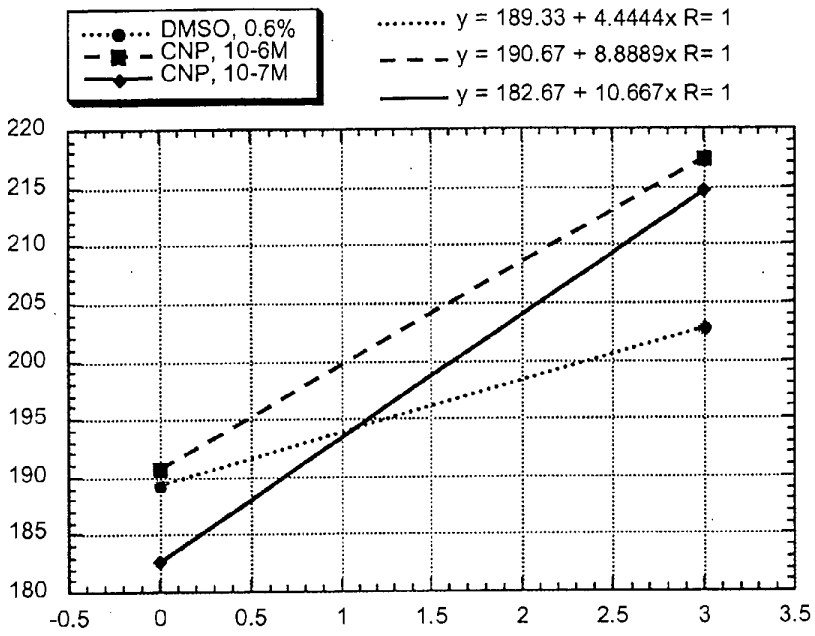



Fig. 1A

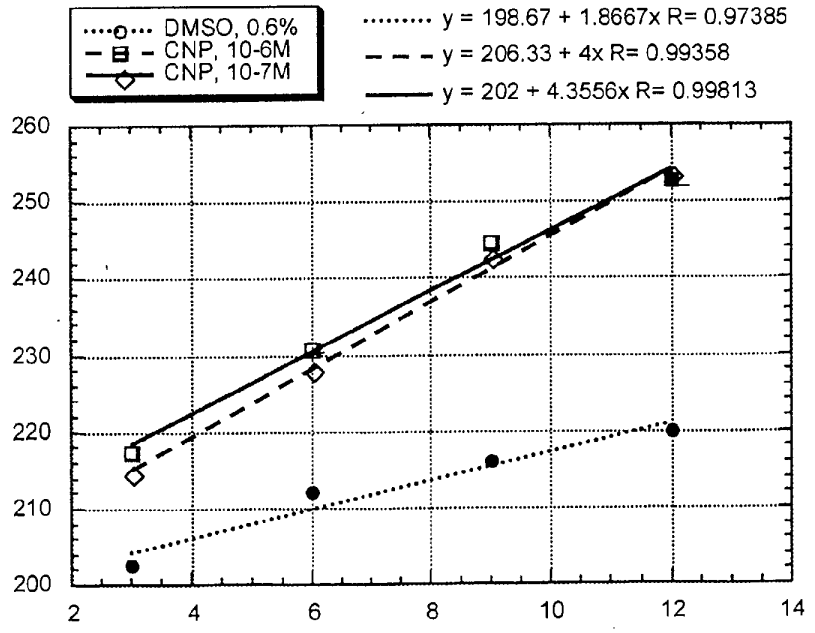



Fig. 1B

Figure 2

Figure 2A

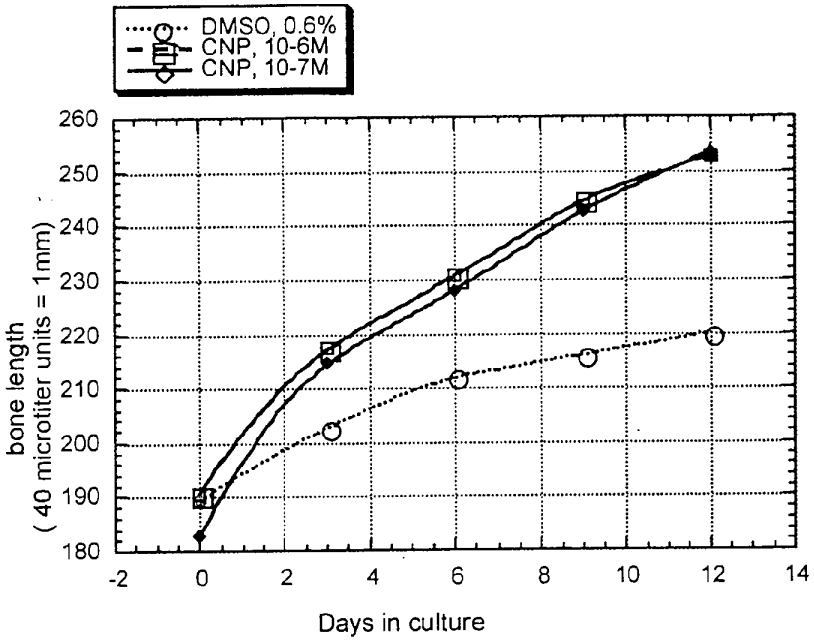
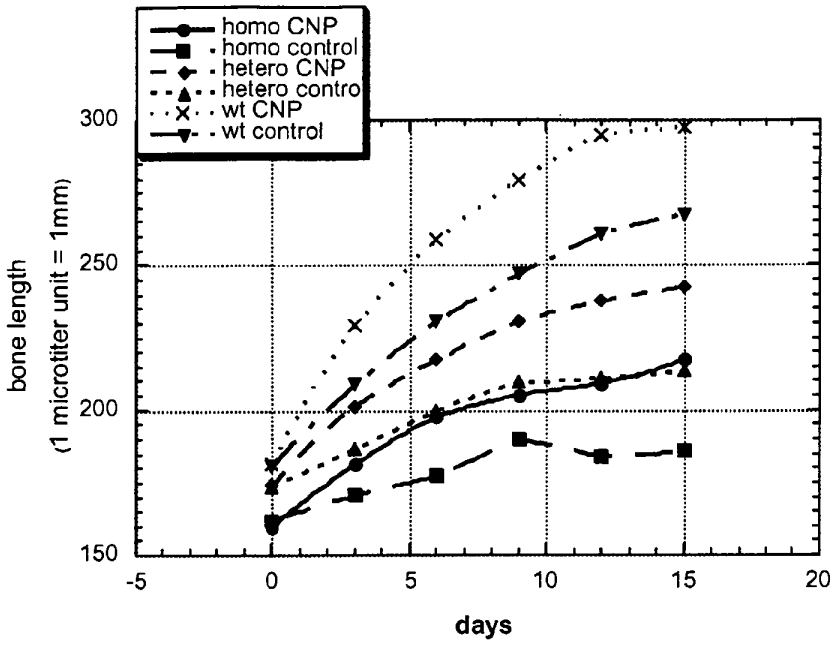


Figure 2 cont.

Figure 2B



## Figure 3

**Figure 3A**

CNP 1-22 SEQ ID NO:1

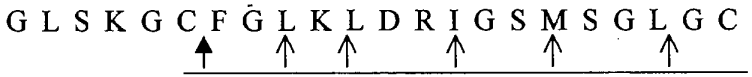



**Figure 3B**

**CNP 5-22** SEQ ID NO:2

**Figure 3C**

Human ANP: SEQ ID NO:3 S L R R S S C F G G R M D R I G A Q S G L G C N S F R Y

Human BNP-32: SEQ ID NO:4 S P K M V Q G S G C F G R K M D R I S S S S G L G C K V L R R H

Human CNP 1-22: SEQ ID NO:1 G L S K G C F G L K L D R I G S M S G L G C

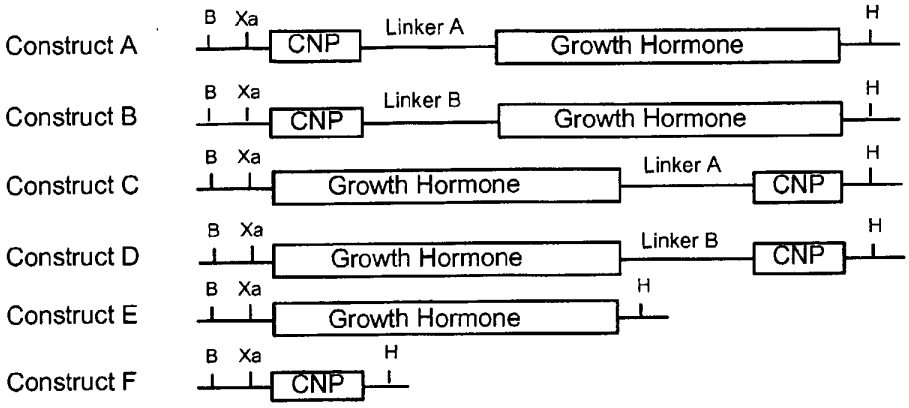
SEQ ID NO:5

**Cys-Phe-Gly-Xaa-Xbb-Xcc-Asp-Arg-Ile-Gly-Xdd-Xee-Ser-Xff-Xgg-Gly-Cys**

**Xaa**=Leu,Ile,Val; **Xbb**=Lys,Leu,Met; **Xcc**=Leu,Ile Ala,Val;

**Xdd**=Ser,Ala,Gly,Thr,Asn; **Xee**=Met,Ala; **Xff**=Gly,Lys,Ala,Leu; **Xgg**=Leu,Met

**Figure 4**


Construct A
B
Xa
CNP
Linker A
Growth Hormone
H
Construct B
B
Xa
CNP
Linker B
Growth Hormone
H
Construct C
B
Xa
Growth Hormone
Linker A
CNP
H
Construct D
B
Xa
Growth Hormone
Linker B
CNP
H
Construct E
B
Xa
Growth Hormone
H
Construct F
B
Xa
CNP
H

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 03/12/2020

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| CAMPELL, BRUCE R | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 03/12/2020 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

jordan.wilson@bmrn.com
lbigornia@bmrn.com
mgbdocket@marshallip.com

| *Applicant-Initiated Interview Summary* | **Application No.** 15/646,822 | **Applicant(s)** Wendt et al. | |
|---|---|---|---|
| | **Examiner** BRUCE R CAMPELL | **Art Unit** 3991 | **AIA (FITF) Status** No |

All participants (applicant, applicants representative, PTO personnel):

(1) BRUCE R. CAMPELL.

(2) Sharon Turner, Timothy Speer.

(3) Kate Neville.

(4) Ajay Singh.

Date of Interview: 24 February 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☑103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: all.

Identification of prior art discussed: all of record.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant argued that the prior art did not suggest the specific CNP analogs which are claimed. Applicant intends to submit a declaration explaining the unpredictability of the art with regard to activity of truncated CNP analogs intermediate in length between CNP22 and CNP53, derivatized (with PEG or HSA) vs. non-derivatiized CNP peptides, and treatment of bone related disorders with CNP. Applicant will also submit a declaration removing the Wendt reference as prior art..

**Applicant recordation instructions:** The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

**Examiner recordation instructions**: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

| /BRUCE R CAMPELL/ /Sharon Turner/<br>Patent Reexamination Specialist, Art Unit 3991 | |
|---|---|

# Summary of Record of Interview Requirements

**Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record**
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

**Title 37 Code of Federal Regulations (CFR) 1.133 Interviews**
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

---

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

**Examiner to Check for Accuracy**

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

PTO/SB/06 (09-11)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/646,822 | Filing Date<br>07/11/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| AMENDMENT | 03/26/2020 | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * 16 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 6 | Minus | *** 6 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| AMENDMENT | | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /PATRICIA F LEWIS/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 Confirmation No.: 7727

Filed: July 11, 2017 Art Unit: 3991

For: Variants of C-Type Natriuretic Peptide Examiner: Bruce Campell

**AMENDMENT IN RESPONSE TO OFFICE ACTION UNDER 37 C.F.R. § 1.111**

MS Re-exam/Re-issue
Central Reexamination Unit
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

**INTRODUCTORY COMMENTS**

This paper is filed in response to an Office Action ("the Action") dated November 26, 2019, in the above-identified U.S. patent application.

This paper is accompanied by a Petition for One-Month Extension of Time and the requisite fee. If any additional fees are deemed necessary, the Commissioner is hereby authorized to charge such fees to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

**Amendments to the Claims** are reflected in the complete listing of the claims begins on page **2** of this paper.

**Remarks** begin on page **16** of this paper.

## AMENDMENTS TO THE CLAIMS

Cancel claim 1.

Cancel claim 2.

Cancel claim 3.

Cancel claim 4.

Cancel claim 5.

Cancel claim 6.

Cancel claim 7.

Cancel claim 8.

Cancel claim 9.

Cancel claim 10.

Cancel claim 11.

Cancel claim 12.

Cancel claim 13.

Cancel claim 14.

15. (New) A macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

16. (New) The macromolecule of claim 15, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

17. (New) The macromolecule of claim 16, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

18. (New) A sustained release CNP variant formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)

(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36).

19. (New) The sustained release formulation of claim 18, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

20. (New) The sustained release formulation of claim 19, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

21. (New) A method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36.

22. (New) A method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36).

23. (New) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a macromolecule capable of releasing a CNP variant comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36), and wherein hydrolysis of the hydrolysable linkage releases the CNP variant.

24. (New) The method of claim 23, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

25. (New) The method of claim 24, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

26. (New) The method of claim 25, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

27. (New) A method of treating a bone-related disorder or skeletal dysplasia, comprising administering a sustained CNP variant release formulation comprising a synthetic polymeric group coupled to the CNP variant through a hydrolysable linkage, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39)
(SEQ ID NO: 159);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38)
(SEQ ID NO: 160);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)
(SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
PEGIK-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);
PE012-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);
and PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36).

28. (New) The method of claim 27, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism and homozygous achondroplasia.

29. (New) The method of claim 28, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

30. (New) The method of claim 29, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

31. (New) The macromolecule of claim 15, wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

32. (New) The macromolecule of claim 15, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

33. (New) The macromolecule of claim 32, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

34. (New) The macromolecule of claim 33, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

35. (New) The macromolecule of claim 15, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

36. (New) The macromolecule of claim 35, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

37. (New) The macromolecule of claim 36, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

38. (New) The macromolecule of claim 15, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

39. (New) The macromolecule of claim 15, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60)

40. (New) The sustained release formulation of claim 18, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

41. (New) The sustained release formulation of claim 18, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

42. (New) The sustained release formulation of claim 41, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

43. (New) The sustained release formulation of claim 42, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

44. (New) The sustained release formulation of claim 18, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

45. (New) The sustained release formulation of claim 44, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

46. (New) The sustained release formulation of claim 45, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

47. (New) The sustained release formulation of claim 18, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

48. (New) The sustained release formulation of claim 18, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

49. (New) The method of claim 21, wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

50. (New) The method of claim 21, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

51. (New) The method of claim 21, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160) .

52. (New) The method of claim 21, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

53. (New) The method of claim 21, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

54. (New) The method of claim 22, wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)(SEQ ID NO: 60).

55. (New) The method of claim 22, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

56. (New) The method of claim 22, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

57. (New) The method of claim 22, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

58. (New) The method of claim 22, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

59. (New) The method of claim 23, wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75)
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)(SEQ ID NO: 60).

60. (New) The method of claim 23, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

61. (New) The method of claim 60, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

62. (New) The method of claim 61, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

63. (New) The method of claim 23, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

64. (New) The method of claim 63, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

65. (New) The method of claim 64, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

66. (New) The method of claim 23, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

67. (New) The method of claim 23, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

68. (New) The method of claim 27, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:145); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75)

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159); and
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37)(SEQ ID NO: 60).

69. (New) The method of claim 27, wherein the CNP variant is LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

70. (New) The method of claim 69, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

71. (New) The method of claim 70, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

72. (New) The method of claim 27, wherein the CNP variant is selected from the group consisting of GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75) and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160).

73. (New) The method of claim 72, wherein the synthetic polymeric group comprises a hydrophilic polymeric moiety.

74. (New) The method of claim 73, wherein the hydrophilic polymeric moiety comprises polyethylene glycol (PEG).

75. (New) The method of claim 27, wherein the CNP variant comprises an amino acid sequence at least 95% identical to GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75).

76. (New) The method of claim 27, wherein the CNP variant comprises an amino acid sequence at least 95% identical to QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60).

## REMARKS

Claims 15-30 are pending and under examination. Claims 31-76 are new.

In the Action, the Examiner variously rejected the claims as follows: claims 15-30 were rejected under 35 USC §102(e) as assertedly anticipated by US Patent No. 8,377,884 ("Wendt"); claims 15-30 were rejected under 35 USC §103(a) as assertedly anticipated by US Patent No. 6,743,425 ("Nakao") in view of Golembo (WO 02/07423), James (US20100216714), Potter et al. (Endocrine Rev. 27:47-72, 2006) ("Potter"), Yeung et al., Peptides 17:101-106, 1996 ("Yeung"), Totsune et al., (Peptides 15:37-40, 1994) ("Totsune"), Eun et al., Enzymology Primer for Recombinant Technology, Chapter 1, pages 1-108, 1996 ("Eun"), Szoka et al., DNA 5:11-20, 1986 ("Szoka") and Huang et al., BMC Biotechnol. 1-11, 2008 ("Huang"), or alternatively claims 15-30 were rejected under 35 USC §103(a) over the above cited references further in view of Wendt. Reconsideration is respectfully requested in light of the following remarks.

### I. Summary of Interview Pursuant to MPEP §713.04 and 37 CFR §1.133

Applicants would like to thank Examiners Campell, Spear and Turner for the courtesy of the phone interview on February 24, 2020.

During the interview, the outstanding §103 rejections were discussed. The Examiners suggested filing a declaration addressing the non-obviousness of the claimed variants. No agreement was reached. Applicants thank the Examiners for the helpful discussion.

### II. Amendments to the claims

Support for the amendments to the claims is found throughout the specification. Claims 15, 18, 21-23 and 27 are amended to recite specific CNP variants. Support for new claims 31-76 is found, for example, in pending claim 15 and at page 51, lines 9-13, and page 53, lines 22-32. The amendments include no new matter.

### III. The rejection of claims 15-30 under 35 USC §102(e) should be withdrawn

The Examiner rejected claims 15-30 under 35 USC §102(e) as assertedly anticipated by Wendt.

Wendt is an earlier publication subject to assignment to the same Assignee as the present application, and having two inventors in common. The priority date of the present application is within 1 year of the filing date of Wendt.

MPEP 2136 states "When a prior U.S. patent, U.S. patent application publication, or international application publication is not a statutory bar, a pre-AIA 35 U.S.C.102(e) rejection can be overcome by antedating the filing date (see MPEP §2136.03 regarding critical reference date of pre-AIA 35U.S.C. 102(e) prior art) of the reference by submitting an affidavit or declaration under 37 CFR 1.131 or by submitting an affidavit or declaration under 37 CFR 1.132 establishing that the relevant disclosure is an inventor's or at least one joint inventor's own work."

Submitted herewith is a Declaration under 37 CFR §1.132 by inventor Daniel Wendt (Exhibit A) describing that the present application and Wendt were under obligation of assignment to the same entity and Wendt is not an invention by another. As such, Wendt is not available as prior art under 35 USC §102(e).

**IV. The rejection of claims 15-30 under 35 USC §103(a) should be withdrawn**

a) The Examiner rejected claims 15-30 as allegedly obvious in view of Nakao '425 in view of Golembo, Nakao '434, James, Potter, Yeung, Eun, Szoka and Huang, asserting that it would have been obvious to modify the method of Nakao '425 to use a CNP analog, in view of the additional state of the art. The Examiner asserted that Yeung shows CNP53 and CNP22 have the same activity, and Golembo shows CNP22 and CNP17 have the same activity. The Examiner asserted that CNP17 variants with an amino acid "tail" (as in Potter), such as CNP22 and CNP53, are active and therefore all peptides with "tails", CNP52, CNP51, etc., will therefore have the same activity (Action, page 6). Applicant respectfully disagrees.

To establish prima facie obviousness, the Examiner must show that all the elements of the claim are taught or suggested in the prior art (MPEP 2143.03 and Examination Guidelines for Determining Obviousness, Section III.A.1, Fed Reg., Vol 72, No. 195, Pages 57526-35, October 10, 2007), and if all the elements of the claim are described in the prior art, the combination of elements must yield predictable results to render a clamed invention obvious. Further, it should be demonstrated that the prior art reference(s) provide a teaching, suggestion or motivation to combine the references, and/or there is a reasonable expectation of success that combining the elements in the art would provide the invention as

claimed (MPEP § 2142 and Federal Register Examination Guidelines for Determining Obviousness, Section III.G, Fed Reg., Vol 72, No. 195, 2007). The court in *KSR v Teleflex* further stated that mere conclusory statements are not sufficient to draw a conclusion of obviousness, but that there must be some articulated reasoning with some rational underpinning to support a legal conclusion of obviousness. *KSR v Teleflex*, 550 U.S. 398 at l, (2007).

Nakao '425 describes treating achondroplasia using CNP22 or CNP53 but does not disclose CNP variants or conjugating a synthetic polymer to the CNP. Nakao '434 (US20070197434) describes treating arthritis using CNP, and suggests that CNP22 or CNP53 can be modified by addition, deletion or substitution of amino acid residues. The disclosure in Nakao '434 is a generic suggestion as to variants (e.g., peptides with 70% to 90% identity) (paragraph [0103]) and there are no changes similar to those recited in the present claims. Nakao '434 proposes only some changes in CNP22, e.g., deletion of amino acids to CNP17 and CNP22 with C-terminal additions (paragraph [0114]). Nakao '434 also does not disclose conjugating a synthetic polymer to the CNP. Neither Nakao '425 nor Nakao '434 disclose any of the CNP variants recited in the claims.

Golembo describes treating skeletal dysplasia by administering natriuretic peptides, CNP22 or CNP17. Golembo discloses possible CNP22 variants, such as substitution of methionine in the CNP ring, and proposes truncated and mutated forms of CNP22/CNP17. Golembo does not disclose any of the CNP variants recited in the claims.

James discloses natriuretic peptides, including ANP, BNP, and CNP, that can comprise polyethylene glycol (PEG), and describes peptide variants that have NPR-A receptor binding activity. James describes that the peptides can have an N-terminal tail (paragraph [0070]) comprising all or part of native protein, but only in the context of addition of a BNP peptide tail. Paragraph [0050] of James describes all possible variants- truncation, insertion, deletion, fusion- that don't eliminate NPR-A binding function, and that can comprise an N- or C-terminal tail. Applicant submits that CNP binds only to the NPR-B receptor and does not bind to the NPR-A receptor as desired by James. As such, the variants of James would not serve the purpose of those CNP variants recited in the claims and therefore, James does not disclose or suggest any of the CNP variants recited in the claims.

Potter is a review of the primary types of natriuretic peptides, ANP, BNP and CNP. Potter describes that CNP126 can be cleaved to CNP53 and CNP22, and describes that FGFR3 mutants leads to dwarfism, proposing that CNP may be useful to treat dwarfism. Potter does not describe CNP variants at all, and therefore does not suggest the variants in the claims.

Yeung describes that CNP22 and CNP53 are equally effective at promoting cGMP production, and that CNP53 is not processed to CNP22. Yeung does not disclose or suggest CNP variants at all, and certainly does not suggest not those variants recited in the claims.

Totsune describes that methionine in the ring portion of CNP22 and CNP53 is susceptible to oxidation. Totsune does not provide any implication as to the effect of this oxidation, and the Examiner implies such oxidation has a negative impact on the peptide and therefore one of skill would make a CNP variant lacking a methionine residue to avoid oxidation.

Eun is a review publication describing factors that influence enzyme turnover. The Examiner cites to passages in Eun describing that susceptibility to degradation can depend on N-terminal residues, and that methionine and glycine (found in some variants in the claims) stabilizes the protein. Eun also describes that C-terminal residues are important for stability, and suggests that polar residues at the C-terminal ends are rapidly degraded.

Huang is cited for its disclosure that peptides can be made as fusion proteins, e.g., with HSA.

Szoka is cited for its disclosure that making recombinant proteins in a particular bacterial system adds a proline to the end, which is assertedly on the N-terminal end of some variants in the claims.

Applicant submits that the Examiner has not established *prima facie* obviousness as not all the elements of the claims are recited in the cited art. Further, the cited art provides no motivation or reasonable expectation of success for arriving at the present variants recited in the claims, not would have predicted the unexpected benefits provided by the recited CNP variants.

Simply because the natural forms of CNP such as CNP22 or CNP53 (e.g., the Examiner's CNP17 with a "tail") have cGMP activity, there is no reasonable expectation from the teachings in the cited art that CNP peptide analogs having lengths between these natural CNP versions would have any activity or be resistant to proteolytic cleavage (Wendt Declaration II, paragraph 8). None of Nakao '425, Nakao '434 or Golembo suggest analogs of CNP having the claimed sequences nor suggest that these analogs would be effective or preferable over the shorter peptides disclosed in Golembo or Nakao '434. Nothing in the cited art suggests that interim-length analogs of CNP would have any such activity. Applicants submit that the cited art did not test even one peptide having a length between CNP22 and CNP53 for activity.

Moreover, Golembo tested variants of CNP17 and showed that many of these variants did not result in cGMP production. Table 2, page 22, of Golembo discloses various mutations, e.g., 17mer or shorter and shows binding to the NPR-B receptor, with most variants showing lower binding compared to CNP-22. Table 3 describes 17mer variants, all had lower binding than the WT CNP17, and most variants were considerably lower, to not detectable. Nakao '434 sets out CNP22 C-terminal variants in SEQ ID NOs: 7, 8, and 9 therein, but these variants were never tested for activity. However, none of Nakao or Golembo tested a CNP variant in vivo because it would have been difficult due to the rapid degradation of CNP22 in vivo (Wendt Declaration II, paragraph 7).

Applicant was the first to discover that CNP variants having an amino acid length between that of CNP22 and CNP53 were resistant to NEP cleavage, often more resistant than CNP22, and were effective for generating cGMP activity (see specification Tables 2-3 and Figures 18-19). Applicants also discovered that the CNP variants are effective to promote long bone growth (see, e.g., Figures 30-32). None of this activity would have been predictable based on the disclosures in the cited art.

The application as filed teaches that CNP22 has a short half-life in vivo and is quickly eliminated from the blood stream (page 3, lines 31-32). Sequences having any amino acid length except for CNP53, CNP22 or CNP17 are not disclosed in the cited art and their resistance to NEP is not taught or suggested in the cited prior art. The Declaration of Daniel Wendt ("Wendt Declaration II") (Exhibit B) describes that it was not known until the present disclosure whether non-natural forms of CNP, e.g., not CNP22 or CNP53, would have been resistant to NEP cleavage or had any activity in vivo (paragraphs 8-9). Wendt

Declaration II also describes that it would not have been predictable that the variants had the recited in vivo activity (paragraphs 9-10).

The specification shows that variants recited in the claims, e.g., CNP37, Pro-Gly-CNP37, Gly-CNP37, have greater resistance to cleavage by neural endopeptidase (NEP) (see page 140, Table 3). Further, it is believed that sequences comprising the CNP37 sequence or having high homology to the CNP37 peptide are also resistant to NEP cleavage (Wendt Declaration II, paragraph 11). Further, Wendt et al., (J Pharmacol Exp Ther 353:132-149, 2015) (Exhibit C) describes that additional CNP variants also have improved properties. Table 1 of Exhibit C shows that CNP variants, e.g., CNP30, CNP33, CNP34, CNP35, CNP38, CNP39 and CNP40 have increased NEP resistance compared to CNP22 and improved or equivalent potency compared to CNP22.

The specification also shows that the variants have therapeutic benefit in vivo, which would not have been known until the present disclosure. Figure 44 describes that Pro-Gly-CNP37 induces long bone growth in vivo. Figure 45 shows that CNP37 and PEGylated CNP27 have a longer half-life in vivo. Examples 12 and 13 describes that Gly-CNP37, Pro-Gly-CNP37, PEO-CNP27 and CNP37 are effective at increasing long bone growth in vivo in wild type mice and mice with achondroplasia and Figures 49-58. Figures 71 to 73 demonstrate that the peptide in SEQ ID NO: 145 (Pro-Gly-CNP37) and another variant, Gly-CNP37 (SEQ ID NO: 75), have similar activity in vivo. Thus, it is expected that the variants recited in the claims with increased NEP resistance and cGMP stimulation will also have similar half-lives and likely activity in vivo. See paragraph 11 of Wendt Declaration II.

With respect to the assertion that certain variants would have been more obvious than others, such as adding Met, Gly or Pro to the N-terminal end (e.g., Eun, Szoka), there is nothing in the cited art that would have led one of skill to these types modifications with these particular truncated CNP variants. For example, there would have been no reason to arrive at a CNP37 variant in view of Nakao or Golembo, so there would have been no reason to arrive at a CNP37 variant having Met, Gly, Pro or multiple residues at the N-terminal end. Further, if Totsune describes that methionine residues can be oxidized and it is implied that it would have a negative impact on the peptide, then why would one of ordinary skill then add a methionine residue to the N-terminal end of a protein as disclosed in Eun. Such an addition would be counter to the purpose for which the Examiner is citing Totsune.

Also, simply because addition of a proline residue to the N-terminal end of recombinant proteins is an artifact of certain bacterial expression systems (Szoka) this does not mean one of ordinary skill would have added it to the end of a peptide which can be made synthetically so then there would be no residual proline as the Examiner assumes. As such, a person of ordinary skill would have needed another motivation to add the proline residue to a CNP peptide as recited in the claims.

With respect to addition of PEG to a peptide or to use of fusion proteins (James, Huang), Applicants reiterate that a person of ordinary skill would not have arrived at the particular CNP variants for the reasons described above, and therefore it would not have been obvious to add PEG to the non-obvious variants or make a fusion protein using the variants.

The combination of cited art neither discloses nor suggests the particular CNP variants described herein, nor demonstrates that the variants have increased NEP cleavage resistance, are effective to promote long bone growth or treat skeletal dysplasia as claimed. Nakao describes that any variety of CNP variants could be made, but Nakao in combination with the cited art provides no motivation to arrive at the CNP variants recited in the claims, and provides no guidance on what changes would be useful to arrive at the particular peptides recited in the claims that are resistant to NEP cleavage, and have cGMP stimulation activity.

For the foregoing reasons, the claims are not obvious over the cited art and the rejection of the claims under 35 USC §103(a) should be withdrawn.

b) The Examiner rejected claims 15-30 as allegedly obvious in view of Nakao in view of Golembo, James, Potter, Yeung, Eun, Szoka and Huang, *further in view of Wendt*.

As set out above, Wendt is a publication subject to assignment to the same entity at the time of filing and is not a publication by another, and therefore cannot be used in the context of a §103(a) obviousness rejection.

See e.g., MPEP 2136, which states “For applications filed on or after November 29, 1999 or pending on or after December 10, 2004, a provisional rejection under 35 U.S.C.103(a) using prior art under pre-AIA 35 U.S.C.102(e) is not proper if the application contains evidence that the application and the prior art reference were owned by the same

person, or subject to an obligation of assignment to the same person, at the time the invention was made.

As such, the rejection herein is the same at that set out in Section III(a) and the claims are not obvious for the same reasons as set out in Section III(a).

## V. Conclusion

Applicants submit that the pending application is in condition for allowance and respectfully request notification of the same.

Dated: March 26, 2020

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| CLAIMS IN EXCESS OF 20 | 1202 | 41 | 100 | 4100 |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Extension-of-Time:** | | | | |
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| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **4300** |

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|---|---|
| **EFS ID:** | 38984228 |
| **Application Number:** | 15646822 |
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| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
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## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Extension of Time | One_Month_Request_for_Extension_of_Time_Under_37_CFR_1136a.PDF | 22616<br>69fde56635172b9f9eae90e8409c5b08e7541054 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Affidavit-traversing rejectns or objectns rule 132 | CURRICULUM_VITAE_DWendt.PDF | 145227<br>8781c5bcf2e39268a262dc015d8ebe15c64e2ae5 | no | 5 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Applicant Arguments/Remarks Made in an Amendment | Wendt_2015.PDF | 1958558<br>3ddda74751d6655a616caa6836c60360ad6734f3 | no | 18 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Affidavit-traversing rejectns or objectns rule 132 | Wendt_I-Declaration_under_1_132.PDF | 1698444<br>49627cf8564aa9aa405443ce9be98b8d3ee6c946 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Affidavit-traversing rejectns or objectns rule 132 | Wendt_II-Declaration_under_37_CFR_1_132.PDF | 4661388<br>f63c8ff3873de4e1ffb24a71cc70c1dfa67c221f | no | 4 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | | 44556C1-R2_response_to__NFOA_dated_11-26-19_OA.PDF | 198672<br>808b458e9a560db8632b5d2ebce498f7e619a394 | yes | 23 |
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| **PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)** | Docket Number (Optional) 30610/44556C1-R2 |
|---|---|
| Application Number 15/646,822-Conf. #7727 | Filed July 11, 2017 |
| For Variants of C-Type Natriuretic Peptide | |
| Art Unit 3991 | Examiner J. F. Railey |

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

| | Fee | Small Entity Fee | Micro Entity Fee | |
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| [x] One month (37 CFR 1.17(a)(1)) | $200 | $100 | $50 | $ 200.00 |
| [ ] Two months (37 CFR 1.17(a)(2)) | $600 | $300 | $150 | $ |
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| [ ] Four months (37 CFR 1.17(a)(4)) | $2,200 | $1,100 | $550 | $ |
| [ ] Five months (37 CFR 1.17(a)(5)) | $3,000 | $1,500 | $750 | $ |

[ ] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

[ ] A check in the amount of the fee is enclosed.

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I am the

[ ] applicant.

[x] attorney or agent of record. Registration number 53,379 .

[ ] attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Katherine L. Neville/ — Signature
March 26, 2020 — Date

Katherine L. Neville — Typed or printed name
(312) 474-6300 — Telephone Number

**NOTE:** This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

[ ] * Total of 1 forms are submitted.

# Daniel J. Wendt

(925) 280-8878 • Walnut Creek, CA • dwendt.biomarin@gmail.com

## Summary

Over 30 years of experience in the pharmaceutical and biotechnology industry with emphasis in structure/function relationships, cellular assay development, enzymology, analytical characterization, and mass spectrometry. Led the preclinical program for Vosoritide and supported CMC efforts throughout IND filing and clinical development. As leader of the preclinical program, I relied on my biochemical and structure function expertise in protein-protein interactions to develop and oversee *in vitro* and *in vivo* studies for the selection of CNP variants with improved pharmacological properties specifically that were optimized for better safety, efficacy and mode of administration. Assays were developed to characterize the strength, potency, purity, stability and safety of Vosoritide as well as to detect this peptide in complex biological fluids. In the Technical Operations organization, CMC lead responsible for writing up multiple investigational new drug applications currently in clinic or on the market. In the Research group, responsible for leading and managing the analytical group, including those related to Gene Therapy projects.

## Professional Experience

**BioMarin Pharmaceutical Inc., San Rafael, CA (2018-2020)** **Principal Scientist**

Positions held at BioMarin in the Research organization:

- Led analytical focus team of 8 employees
- Research project lead, Core team representative. Cross-functional team and collaboration with KOLs tasked with finding short and long-term next generation indications.
- Analytical lead for GT projects. Responsible for developing analytical processes to ensure consistent and reliable dosing in animals; well-characterized therapeutic products.
- In vitro/analytical support of various programs.

**External Roles/Business Collaborations**

Managed collaboration with various outside technology providers

**BioMarin Pharmaceutical Inc., Novato, CA (2012-2018)** **Principal Scientist**

Positions held at BioMarin in the TOPS organization:

- Led and active research member of the CNP for ACH preclinical program. Also served as research core team lead after program went into the clinic
- Analytical lead/in vitro support for various programs
- CMC analytical characterization lead for Naglazyme and CNP (BMN 111) projects
- Led a group of scientists responsible for developing and qualifying assays for IND enabling studies
- Responsible for writing multiple characterization sections of INDs for BMN111 (CNP for ACH) and NAGLU (for MPS IIIB) projects
- Supervised three people (1 PhD and 2 Research Associates) and other project management experience

BioMarin Pharmaceutical Inc., Novato, CA (2008-2011) Sr Scientist II

Positions held at BioMarin in the TOPS organization

- Project Leader of BMN 111 for ACH program
- Led a proteomic platform technology for discovery of novel "shuttle" proteins to take drugs across the blood-brain barrier; developed a purification procedure for novel fusion proteins designed to cross the blood-brain barrier; developed an in-vitro transport assay, which consisted of either a monolayer or co-culture on a membrane insert for trancytosis experiments
- Primary scientist involved with discovering the mechanism of inactivation of iduronidase (lysosomal enzyme)
- Point person for collaborations with crystallography lab for structure activity relationship
- Supervised two Research Associates

BioMarin Pharmaceutical Inc., Novato, CA (2005-2008) Sr Scientist I
BioMarin Pharmaceutical Inc., Novato, CA (2003-2005) Scientist II
BioMarin Pharmaceutical Inc., Novato, CA (1998-2003) Scientist

Genencor International Inc., Palo Alto, Ca (1989-1998) Sr Research Associate

- Discovery, cloned and purified a novel enzyme isatin hydrolase
- Performed enzyme kinetic studies
- Designed novel enzyme approaches to channel carbon and electrons down the desired pathway
- Devised methods for purification and characterization of polysaccharide degrading enzymes including cellulase, pectinase and xylanase
- Analyzed microbial fermentation for secreted enzymes and end products
- Experience with stability, folding and interaction of proteins and the thermodynamics of these processes at a molecular level using a combination of site-directed mutagenesis and biophysical measurement

## Academic Experience

- Biochemical analysis of brain lesions in AIDS patients
  Advisor: Dr. Satya Dandekar (Department of Medical Microbiology and Immunology at UC Davis)
  Studied HIV pathogenesis: mucosal immunology and genomics of host-virus interactions at the mucosal sites and systemic impact

## Technical Skills

- Over 30 years of pharmaceutical experience developing, qualifying, transferring, and supporting analytical methods in a regulated environment
- Mass spectrometry (MS) including ESI-MSD, ESI-TOF and MALDI-TOF
- Structure activity relationships (SAR) of proteins for identifying biological mechanism of action, substrate binding/catalysis and for formulation stability
- Enzyme kinetics (Michaelis-Menton) using ultraviolet and fluorescent detection
- Adept at analysis of biomolecules, including recombinant de novo proteins, peptides, fusion proteins, pegylated proteins, proteomic samples, genomic samples and carbohydrates
- Familiar with both bacterial and mammalian expression systems, including translational errors and relationships between cell culture/fermentation conditions and product quality attributes

- High-performance liquid chromatography (HPLC) and fast-protein liquid chromatography (FPLC) including reverse phase (RP HPLC), ion exchange (IEX), size-exclusion (SEC) and affinity chromatography
- Experience with capillary electrophoresis (CE) including capillary zone electrophoresis (CZE), capillary gel electrophoresis (SDS-CGE) and capillary isoelectric focusing (cIEF)
- Physical detection methods including multi-angle light scattering (MALS), differential scanning calorimetry (DSC), circular dichroism (CD) and nuclear magnetic resonance (NMR)
- Extensive experience with bioassays and cell fluorescent staining techniques
- Experience with flow cytometry, surface plasmon resonance, high throughput screening, phage display and site-directed mutagenesis in mammalian cells

## Education

**BS Genetics** **June 1989**
University of California at Davis

## Publications

1. Bell SM, **Wendt DJ**, Zhang Y, Taylor TW, Long S, Tsuruda L, Zhao B, Laipis P, Fitzpatrick PA, "Formulation and PEGylation optimization of the therapeutic PEGylated phenylalanine ammonia lyase for the treatment of phenylketonuria," PLoS ONE, **2017**, 12(3).

2. **Wendt DJ**, Dvorak-Ewell M, Bullens S, Lorget F, Bell SM, Peng J, Castillo S, Aoyagi-Scharber M, O'Neill CA, Krejci P, Wilcox WR, Rimoin DL, Bunting S, "Neutral endopeptidase-resistant C-type natriuretic peptide variant represents a new therapeutic approach for treatment of fibroblast growth factor receptor 3-related dwarfism," J Pharmacol Exp Ther, **2015**, 353(1): 132-49.

3. Kan SH, Aoyagi-Scharber M, Le SQ, Vincelette J, Ohmi K, Bullens S, **Wendt DJ**, Christianson TM, Tiger PM, Brown JR, Lawrence R, Yip BK, Holtzinger J, Bagri A, Crippen-Harmon D, Vondrak KN, Chen Z, Hague CM, Woloszynek JC, Cheung DS, Webster KA, Adintori EG, Lo MJ, Wong W, Fitzpatrick PA, LeBowitz JH, Crawford BE, Bunting S, Dickson PI, Neufeld EF, "Delivery of an enzyme-IGFII fusion protein to the mouse brain is therapeutic for mucopolysaccharidosis type IIIB," Proc Natl Acad Sci, **2014**, 111(41): 14870-5.

4. **Wendt**, Zankel, Alferness and Prince. Chemical Modification of Recombinant Human alpha-L-Iduronidase with Diethyl Pyrocarbonate and N-Ethylmaleimide
   Evidence for an Active Site Histidine and Cysteine. Publication pending.

5. Lorget, Kaci, Peng, Benoist-Lasselin, Mugniery, Oppeneer, **Wendt**, Bell, Bullens, Bunting, Tsuruda, O'Neill, Di Rocco, Munnich and Legeai-Mallet, "Evaluation of the Therapeutic Potential of a CNP Analog in a Fgfr3 Mouse Model Recapitulating Achondroplasia," Am J Hum Genet., **2012,** 91(6), 1108–1114.

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9. Weyler, Walter; **Wendt**, Dan; Whited, Gregory; Le, Huan. Identification and cloning of isatin hydrolase, a novel indole assimilatory pathway enzyme. Abstracts of the General Meeting of the American Society for Microbiology, (1996) Vol. 96, No. 0, pp. 555. Meeting Info.: 96th General Meeting of the American Society for Microbiology. New Orleans, Louisiana, USA. May 19-23, 1996.

## Patents

1. Mika Aoyagi-Scharber, Teresa Margaret Christianson, Melita Dvorak-Ewell, **Daniel J. Wendt**, Shinong Long, Jonathan LeBowitz, Daniel Solomon Gold. Targeted therapeutic lysosomal enzyme fusion proteins and uses thereof. Patent number: 9845346. Filed: October 14, 2015. Date of Patent: December 19, 2017.

2. Okhamafe, Bell, Zecherle, Antonsen, Zhang, Tran, Fitzpatrick, Kakkis, Vellard, **Wendt**, and Muthalif, "Compositions of prokaryotic phenylalanine ammonia-lyase variants and methods of using compositions thereof," Patent Application 20130039898. February 14, 2013.

3. **Daniel J. Wendt**, Shinong Long, Sianna Castillo, Christopher P. Price, Mika Aoyagi-Scharber, Michel Claude Vellard, Augustus O. Okhamafe, "Variants of c-type natriuretic peptide," Publication number: 20170051033. Publication date: February 23, 2017.

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The Journal of Pharmacology and Experimental Therapeutics

# Neutral Endopeptidase-Resistant C-Type Natriuretic Peptide Variant Represents a New Therapeutic Approach for Treatment of Fibroblast Growth Factor Receptor 3–Related Dwarfism

Daniel J. Wendt, Melita Dvorak-Ewell,[1] Sherry Bullens, Florence Lorget,[2] Sean M. Bell, Jeff Peng, Sianna Castillo, Mika Aoyagi-Scharber, Charles A. O'Neill, Pavel Krejci,[3] William R. Wilcox,[4] David L. Rimoin, and Stuart Bunting

*BioMarin Pharmaceutical Inc., Novato, California (D.J.W., M.D.-E., Sh.B., F.L., S.M.B., J.P., S.C., M.A.-S., C.A.O., St.B.); and Cedars-Sinai Medical Center, Los Angeles, California (P.K., W.R.W., D.L.R.)*

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## ABSTRACT

Achondroplasia (ACH), the most common form of human dwarfism, is caused by an activating autosomal dominant mutation in the fibroblast growth factor receptor-3 gene. Genetic overexpression of C-type natriuretic peptide (CNP), a positive regulator of endochondral bone growth, prevents dwarfism in mouse models of ACH. However, administration of exogenous CNP is compromised by its rapid clearance in vivo through receptor-mediated and proteolytic pathways. Using in vitro approaches, we developed modified variants of human CNP, resistant to proteolytic degradation by neutral endopeptidase, that retain the ability to stimulate signaling downstream of the CNP receptor, natriuretic peptide receptor B. The variants tested in vivo demonstrated significantly longer serum half-lives than native CNP. Subcutaneous administration of one of these CNP variants (BMN 111) resulted in correction of the dwarfism phenotype in a mouse model of ACH and overgrowth of the axial and appendicular skeletons in wild-type mice without observable changes in trabecular and cortical bone architecture. Moreover, significant growth plate widening that translated into accelerated bone growth, at hemodynamically tolerable doses, was observed in juvenile cynomolgus monkeys that had received daily subcutaneous administrations of BMN 111. BMN 111 was well tolerated and represents a promising new approach for treatment of patients with ACH.

## Introduction

Achondroplasia (ACH), the most common form of human dwarfism with an estimated prevalence between 1 in 16,000 to 1 in 26,000 live births (Foldynova-Trantirkova et al., 2012), is an autosomal dominant condition with the majority of new cases (80%–90%) originating de novo from parents of normal stature (Murdoch et al., 1970; Rousseau et al., 1994). The hallmark of ACH is defective endochondral ossification, resulting in rhizomelic dwarfism, as well as skull and vertebral dysmorphism. Neurologic complications in infants due to foramen magnum stenosis and cervicomedullary compression may lead to potentially lethal hydrocephalus, hypotonia, respiratory insufficiency, apnea, cyanotic episodes, feeding problems, and quadriparesis. Mortality is increased in the first 4 years of life and in the fourth to fifth decades (Trotter and Hall, 2005; Wynn et al., 2007). Current treatments include neurosurgery and orthopedic interventions; limb lengthening to increase stature requires multiple operations over 2–3 years and remains controversial (Horton et al., 2007; Shirley and Ain, 2009). There are currently no approved pharmacologic interventions.

ACH is most commonly caused by a G380R gain-of-function mutation in the fibroblast growth factor receptor-3 (*FGFR3*) gene, resulting in sustained activation of the downstream extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, among others (Foldynova-Trantirkova et al., 2012), that cause supraphysiologic negative

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[1]Current affiliation: Ultragenyx Pharmaceutical Inc., Novato, California.
[2]Current affiliation: Safety Assessment, Genentech Inc., South San Francisco, California.
[3]Current affiliation: Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[4]Current affiliation: Department of Human Genetics, Emory University, Atlanta, Georgia.
David L. Rimoin died May 2012.
dx.doi.org/10.1124/jpet.114.218560.

**ABBREVIATIONS:** ACH, achondroplasia; BMN 111, recombinant variant of C-type natriuretic peptide; BMN 1B2, chemically synthesized variant of C-type natriuretic peptide; BP, blood pressure; CNP, C-type natriuretic peptide; ECG, electrocardiography; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR3, fibroblast growth factor receptor-3; HR, heart rate; HSA, human serum albumin; IHC, immunohistochemistry; MAP, mean arterial pressure; MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; NEP, neutral endopeptidase; NPR, natriuretic peptide receptor; PBS, phosphate-buffered saline; PD, pharmacodynamics; PEG, polyethylene glycol; PEO, polyethylene oxide; PK, pharmacokinetics; sFGFR3, soluble fibroblast growth factor receptor-3; TDI, thanatophoric dysplasia type I; TDII, thanatophoric dysplasia type II.





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regulation of chondrocyte proliferation and differentiation as well as decreased extracellular matrix synthesis (Murakami et al., 2004; Yasoda et al., 2004; Sebastian et al., 2011). In addition, stenosis of the foramen magnum and the spinal canal, caused by premature synchondrosis closure and fusion of ossification centers, is regulated by the same pathway (Hecht and Butler, 1990; Modi et al., 2008; Matsushita et al., 2009). Paracrine/autocrine factor C-type natriuretic peptide (CNP) signals through natriuretic peptide receptor (NPR) B and modulates the activity of FGFR3 through inhibition of the ERK/MAPK pathway at the level of rapidly accelerated fibrosarcoma protein kinase (RAF-1) (Krejci et al., 2005; Horton et al., 2007). CNP knockout mice, as well as those expressing mutant CNP receptors, exhibit dwarfism and have growth plates histologically similar to ACH (Rimoin et al., 1970; Naski et al., 1998; Chusho et al., 2001), whereas overexpression of CNP in mice (Kake et al., 2009) and humans (Bocciardi et al., 2007; Moncla et al., 2007) is characterized by skeletal overgrowth. The dwarfism in mice overexpressing FGFR3 with a mutation analogous to human G380R (*Fgfr3*$^{ACH/+}$) under the control of the type II collagen promoter is corrected by endogenous CNP overproduction (Yasoda et al., 2004) or the continuous infusion of exogenous CNP (Yasoda et al., 2009), giving credence to the hypothesis that systemic administration of CNP should stimulate growth in pediatric ACH patients with open growth plates.

CNP, expressed as a 126–amino acid protein precursor (prepro-CNP), is processed to an active 53–amino acid cyclic peptide by furin and further processed to a 22–amino acid peptide by unknown proteases (Potter et al., 2006). It has been reported that only the 17–amino acid cyclic domain residues (Cys6–Cys22 of CNP22), formed by an intramolecular disulfide linkage, are required for activity (Furuya et al., 1992). Native CNP (CNP22) is rapidly cleared from the circulation by the natriuretic clearance receptor (NPR C) and neutral endopeptidase (NEP) (EC 3.4.24.11; metalloendopeptidase; enkephalinase; neprilysin; CD10, CALLA) (Brandt et al., 1995, 1997). As a result, CNP22 has a short half-life in serum of less than 2 minutes in mice and humans, thereby requiring a lengthy infusion process to result in a pharmacological benefit (Hunt et al., 1994; Yasoda et al., 2009). In fact, mice given intravenous bolus or subcutaneous administrations of CNP22 demonstrated no pharmacological benefit.

We recently described the pharmacological activity of a 39–amino acid CNP variant (BMN 111; recombinant variant of C-type natriuretic peptide), which has an extended serum half-life due to its resistance to NEP digestion (Lorget et al., 2012). We demonstrated that daily subcutaneous administrations of BMN 111 in an ACH mouse model resulted in increased axial and appendicular skeletal lengths, improvements in dwarfism-related clinical features including flattening of the skull, straightening of the tibias and femurs, and correction of the growth plate defect. Here, we report the development of BMN 111, through in vitro and in vivo approaches, which is resistant to degradation by NEP and designed to elicit the growth-promoting effects of native CNP through a subcutaneous route of administration. We also examined the cardiovascular effects of BMN 111, since it is well established that natriuretic peptides, including CNP, induce vasodilation (Clavell et al., 1993; Charles et al., 1995; Igaki et al., 1998; Scotland et al., 2005; Pagel-Langenickel et al., 2007), and then evaluated the growth-potential at doses that were considered hemodynamically acceptable [<10% drop in blood pressure (BP) and <25% increase in heart rate (HR)] in mice and monkeys. This article focuses on the pharmacological effects of daily subcutaneous administrations of BMN 111 in mice (normal and ACH models) and normal juvenile cynomolgus monkeys.

## Materials and Methods

**Native CNP and Variants.** Native CNP and variants were chemically synthesized using standard Fmoc chemistry (AnaSpec Inc., Fremont, CA; and GenScript USA, Inc., Piscataway, NJ). Protein sequences for coded samples were as follows: $NH_2$-GLSKGCFGLKLDRIGSMSGLGC-COOH [native CNP; CNP22], $NH_2$-DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-COOH [CNP53], $NH_2$-GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-COOH [BMN 1B2 chemically synthesized variant of C-type natriuretic peptide], $NH_2$-GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC-COOH [chimeric peptide of human serum albumin (HSA) and CNP27; the HSA sequence (AC P02768; amino acids 27–36) is underlined] [$HSA_{(27–36)}$-CNP27], $NH_2$-GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC-COOH [BMN 1B2 (QQ)], $NH_2$-GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC-COOH [chimeric peptide of HSA and CNP22; the HSA sequence (AC P02768; amino acids 231–245) is underlined] [$HSA_{(231–245)}$-CNP22], $NH_2$-GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC-COOH [chimeric peptide of human IgG and CNP22; the IgG sequence (AC P01857; amino acids 224–237) is underlined] [$IgG_{(224–237)}$-CNP22], and $NH_2$-GQPREPQVYTGANQQGLSKGCFGLKLDRIGSMSGLGC-COOH [chimeric peptide of human IgG and CNP27; the IgG sequence (AC P01857; amino acids 224–233) and the KK to QQ CNP27 variant sequence are underlined] [$IgG_{(224–233)}$-CNP27(QQ)]. BMN 111 was recombinantly expressed in *Escherichia coli* (Long et al., 2012) and has the following protein sequence: $NH_2$-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-COOH. CNP22 and all variant constructs have been oxidized to form one intramolecular disulfide bond. All peptides were ≥90% pure and masses were confirmed by liquid chromatography/mass spectrometry.

**NEP Resistance.** Native CNP (CNP22) and variants (100 μM) were incubated in the presence of purified recombinant human NEP (no. 1182-ZN-010, 1 μg/ml; R&D Systems, Minneapolis, MN) in phosphate-buffered saline (PBS) buffer at 37°C for 140 minutes. Throughout the incubation, a portion of the sample was removed and quenched with EDTA (10 mM). Reactions were reduced with dithiothreitol (10 mM) for 30 minutes at 37°C and then analyzed by liquid chromatography/mass spectrometry. All concentrations listed are final. Results were reported as percentage of intact peptide remaining compared with time zero. All assays were repeated at least once for candidates that demonstrated native potency.

**Potency (cGMP) Assay.** Potency was determined in a cell-based assay using murine NIH3T3 fibroblasts, which endogenously express NPR B but not the NPR A nor NPR C receptors (Abbey and Potter, 2003). Briefly, 50%–80% confluent fibroblasts were pretreated with a phosphodiesterase inhibitor (0.75 mM isobutylmethylxanthine) in Dulbecco's modified Eagle's medium/PBS (1:1) for 15 minutes at 37°C/5% $CO_2$. Next, CNP22 or variants ($10^{-11}$ M to $10^{-5}$ M) were added to the cells without media exchange in duplicate and incubated for an additional 15 minutes. Cells were detergent lysed (0.1% Triton X-100) and cGMP concentration was determined using a competitive immuno-based assay (CatchPoint; Molecular Devices, Sunnyvale, CA).

**PEGylation.** PEGylation reaction conditions were optimized to facilitate specific conjugation of polyethylene glycol (PEG) moiety at the $NH_2$ terminus of CNP or its variant, such as CNP27. Briefly, *N*-hydroxysuccimimide–activated PEGs of varying size (NOF America Corporation, White Plains, NY; and Thermo Fisher Scientific, Waltham, MA) were incubated with CNP22 or CNP27 at a 1:1 molar ratio in 0.1 M $KPO_4$ pH 6 for 1 hour at room temperature. $NH_2$-terminal lysines (i.e., nonring lysines) of CNP27 were changed to arginines to eliminate additional PEGylation sites without affecting NPR B binding and signaling activity (data not shown). Mono-PEGylated species were

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purified by C5 reverse-phase high-performance liquid chromatography using an acetonitrile gradient containing 0.1% formic acid.

**Pharmacokinetics.** The pharmacokinetics (PK) profile of various CNP variants and their time courses of plasma cGMP concentrations were determined in 7- to 8-week-old male wild-type rats [Crj:CD (SD) IGS] or wild-type mice [FVB/nJ] (Charles River Laboratories, Inc., Wilmington, MA) after a single intravenous (20 nmol/kg in rats, $n = 3$; 25 nmol/kg in mice, $n = 4$) or subcutaneous (50 nmol/kg in rats, $n = 3$; 70 nmol/kg in mice, $n = 4$) administration. All peptides were formulated in 30 mM acetic acid pH 4.0 containing 10% sucrose and 1% benzyl alcohol. Plasma CNP immunoreactivity was determined using a competitive radioimmunoassay and a commercially available polyclonal antibody against the cyclic ring portion of CNP (Bachem, Bubendorf, Switzerland). Plasma cGMP concentration was determined by a competitive radioimmunoassay (Yamasa Corporation, Salem, OR).

**Activity, Accumulation, and Clearance of BMN 111 at the Growth Plate.** Mice were dosed and anesthetized at 15 minutes postdose, which was previously determined to coincide with the maximum cGMP response time, unless otherwise noted. Blood was collected from the heart via intracardiac puncture. Femurs with complete knee cartilage were harvested and immediately frozen in liquid nitrogen. Distal epiphysis sections from each mouse were separated for either cGMP or immunohistochemistry (IHC) experiments. For cGMP experiments, the epiphysis was pulverized using a Covaris CPO2 cryoPREP tissue homogenizer (Covaris, Inc., Woburn, MA). cGMP was extracted from the frozen pulverized epiphysis in PBS buffer containing 0.8 mM phosphodiesterase inhibitor (isobutylmethylxanthine) and quantified by competitive enzyme-linked immunosorbent assay (CatchPoint cGMP fluorescent assay kit; Molecular Devices). For IHC, tissues were fixed in 4% paraformaldehyde immediately after dissection, decalcified in 10% formic acid/PBS until no calcium oxalate precipitate formed with 5% ammonium oxalate, then dehydrated, paraffin embedded, and sectioned at 7 $\mu$m. Sections were deparaffinized and rehydrated prior to antigen retrieval in 10 mM citrate (30 minutes, 80°C), then blocked (1% normal donkey serum, 0.1% bovine serum albumin, 0.1% $NaN_3$, 0.3% Triton X-100 in PBS; 1 hour, room temperature) and incubated in monoclonal CNP antibodies (4°C, overnight). Secondary donkey anti-mouse antibodies, conjugated to Alexa Fluor 488 were applied (1 hour, room temperature; Invitrogen, Carlsbad, CA). For quantification of signal intensity, confocal stacks were acquired using a Zeiss LSM 510 NLO laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a 40× objective, 2× zoom, and a 0.53-$\mu$m $z$ increment were used for IHC experiments. All experiments were performed in duplicate ($n = 2$).

**Dose Regimen.** Three-week-old wild-type (FVB/nJ; Charles River Laboratories, Inc.) male mice were given subcutaneous injections of BMN 111 (20 nmol/kg) daily on alternating weeks (weeks 1, 3, and 5) or vehicle [30 mM acetic acid pH 4.0 containing 10% sucrose (w/v) and 1% (w/v) benzyl alcohol] daily for 5 weeks ($n = 10$/group). Tail measurements were collected at study initiation. Growth was monitored during the in-life treatment period by weekly tail measurements. At necropsy, final X-ray and naso-anal and tail measurements were obtained. Long bones were collected and measured for length, and the femur and tibia were fixed for histology and archived.

**Pharmacological Effects of CNP Variants in Wild-Type Mice.** FVB/nJ wild-type mice (Charles River Laboratories, Inc.) were administered daily subcutaneous injections at varying dose levels (20–200 nmol/kg; $n = 8$/group) over 35 days. All CNP variants were formulated in vehicle [30 mM acetic acid buffer solution pH 4.0, containing 10% (w/v) sucrose and 1% (w/v) benzyl alcohol]. Mice (± 1 S.D. of the average body weight) were randomized at 3 weeks ± 2 days of age. Doses were given at approximately the same time each day, 2 hours prior to the dark cycle, and were based on the most recently collected body weight. The lengths of the tibia, femur, humerus, ulna, and lumbar vertebra 5 were measured with a caliper. Treated groups were compared with the vehicle control group at common time points by analysis of variance with a post hoc Dunnett's $t$ test (Dunnett and Crisafio, 1955) or other appropriate test.

**Pharmacological Effects of BMN 111 in *Fgfr3*$^{ACH/+}$ Nice.** *Fgfr3*$^{ACH/+}$ mice were kindly provided by David M. Ornitz (Washington University in St. Louis, St. Louis, MO) and bred at Jackson Laboratories (West Sacramento, CA). Expression of activated FGFR3 was targeted to growth plate cartilage using regulatory elements from the collagen 2 gene (Naski et al., 1998). Three-week-old *Fgfr3*$^{ACH/+}$ male mice (FVB/nJ. *Fgfr3*$^{ACH/+}$ JAX West; $n = 8$/group) were administered daily subcutaneous injections over 35 days (5, 20, and 70 nmol/kg). *Fgfr3*$^{ACH/+}$ mice and their wild-type littermates were anesthetized and randomized by body weight into treatment groups. Prior to the study, mice were monitored for body weight, general health, and tail length. On day 37, all mice were euthanized by terminal anesthesia. Left and right tibia, femur, humerus, and ulna were collected and measured using a digital caliper. The left bones were fixed in 10% neutral-buffered formalin overnight, and then stored in ethanol at 2–8°C.

**Hemodynamic Effects of CNP Variants in Wild-Type Mice.** Mouse studies were performed at LAB Research, Inc. (Dorval, QC, Canada). An isoflurane gas–anesthetized mouse model was used to reduce background variability in hemodynamic readouts, and to provide greater sensitivity to reduction in BP by blunting the compensatory increase in HR. CNP variants were tested over a dose range of 20–200 nmol/kg (2000 nmol/kg additional dose for BMN 111). Mice (6- to 7-week-old FVB/nJ; Charles River Laboratories, Saint-Constant, QC, Canada) were anesthetized with isoflurane gas. A pressure-monitoring catheter connected to a telemetry transmitter (PA-C10 or PXT-C50; Data Sciences International, New Brighton, MN) was placed in the aorta for arterial BP measurements. The position of the catheter was confirmed by analysis of pressure tracings. Hemodynamic parameters were recorded continuously, and were allowed to stabilize for at least 15 minutes prior to subcutaneous administration of CNP variants or vehicle control. At least 30 minutes were allowed to elapse before administration of successive doses. The mean of parameter values in the 15 minutes before dosing was compared with the mean of parameter values in the 15 minutes immediately after dosing ($n = 3$–5/group).

**Hemodynamic Effects of BMN 111 in Cynomolgus Monkeys.** All nonhuman primate studies were performed at LAB Research, Inc. At least 2 weeks prior to experimentation, animals were implanted with a cardiovascular transmitter (Data Sciences International) by which electrocardiography (ECG) data and systolic BP, diastolic BP, and mean arterial pressure (MAP) were recorded continuously via telemetry (Dataquest A.R.T.; Data Sciences International). Experiments were conducted first in isoflurane gas–anesthetized monkeys to establish the hemodynamically active dose range of BMN 111 (doses tested ranged from 0.35 to 17 nmol/kg). After anesthetic induction, hemodynamic and ECG readouts were allowed to stabilize for at least 15 minutes before administration of BMN 111. The hemodynamically active dose range was then confirmed in conscious animals (7–35 nmol/kg). In conscious monkeys, to minimize derangement of HR and BP due to animal handling, BMN 111 was administered via a long subcutaneously implanted catheter, which allowed "remote" administration without removing the animal from the cage. Mean HR and MAP values from 10 to 20 minutes postdose (covering the time of BP nadir) were compared with mean values in the 15 minutes just prior to dosing. ECG data were evaluated from 15 minutes prior to each dose through 60 minutes postdose ($n = 1$–4/group).

**Pharmacological Effects of BMN 111 in Cynomolgus Monkeys.** The effect of BMN 111 on growth was investigated in normal, growing, juvenile male cynomolgus monkeys (aged 2–4 years at the onset of treatment; 2.2–2.9 kg body weight). BMN 111 (either 2.25 or 8.25 nmol/kg, or vehicle control; $n = 4$/group) was administered by daily subcutaneous injection for 181 days. Throughout the study, animals were monitored for mortality and clinical signs. Hematology and clinical chemistry parameters were measured on days −7, −1, 7, 21, 35, 49, 63, 77, 91, 105, 133, 161, and 182. Total serum alkaline phosphatase was measured on an automated chemistry analyzer (CiToxLAB, Laval, QC, Canada). Bone-specific alkaline phosphatase was measured using the Ostase BAP assay (Immunodiagnostic Systems Inc., Gaithersburg, MD). During pretreatment and weeks 4, 8, 13, and 23, assessments of

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tibial length and growth plate width were made by digital radiographs, proximal tibial growth plate volume and width were evaluated by magnetic resonance imaging (MRI), and lengths of limbs and tail were measured with a tape measure. One day after their last BMN 111 dose, the animals were euthanized and subjected to necropsy.

**MRI.** Sagittal, three-dimensional, fat-suppressed spoiled gradient recalled echo imaging sequences of each knee were acquired with an eight-channel knee coil, using a high-resolution 1.5 Tesla system (GE HDx, Mississauga, ON, Canada). Sequence parameters were as follows: echo time, 15 milliseconds; repetition time, 47 milliseconds; number of averages, 3; slice thickness/gap, 1.5 mm/0; matrix, 512 × 512; and field of view, 10 cm. All measures were performed by the same veterinary radiologist. The maximal height of the proximal physis of the right and left tibia was measured in its central third using OsiriX 3.7.1 software (Pixmeo, Geneva, Switzerland). Using the brush selection tool available in the software, the surface of the physis of the right proximal tibia was then selected to include the hyperintense layer between the adjacent hypointense bone. This was repeated on all consecutive images on which the growth plate was well demarcated and surrounded with hypointense layers of bone. This technique aimed to only select the plate itself and exclude the peripheral cartilage. To avoid inclusion of this peripheral cartilage, the selection solely included the portions of the plate that presented parallel borders and excluded more peripheral portions that presented diverging margins. When the surface of the growth plate was selected on all consecutive images, its volume was calculated using the automated volume calculation plug-in included in the software ($n$ = 4/group).

**Radiographic Evaluation of Tibial Length.** Posteroanterior projections collimated to include each of the lower limbs and centered on the knees were performed with digital computed radiography (Agfa CR-DX, Toronto, ON, Canada) and taken while the animals were under general anesthesia. Mediolateral projections of the right tibia, centered on the proximal tibial physis, were also performed. Right tibial lengths (in millimeters) were measured manually on posterior–anterior projections with dedicated image analysis software (OsiriX 3.7.1; Pixmeo). The system was calibrated and the monkey legs were placed directly on the phosphorus plates to limit magnification effects. All images were interpreted and measured by the same veterinary radiologist who remained blinded to the treatment groups ($n$ = 4/group).

**Postmortem Microcomputed Tomography of Lumbar Vertebrae.** Lumbar vertebrae 2, 3, and 4 were excised at necropsy, fixed in formalin, and scanned using the SkyScan 1176 microCT instrument (Micro Photonics, Inc., Allentown, PA), at a resolution of 35 $\mu$m, with the X-ray source set to 80 kV, 300 $\mu$A, and using a Cu + Al filter. Images were reconstructed by NRecon (Bruker MicroCT, Kontich, Belgium). To measure the foramen area of each vertebra, images were processed using the SkyScan-associated Data Viewer and the bone position was optimized. For each vertebra, the area was computed from the transaxial image corresponding to the narrowest part of the foramen in the coronal aspect ($n$ = 4/group). The relevant transaxial image was saved as a single image and the foramen area measured using CTan software (Bruker MicroCT).

**Histomorphometric Analysis of the Growth Plate in Cynomolgus Monkeys.** For dynamic histomorphometry, calcein (10 mg/kg) was administered 14 days prior to necropsy, and oxytetracycline (40 mg/kg) was administered 6 days prior to necropsy. Left tibias were dissected, formalin fixed, dehydrated, and embedded in methyl methacrylate. Five 7-$\mu$m sections were obtained from the 50% level of the bone for analysis of the proximal growth plates and trabecular bone. Sections were stained with von Kossa, Goldner trichrome, and tartrate-resistant acid phosphatase stain ($n$ = 4/group). Rate of growth was determined from the slope of length measurements plotted over time, and from fluorescent labeling of new bone.

**Histomorphometric Analysis of the Bone of Cynomolgus Monkeys Treated with BMN 111.** Left tibias, with growth plates intact, were harvested at necropsy, formalin fixed, and stored in 70% ethanol. Tibias were trimmed, dehydrated, and embedded in methyl methacrylate for plastic histology. Five 7-$\mu$m sections were obtained from the 50% level of the bone for analysis of the proximal growth plates and trabecular bone. Tibias were stained with von Kossa, Goldner trichrome, and tartrate-resistant acid phosphatase stains. The combination of these three stains allowed analysis of the growth plate morphology, trabecular bone volume and architecture, quantification of unmineralized matrix (osteoid), and quantification of osteoblast and osteoclast numbers. Unstained sections were mounted for visualization of fluorescent labels for dynamic histomorphometry. Two operators measured total growth plate thickness of the right proximal tibial plate at six randomly chosen spots; 12 measurements were thereafter averaged for each sample. For each of the 12 fields, three columns of proliferating cells were assessed to determine the average number of proliferating cells per proliferating column. In addition, four regions of cuboidal chondrocytes in each field were assessed for mean cell volume of hypertrophic chondrocytes. For assessment of proliferating zone thickness and hypertrophic zone thickness, five measurements were made and averaged for each sample. Trabecular bone histomorphometry was evaluated within two 3500 $\mu$m × 3500 $\mu$m regions of interest by two operators.

All procedures described herein were conducted in accordance with the principles and procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice and rats were humanely euthanized via anesthesia with carbon dioxide (performed in accordance with accepted American Veterinary Medical Association Guidelines on Euthanasia, June 2007). The monkeys were sedated with a combination of ketamine hydrochloride and acepromazine given intramuscularly, followed by an overdose of sodium pentobarbital, followed by exsanguination.

## Results

**Rational Design and In Vitro Screening of Potential NEP-Resistant CNP Variants.** Watanabe et al. (1997) reported that proteolysis of CNP22 by NEP occurred after initial attack at the Cys6–Phe7 bond. To test this, we synthesized peptidomimetics of CNP22 that contained either a reduced or methylated amide bond between Cys6 and Phe7 (Cys-methylene and *N*-methyl-Phe7, respectively) of CNP22 and incubated in the presence of purified human NEP. Analysis of the digestion products revealed that the Cys–Phe peptidomimetic bond was resistant to NEP in both variants (data not shown). However, when measuring the rate of disappearance of the intact molecule, these variants were indistinguishable from CNP22, indicating that proteolysis occurred at other sites of CNP22 and does not depend on initial cleavage of the Cys–Phe bond (Table 1).

Oefner et al. (2000) proposed that the size-limited active site cavity of NEP restricts substrates based on their size (<3 kDa), a claim that is supported by natural substrate data (Kerr and Kenny, 1974; Erdös and Skidgel, 1989; Vijayaraghavan et al., 1990). To test this, we made larger variants of CNP through PEG conjugation, native CNP amino acid extensions, or by fusing CNP to other peptide sequences (chimeras). CNP variants, produced by chemically conjugating PEG units to the peptide $NH_2$ terminus, exhibited size-dependent resistance to NEP proteolysis. Specifically, NEP resistance was observed in PEGylated CNP22 variants in which the molecular mass of the PEG unit was ≥1 kDa or when the total molecular mass of the PEG-CNP22 conjugate exceeded 3.2 kDa. However, these PEG-CNP conjugates were poor agonists of NPR B (≥16-fold increase in $EC_{50}$). Interestingly, PEGylation of a longer native CNP sequence (CNP27) reclaimed the lost potency, while maintaining NEP resistance (Table 1).

Similar size-dependent results were observed by increasing the size of CNP22 through amino acid extensions. Here, we

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TABLE 1
In vitro potency and NEP resistance for CNP variants
Data are expressed ± S.D. values as applicable.

| Description | Molecular Mass | Potency[a] ($EC_{50}$) | NEP Resistance[b] |
|---|---|---|---|
| | *kDa* | *nM* | *% Intact* |
| CNP22 | 2.2 | 13 ± 5.4 | 2.4 ± 1.8 |
| CNP22, K4R[c] | 2.2 | 12 ± 1.4 | <5 |
| CNP27, K4R, K5R, K9R[c] | 2.8 | 8.7 ± 1.8 | <5 |
| ANP28 | 3.1 | >2000 | NT |
| CNP22, Cys6-methylene[d] | 2.2 | 44 ± 6.2 | <5 |
| CNP22, *N*-methyl-Phe7[d] | 2.2 | 860 ± 380 | <5 |
| CNP22, 20 kDa PEG | 22 | >2000 | 100 |
| CNP22, 5 kDa PEG | 7.2 | >2000 | 84 |
| CNP22, 2 kDa PEG | 4.2 | >2000 | 100 |
| CNP22, 1 kDa PEO24 | 3.2 | 640 ± 320 | 90 |
| CNP22, 0.6 kDa PEO12 | 2.8 | 210 ± 30 | 40 |
| CNP27, 2 kDa PEG | 4.8 | >2000 | 100 |
| CNP27, 1 kDa PEO24 | 3.8 | 16 ± 2.8 | 103 ± 2.7 |
| CNP27, 0.6 kDa PEO12 | 3.4 | 7.8 ± 1.4 | 69 ± 1.6 |
| CNP30 | 3.1 | 8.4 ± 3.9 | 36 ± 1.9 |
| CNP33 | 3.5 | 11 ± 0.1 | 99 ± 1.2 |
| CNP36 | 3.8 | 5.8 ± 3.5 | 98 ± 2.2 |
| CNP37 | 3.9 | 11 ± 2.0 | 97 ± 8.3 |
| CNP38 | 4.1 | 6.8 ± 0.4 | 105 ± 7.7 |
| CNP39 | 4.2 | 17 ± 1.6 | 95 ± 6.8 |
| CNP40 | 4.3 | 10 ± 2.6 | 101 ± 6.3 |
| CNP53 | 5.8 | 7.1 ± 0.5 | 106 ± 20 |
| BMN 1B2 | 4.0 | 8.7 ± 0.5 | 110 ± 0.02 |
| BMN 1B2(QQ) | 4.0 | 130 ± 20 | 102 |
| BMN 111[e] | 4.1 | 4.9 ± 1.5 | 99 ± 0.6 |
| $HSA_{(231–245)}$-CNP22[f] | 3.9 | 11 ± 3.2 | 20 ± 0.6 |
| $IgG1_{(224–237)}$-CNP22[f] | 3.7 | 72 ± 5.9 | 75 |
| $IgG1_{(224–233)}$-CNP27(QQ)[f] | 3.9 | 920 ± 50 | 40 |
| $HSA_{(27–36)}$-CNP27[f] | 4.0 | 6.9 ± 2.1 | 105 ± 6.4 |

NT, not tested.
[a]Mean $EC_{50}$ ($n \geq 2$) of cGMP production in murine NIH3T3 fibroblasts after 15-minute exposure to CNP variants ($10^{-10}$ M to $10^{-5}$ M), with nonlinear curve fit using the Hill equation (Erithacus Software).
[b]NEP resistance was determined by measuring the amount of intact peptide remaining after exposure to human NEP for 140 minutes in PBS at 37°C ($n = 2$, for variants with near native potency; $n = 1$ for all other variants). Peptide digests were analyzed by liquid chromatography/mass spectrometry.
[c]Peptides used for PEGylation variants.
[d]Non-native Cys6-Phe7 peptide bond analogs were synthesized based on reported initial NEP cleavage site (Watanabe et al., 1997).
[e]Biologic synthesis (all other analogs in this table were prepared by chemical synthesis).
[f]Chimeric sequences were synthesized on the amino terminus of CNP (IgG, Ac P01857, PDB ID 2IWG; HSA Ac P02768, PDB ID 1BM0).

synthesized native amino acids on the $NH_2$ terminus of CNP22 based on CNP53 sequence (active tissue expressed form of CNP). NEP resistance was observed when the total number of residues was ≥33 amino acids (>3.4 kDa) and all retained CNP22 potency ($EC_{50}$ = 7–18 nM). Variants of CNP37 designed for enhanced serum stability, BMN 1B2 (approximately 4.0 kDa) and BMN 111 (approximately 4.1 kDa), also demonstrated NEP resistance and equivalent potency to CNP22. However, glutamine substitutions at the native processing site [Lys30 to Lys31 of CNP53, BMN 1B2(QQ)], designed to mitigate generation of CNP22 after parenteral injection, were 10-fold less potent than CNP22 ($EC_{50}$ = 130 nM; Table 1).

Finally, to address the potential proteolytic vulnerability issues of native CNP sequence to unknown protease(s) in vivo, we designed a variety of chimeric CNP variants derived from short sequences of albumin and IgG. Non-native CNP sequences were chemically synthesized to the $NH_2$ terminus of CNP22 and CNP27 and were selected based on their homology between species (>70%), abundance in serum (>1 mg/ml), and exposure to solvent (>90%) using crystal structure data (PDB IDs 1BM0 and 2IWG). In silico database programs (http://www.syfpeithi.de and http://www.imtech.res.in/raghava/hlapred) were used to avoid introducing human leukocyte antigen binding sites at the chimeric junction to reduce the potential of an immunogenic response. Of the four chimeras made, three were sensitive to NEP, despite having molecular masses ≥3.7 kDa (Table 1). This suggested that structural components may also influence NEP resistance, since native sequence constructs smaller in size (3.4 kDa) were completely resistant to NEP. Moreover, divergence away from native CNP sequence resulted in a significant decrease in potency. One chimeric variant, $HSA_{(27–36)}$-CNP27, demonstrated NEP resistance and equivalent potency to CNP22. In vitro NEP resistance and potency profiles of five CNP variants chosen for further in vivo evaluation are shown in Fig. 1.

**NEP-Resistant CNP Variants Exhibit Longer Serum Half-Lives than Native CNP.** NEP-resistant variants demonstrated an increase in serum half-life (approximately 7- to 16-fold after intravenous administration and 2- to 7-fold after subcutaneous administration in rats and mice) compared to CNP22, with half-life ($t_{1/2}$) = 14–23 minutes for NEP-resistant variants versus ≤2 minutes for CNP22 when dosed intravenously and $t_{1/2}$ = 12–25 minutes for NEP-resistant variants versus 3–5 minutes for CNP22 when dosed subcutaneously (Fig. 2; Table 2). The PK profiles were similar for

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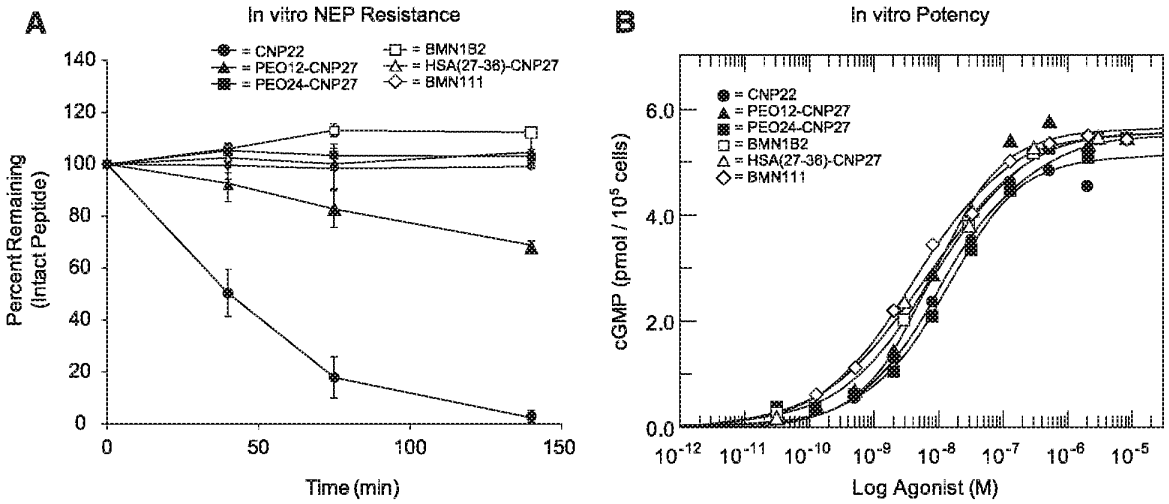


**Fig. 1.** NEP resistance and potency of CNP variants. (A) Plot of intact CNP22 and its variants remaining after incubation with recombinant human NEP in PBS at 37°C for 140 minutes. The mean percentage of intact peptide remaining was determined at designated times by liquid chromatography/mass spectrometry ($n$ = 2). Error bars indicate the S.D. (B) Mean cGMP production in murine fibroblasts (NIH3T3; $n \geq 2$; error bars omitted for comparison clarity). The $EC_{50}$ value was determined after 15-minute exposure to CNP22 or variants ($10^{-11}$ M to $10^{-5}$ M) using a nonlinear curve fit (Hill equation; Erithacus Software Ltd., Surrey, UK). Symbols indicate the following: CNP22 (solid circle), PEO24-CNP27 (solid square), PEO12-CNP27 (solid triangle), BMN 1B2 (open square), BMN 111 (open diamond), and $HSA_{(27-36)}$-CNP27 (open triangle).

most NEP-resistant variants tested, with the exception that the polyethylene oxide (PEO) PEGylated variant (PEO24-CNP27) demonstrated a 3-fold longer serum half-life than the other variants after subcutaneous administration (Fig. 2B). Plasma cGMP profiles, a pharmacodynamics (PD) marker of NPR B activation (Wielinga et al., 2003), correlated well with the PK profiles of the CNP variants, demonstrating a clear PK/PD relationship (Fig. 2, C and D). Interestingly, cGMP concentration is not maintained for the PEGylated variant, despite the elevated exposure of this variant at the later time points (60–180 minutes; Fig. 2D). This could be caused by receptor desensitization of NPR B, which is known to occur upon prolonged exposure to CNP (Potter and Hunter, 2001). BMN 111, the recombinant version of BMN 1B2 containing one additional proline residue at the amino terminus, also demonstrated a prolonged half-life compared with CNP22 in wild-type murine studies (Fig. 2, E and F). Importantly, our data are consistent with a model whereby NEP functions as one of the major clearance pathways of CNP and supports our hypothesis that NEP-resistant variants should have longer serum half-lives.

**CNP Variant Selection Based on Stimulation of Bone Growth and Hemodynamic Effects in Wild-Type Mice.** Studies in rat chondrocytes using the method developed by Krejci et al. (2005) indicated that daily 1-hour exposure to CNP22 significantly reversed the growth arrest induced by FGFR3 activation, comparable to cells continuously exposed to CNP22; these results support daily administration of CNP variants in wild-type mice (data not shown). Although PEO24-CNP27 demonstrated a superior PK profile, it failed to provide a significant growth benefit in wild-type mice compared with the placebo control in preliminary range-finding studies (data not shown). For this reason, we decided to evaluate a smaller, more potent PEG variant, PEO12-CNP27, in the comparative study.

Three-week-old wild-type FVB/nJ male mice ($n$ = 3–9/group) were given daily subcutaneous injections of CNP variants BMN 1B2, BMN 111, PEO12-CNP27, or $HSA_{(27-36)}$-CNP27 at 20, 70, or 200 nmol/kg or vehicle for 36 days. The growth of the appendicular and axial skeletons was dose related for most of the variants tested; however, growth effects were more pronounced in mice treated with BMN 111 (Fig. 3). A significant increase in naso-anal length was detected as early as 8 days after the start of BMN 111 treatment (data not shown). The PEGylated CNP variant, PEO12-CNP27, was the least pharmacologically active of the variants tested, potentially due to poor tissue bioavailability associated with PEGylated proteins (Veronese and Pasut, 2005; Ryan et al., 2008), and performed similarly to PEO24-CNP27 in our preliminary range-finding study. After 2 weeks of treatment, axial growth (naso-anal and tail length) was evident in mice treated with the chimeric CNP variant; however, the response was not sustained beyond 3 weeks (data not shown).

Additional studies designed to look at accumulation and clearance of BMN 111 at the growth plate demonstrated that consecutive daily administrations of BMN 111 augmented the cGMP levels in the distal femur growth plate, but not in the kidney, 15 minutes after the last injection (Fig. 4A). Consistent with this augmented cGMP response, immunoreactive CNP persists for several days after the last injection in wild-type mice (Fig. 4B, right). However, the accumulated BMN 111 appears to be inactive because the cGMP response was reduced to background levels by 24 hours after administration (Fig. 4B, left). On the basis of the augmented activity response we observed after consecutive daily administrations (Fig. 4A), it is unlikely that the immunoreactive BMN 111 has caused receptor desensitization. Rather, it is more likely that BMN 111 has been inactivated through a proteolytic event. In agreement with these data, in vivo dose regimen studies in wild-type mice demonstrated that accelerated growth was observed only during the week when mice received daily dosing. Discontinuation of treatment at 1-week intervals resulted in a return to normal growth rate (Fig. 5).

CNP produces hemodynamic effects in mice (Lopez et al., 1997), nonhuman primates (Seymour et al., 1996), rats, dogs, and humans (Barr et al., 1996); therefore, we decided to examine

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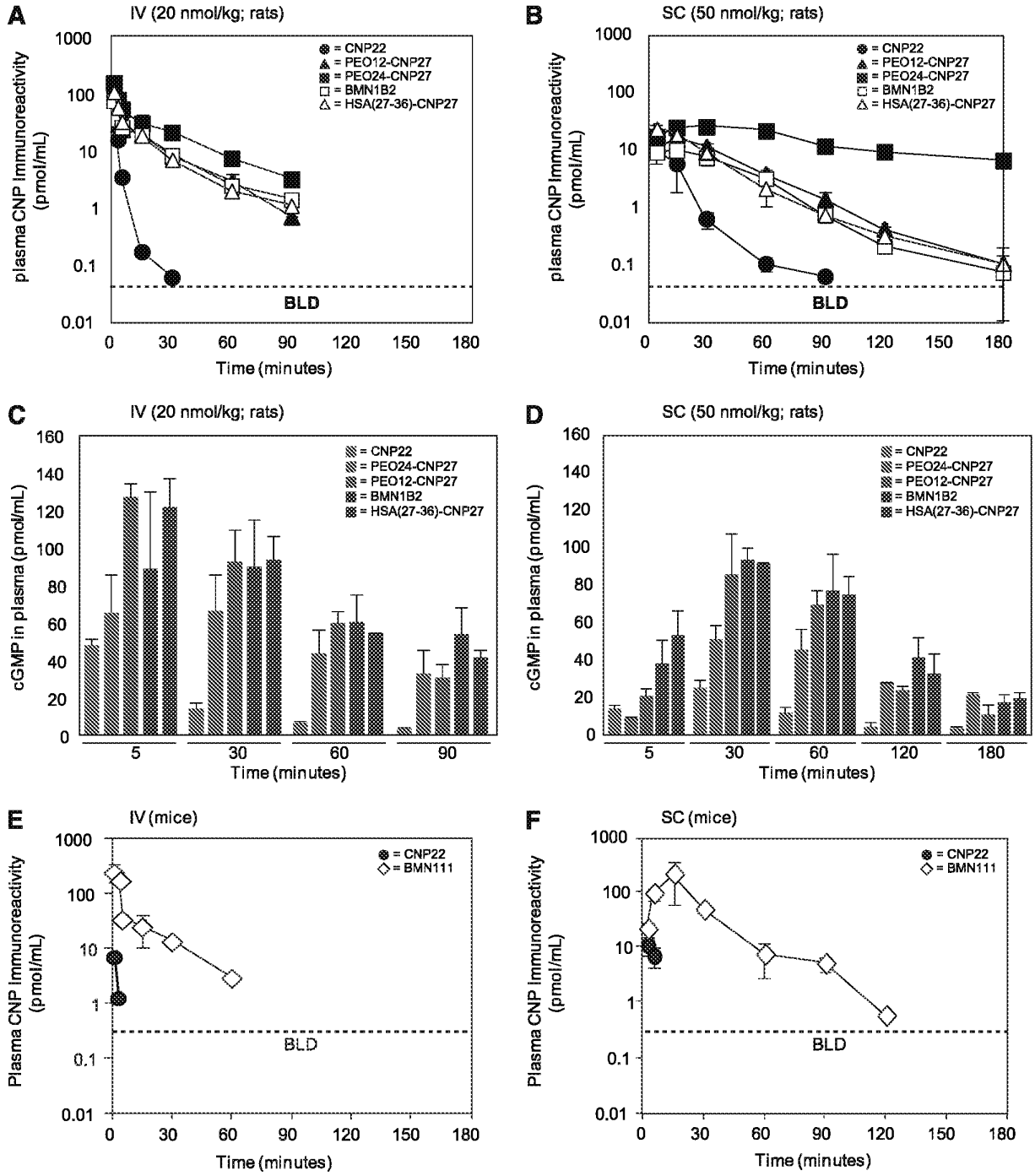


**Fig. 2.** PK and PD evaluation of NEP-resistant CNP variants. (A and B) Plasma CNP levels after a single intavenous (20 nmol/kg) or subcutaneous (50 nmol/kg) administration of CNP22 or variants in normal rats ($n$ = 3). CNP immunoreactivity was determined using an anti-CNP rabbit polyclonal antibody in a competitive radioimmunoassay. (C and D) Plasma cGMP concentration in response to CNP binding to NPR B. cGMP concentration was determined by radioimmunoassay ($n$ = 3). (E and F) Plasma CNP levels after a single intravenous (50 or 25 nmol/kg) or subcutaneous (130 or 70 nmol/kg) administration of CNP22 or BMN 111 in normal mice ($n$ = 4). Error bars indicate the S.D. Symbols indicate the following: CNP22 (solid circle), PEO24-CNP27 (solid square), PEO12-CNP27 (solid triangle), BMN1B2 (open square), BMN111 (open diamond), and $HSA_{(27-36)}$-CNP27 (open triangle). BLD, below limit of detection; IV, intravenous; SC, subcutaneous.

cardiovascular effects of the CNP variants (20–200 nmol/kg) in anesthetized wild-type FVB/nJ male mice fitted with a pressure-monitoring catheter connected to a telemetry transmitter. All variants showed similar BP-reducing and HR-increasing activity (Fig. 6). In most animals, effects were observed within 5 minutes of subcutaneous administration, with maximal drop in MAP occurring between 5 and 20 minutes postdose. This timing correlated well with the maximum concentration of the CNP

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TABLE 2
PK parameters of CNP variants in wild-type rats [Crj:CD (SD) IGS] and wild-type mice [FVB/nJ]
Data are expressed with the S.D. in parentheses if applicable.

| Group | Animal | Dose | Route | $C_{max}$ | $t_{max}$ | $t_{1/2}$ | Bioavailability |
|---|---|---|---|---|---|---|---|
| | | *nmol/kg* | | *pmol/ml* | *min* | *min* | % |
| CNP22 | Rat | 20 | i.v. | NA | NA | 1.4 (0.5) | NA |
| PEO24-CNP27 | Rat | 20 | i.v. | NA | NA | 22 (1.5) | NA |
| PEO12-CNP27 | Rat | 20 | i.v. | NA | NA | 17 (1.3) | NA |
| BMN 1B2 | Rat | 20 | i.v. | NA | NA | 23 (3.4) | NA |
| $HSA_{(27-36)}$-CNP27 | Rat | 20 | i.v. | NA | NA | 23 (1.1) | NA |
| CNP22 | Mice | 50 | i.v. | 7.3 (1.1) | 1 (0) | ≤2 | NA |
| BMN 111 | Mice | 25 | i.v. | 250 (86) | 1.5 (1) | 14 | NA |
| CNP22 | Rat | 50 | s.c. | 9.0 (3.7) | 5.0 (0.0) | 10 (5.0) | 19 (9.0) |
| PEO24-CNP27 | Rat | 50 | s.c. | 24 (1.9) | 25 (8.7) | 78 (16) | 60 (6.0) |
| PEO12-CNP27 | Rat | 50 | s.c. | 15 (1.8) | 12 (5.8) | 25 (4.4) | 24 (1.0) |
| BMN 1B2 | Rat | 50 | s.c. | 9.4 (2.2) | 12 (5.8) | 19 (4.3) | 19 (4.0) |
| $HSA_{(27-36)}$-CNP27 | Rat | 50 | s.c. | 22 (4.4) | 5.0 (0.0) | 25 (8.5) | 25 (3.0) |
| CNP22 | Mice | 130 | s.c. | 10 (3.2) | 2.8 (1.5) | ≤5 | 100 |
| BMN 111 | Mice | 70 | s.c. | 200 (140) | 13 (5) | 15 | 98 |

NA, not available.

variants, and demonstrated a clear PK/PD relationship for this physiologic response. Because the hemodynamic responses were similar between the doses and variants tested, cardiovascular activity was not a differentiating property and no further experiments or statistical analyses were performed.

BMN 111 demonstrated an increased pharmacological activity compared with the PEGylated and chimeric CNP variants in wild-type mice, whereas the transient hemodynamic response was very similar for the non-PEGylated CNP variants. Histomorphometric analysis of long bones showed no observable changes in trabecular and cortical architecture associated with the 5-week daily treatment of BMN 111 (data not shown), indicating that although longitudinal growth was stimulated, de novo bone formation was unaffected and normal. Based on potency and similarity to native sequence, BMN 111 was selected for studies in ACH mice and cynomolgus monkeys.

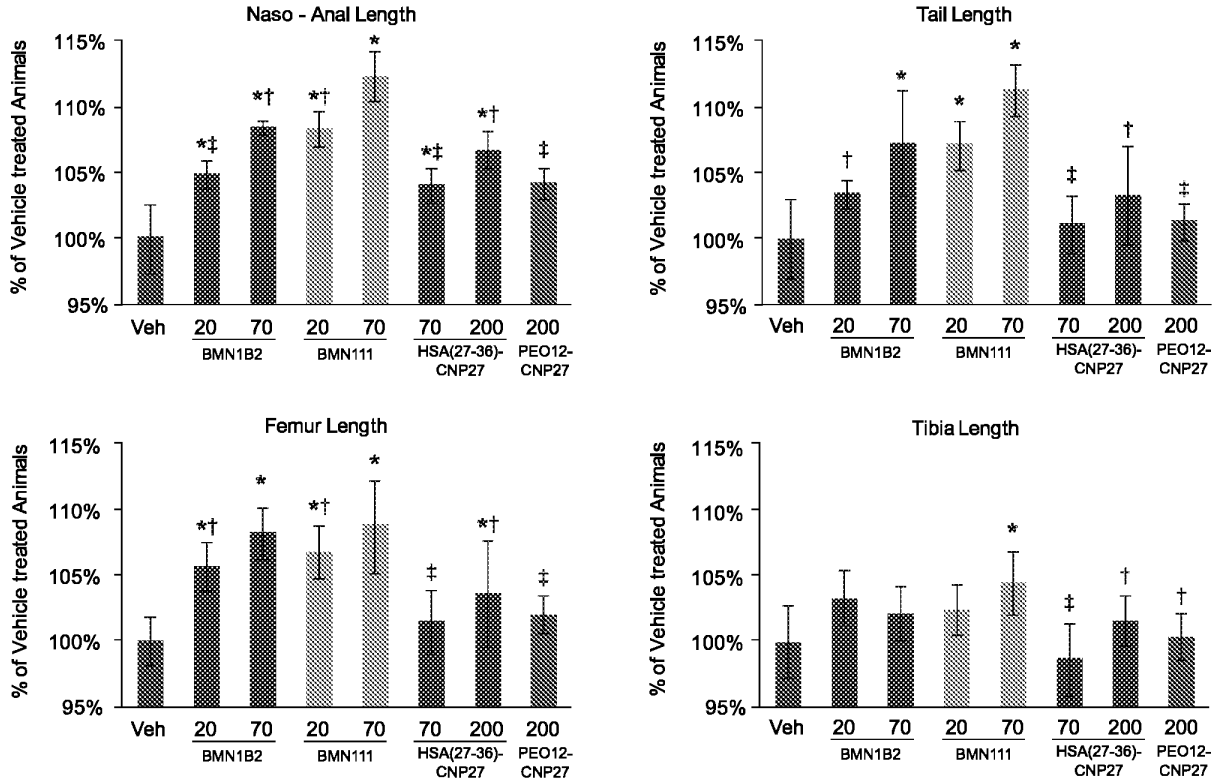


**Fig. 3.** Wild-type (FVB/nJ) mice treated with various NEP-resistant CNP variants. Growth of the appendicular and axial skeletons of wild-type mice (FVB/nJ) treated with CNP variants. Three-week-old wild-type mice were given daily subcutaneous administrations of CNP variants (20, 70 or 200 nmol/kg; $n$ = 8/group) or vehicle for 5-weeks. The asterisk denotes statistical significance compared with the vehicle control ($P < 0.05$; ANOVA with post hoc Dunnett's $t$ test). The dagger denotes significance compared with BMN 111 at 70 nmol/kg. The double dagger denotes significance compared with BMN 111 at both 20 and 70 nmol/kg (one-way ANOVA, post hoc Tukey's test). ANOVA, analysis of variance; Veh, vehicle. Error bars indicate the S.D.

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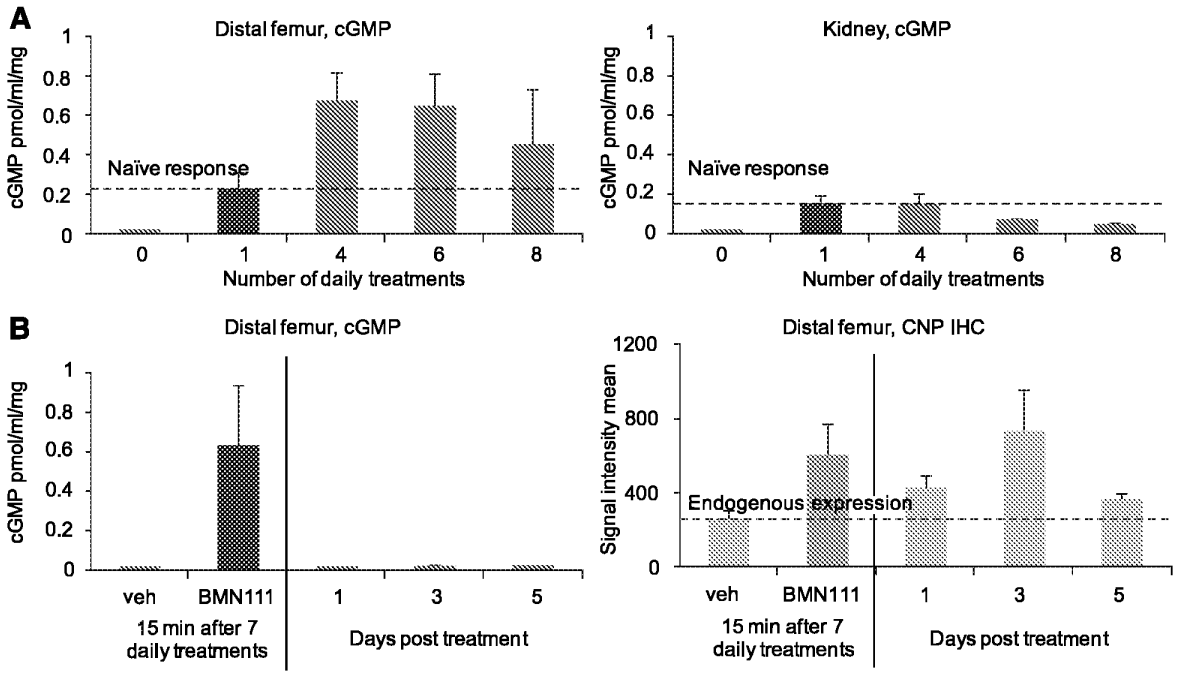


**Fig. 4.** Activity, accumulation, and clearance of NEP-resistant CNP variant, BMN 111, at the growth plate. (A) cGMP production during daily treatments. Wild-type CD1 mice were treated with 200 nmol/kg BMN 111 daily for as long as 8 days. Distal femura, containing the growth plate, and kidneys were dissected 15 minutes after the first, fourth, sixth, and eighth doses and cGMP was extracted and quantified ($n$ = 2). (B) BMN 111 residence and activity during after treatment withdrawal. Wild-type CD1 mice were treated daily with 200 nmol/kg BMN 111 for 7 days. Samples were obtained after treatment withdrawal. Distal femora, containing the growth plate, were dissected 15 minutes after the last treatment and 1, 3, and 5 days thereafter ($n$ = 2). Tissues were used for cGMP analysis or CNP IHC. Confocal microscopy allowed for detection of accumulated CNP signal in defined regions of interest of the growth plate. cGMP was quantified by a competitive enzyme-linked immunosorbent assay and normalized for tissue weight. veh, vehicle. Error bars indicate the S.D.

**Pharmacological Effects of BMN 111 in ACH Mice.** Targeted expression of an activated FGFR3 in the growth plate cartilage of mice was achieved using regulatory elements of the collagen 2 gene (Naski et al., 1998). Three-week-old

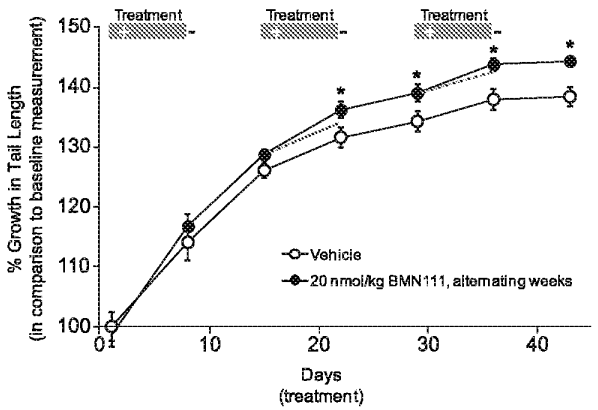


**Fig. 5.** Effect of discontinuous BMN 111 dose intervals on axial skeletal growth. Three-week-old wild-type (FVB/nJ) male mice were given subcutaneous injections of BMN 111 (20 nmol/kg) daily on alternating weeks (weeks 1, 3, and 5) or vehicle daily for 5 weeks. Tail measurements were collected at study initiation. A normal growth pattern resumes after discontinuation of treatment. Statistical significance (*$P$ < 0.05 versus vehicle) was noted for all end points beginning at day 22 through the end of the study (analysis of variance with post hoc Dunnett's test). The red dotted lines depict normal growth and were added to illustrate accelerated growth during the treatment period ($n$ = 10/group). Error bars indicate the S.D.

*Fgfr3*$^{ACH/+}$ male mice and their wild-type (FVB/nJ) littermates ($n$ = 8–10/group) were given daily subcutaneous injections of BMN 111 at 5, 20, or 70 nmol/kg (20, 80, or 280 $\mu$g/kg) or vehicle for 36 days. Significant growth in the appendicular and axial skeletons was observed in BMN 111–treated *Fgfr3*$^{ACH/+}$ mice (Fig. 7A). Although this *Fgfr3*$^{ACH/+}$ mouse model represents a mild phenotype, naso-anal and femur lengths of *Fgfr3*$^{ACH/+}$ mice were significantly shorter than wild-type mice at the start of the study ($P < 0.05$). Correction of the tail length was observed after 36 days of BMN 111 daily subcutaneous administrations at the 70 nmol/kg dose level. Naso-anal lengths were corrected at 20 nmol/kg after daily subcutaneous administration of BMN 111 for 36 days. Femur and tibia lengths were corrected at 5 and 20 nmol/kg by the end of the study. Histologic examination revealed a statistically significant increase in growth plate expansion in *Fgfr3*$^{ACH/+}$ mice treated with 70 nmol/kg BMN 111 (Fig. 7B), including increased area and/or height in the zones of resting cartilage, proliferation, and hypertrophy (data not shown). These data indicate that BMN 111 activation of NPR B corrects growth plate abnormalities secondary to the *Fgfr3* mutation that results in ACH dwarfism.

**Hemodynamic Effects of BMN 111 in Cynomolgus Monkeys.** After initial dose-ranging studies were performed in mice (Fig. 6), a pilot study was performed in normal juvenile, anesthetized or conscious, cynomolgus monkeys after a single subcutaneous administration (dose range, 0–35 nmol/kg), measuring acute cardiovascular effects of BMN 111, to determine the doses to be used in a long-term (6-month) study looking at growth and tolerability parameters (Fig. 8). The aim

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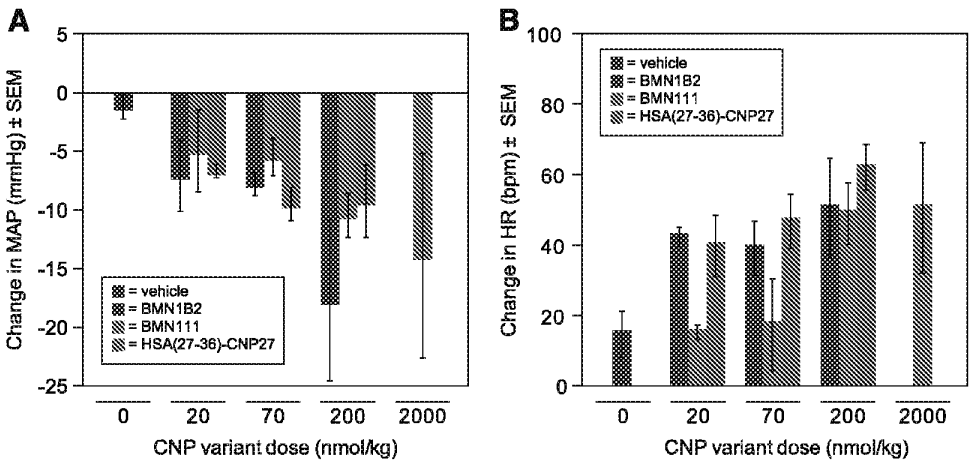


**Fig. 6.** (A and B) Change in MAP (A) and HR (B) in anesthetized mice treated with NEP-resistant CNP variants. CNP variants were tested over a dose range of 20–200 nmol/kg (2000 nmol/kg for BMN 111) in 6- to 7-week-old wild-type mice ($n$ = 3/group; vehicle $n$ = 5/group). The difference between mean values over the 15 minutes predose and 15 minutes postdose is shown; this encompassed the time of BP nadir and HR zenith. bpm, beats per minute.

was to find a tolerable dose that yielded a ≤10-mm Hg (approximately 10%) decrease in MAP or a ≤50-beats per minute increase (approximately 25%) in resting HR. It was observed that HR increase was the most sensitive parameter, probably due to reflex tachycardia, and a dose of 7 nmol/kg gave an approximately 25% increase in HR in conscious monkeys, with little or no effect on MAP (Fig. 8, B and D). The increase in HR was transient, with the maximal increase observed at 10–20 minutes postdose (Fig. 8, E and F). Multiple subcutaneous daily dosages (7 and 17.5 nmol/kg) for 7 consecutive days were well tolerated. ECG parameters were unaffected at any dose of BMN 111 tested (data not shown). The drop in MAP after BMN 111 administration was inconsistent and often somewhat less marked after subsequent doses (data not shown). On the basis of these data, the highest dose chosen for the long-term study was 8.25 nmol/kg. A lower dosage of 2.25 nmol/kg per day, which gave little or no cardiovascular effect, was also tested.

**Pharmacological Effects of BMN 111 in Cynomolgus Monkeys.** BMN 111 was administered subcutaneously to growing (2- to 4-year-old) cynomolgus male monkeys at 2.25 or 8.25 nmol/kg once daily for 6 months ($n$ = 4/group). Although BP was not monitored, no clinical signs of hypotension or distress were noted in any animal at any time during the study. The effect on proximal tibial growth plate size was observed by MRI imaging performed during the fourth week of dosing (Fig. 9A). Mean growth plate volume increased approximately 40% for the high-dose group versus the pretreatment volume. This was the peak growth plate volume noted. Volume receded thereafter, but remained greater than baseline throughout the remainder of the 6-month study. Treatment with BMN 111 resulted in a dose-dependent increase in total tibial length (measured from digital radiographs) and rate of growth (Fig. 9b) as well as increased lengths of arms, legs, and tail when measured externally (data not shown). Treated animals maintained their height/length advantage through the end of the study period. Clinical chemistry and hematology parameters remained normal and unchanged throughout the 6-month study with the noted exception of increased serum levels of total and bone-specific alkaline phosphatase associated with the increase in bone formation (Fig. 9C).

Growth plate expansion, evaluated post mortem after 6 months of treatment, was evident at the histologic level (Fig. 10B, upper), with significant expansions in total growth plate thickness, proliferating zone thickness, and hypertrophic zone thickness, changes that are associated with inhibition of FGFR3 signaling (Iwata et al., 2000; Ornitz and Marie, 2002) (Table 3). Similar histologic and growth plate expansion results were observed in wild-type and $Fgfr3^{ACH/+}$ murine studies (Fig. 10A; Table 3). Double fluorochrome labeling of newly formed mineralized bone performed during the final 14 days of the in vivo study illustrated that growth plate expansion in response to 8.25 nmol/kg per day BMN 111 translated into increased longitudinal growth of mineralized bone (Fig. 10B, lower panel). Static and dynamic measurements of trabecular bone architecture and turnover were not affected by BMN 111 treatment, indicating that normal bone was formed (Table 4).

To assess the effects of BMN 111 treatment on vertebral foramen area, post mortem microcomputed tomography was performed on excised lumbar vertebrae 2–4. For the high-dose group (8.25 nmol/kg per day), mean vertebral foramen area increased approximately 10%–17% going up the spine (L4 to L2) versus the vehicle control group, and was statistically significant in L2 ($P$ = 0.03 versus vehicle by two-tailed $t$ test) (Fig. 9D).

## Discussion

In ACH, mutations in FGFR3 result in constitutive activation, suppressing the proliferation and differentiation of chondrocytes resulting in improper cartilage to bone conversion in the growth plate (Laederich and Horton, 2010). ACH is associated with significant morbidity and increased mortality, and current treatments are mostly surgical (Trotter and Hall, 2005; Wynn et al., 2007). BMN 111, a CNP variant, offers a potential treatment for ACH that addresses the underlying biochemical defect. By signaling through NPR B, BMN 111 suppresses downstream signals in normal and mutated FGFR3 pathways to enhance or restore chondrocyte proliferation and differentiation resulting in bone growth. Specifically, BMN 111 inhibits the ERK/MAPK pathway through phosphorylation of Raf-1 by cGMP-dependent protein kinase 2 (Krejci et al., 2005).

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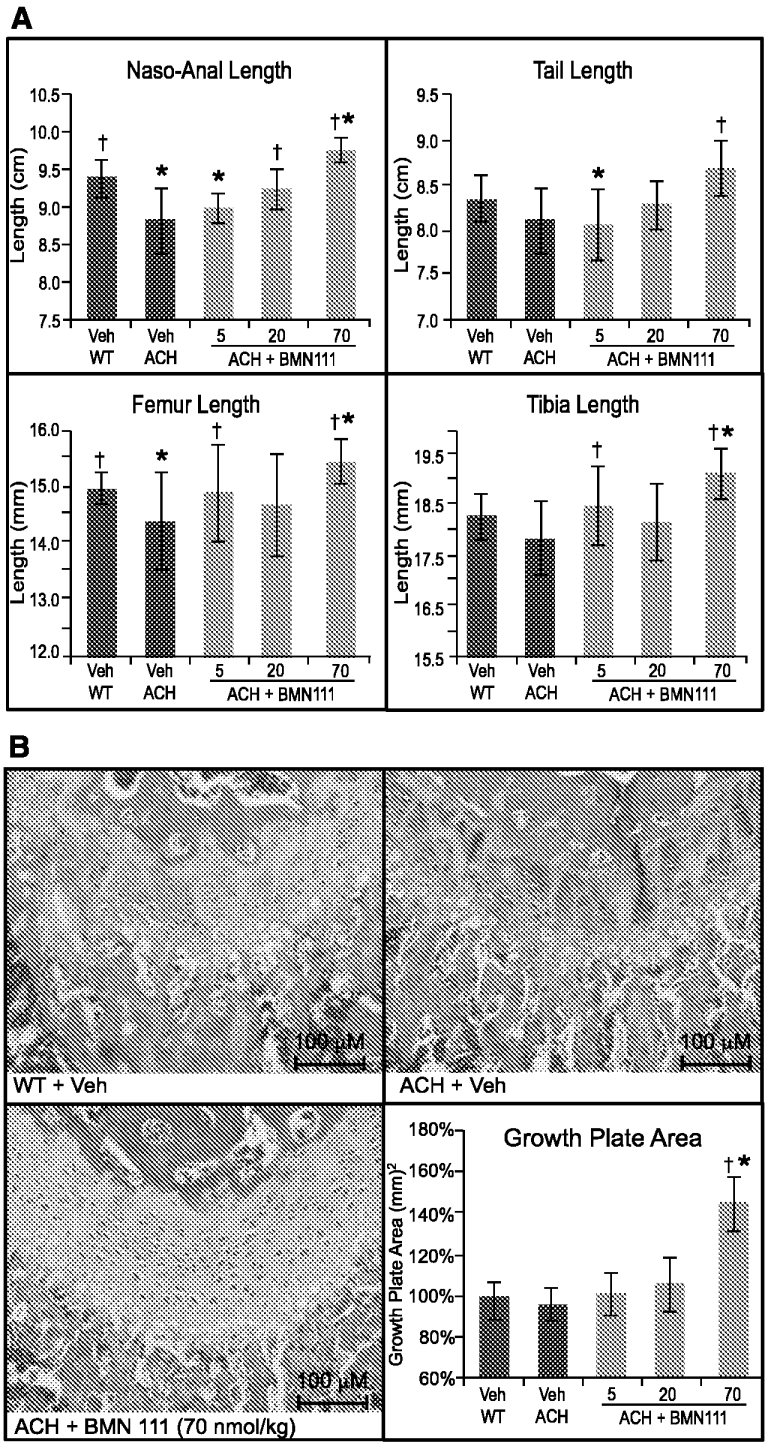


**Fig. 7.** $Fgfr3^{ACH/+}$ mice treated with BMN 111. (A) Growth of the appendicular and axial skeletons of $Fgfr3^{ACH/+}$ mice after treatment with BMN 111. Three-week-old $Fgfr3^{ACH/+}$ mice given daily subcutaneous administrations of BMN 111 (5, 20, or 70 nmol/kg) or vehicle for 5 weeks ($n$ = 8/group). Wild-type vehicle controlled mice (FVB/nJ) were included to assess the degree of phenotype and normalization for each growth parameter ($n$ = 8). The asterisk denotes statistical significance ($P < 0.05$) against vehicle-treated wild-type mice. The dagger denotes statistical significance against vehicle-treated $Fgfr3^{ACH/+}$ mice (analysis of variance with post hoc Dunnett's $t$ test). (B) Distal femoral growth plates of mice treated with vehicle or BMN 111 (trichrome stained). Significant growth plate expansion was observed in $Fgfr3^{ACH/+}$ mice treated with 70 nmol/kg BMN 111. Error bars indicate the S.D. Original magnification, 10× in B. Veh, vehicle; WT, wild type.

Because CNP is rapidly cleared from the circulation through receptor-mediated (NPR C) and proteolytic (NEP) pathways (Potter, 2011), CNP requires continuous infusion to be effective in ACH murine studies (Yasoda et al., 2009); however, this is not a desired therapy by physicians or patients. To overcome these limitations, we developed a CNP variant, BMN 111, which resists degradation by NEP at the site of subcutaneous administration and at the growth plate (Ruchon et al., 2000; Yamashita et al., 2000; Nakajima et al., 2012). Here, we demonstrate that BMN 111 is effective as a subcutaneous injectable therapeutic that promotes bone growth in juvenile wild-type mice and juvenile cynomolgus monkeys and corrects the ACH phenotype in $Fgfr3^{ACH/+}$ mice.

NEP prefers small peptides based on physiologic substrate and crystal structure data. Larger CNP variants (>3 kDa) demonstrated in vitro NEP resistance and a subset retained

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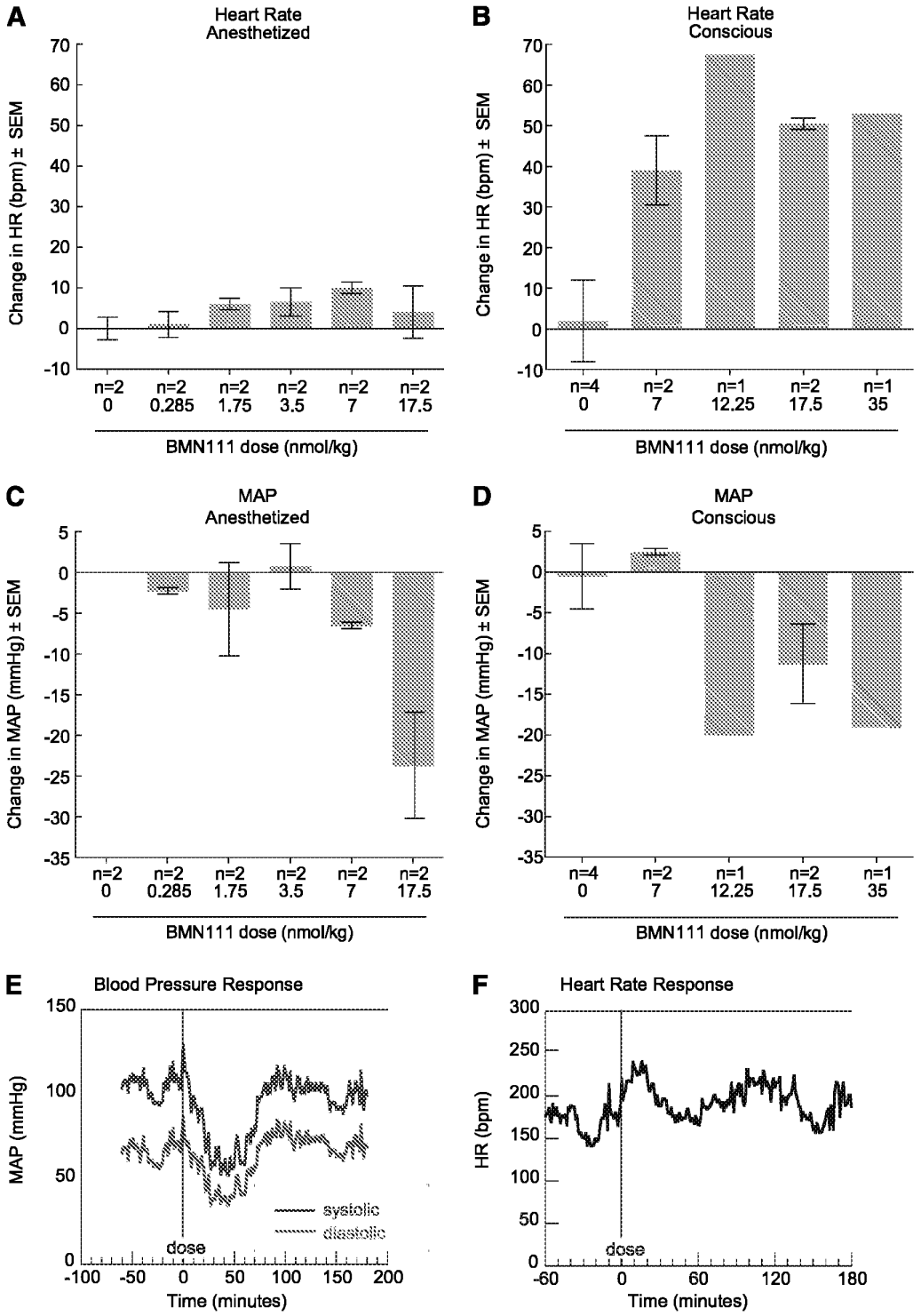


**Fig. 8.** Effect of BMN 111 on BP and HR in cynomolgus monkeys. (A–D) In both anesthetized (A and C) and conscious monkeys (B and D), BMN 111 decreased MAP in a dose-dependent manner ($n$ = 1–4/group). In conscious animals, there was a concomitant increase in HR. The HR response was blunted in the anesthetized animals. (A and B) Change in average HR over 10–20 minutes postdose (encompassing time of HR zenith) and baseline (15 minutes just prior to dosing). (C and D) Change in average MAP over 10–20 minutes postdose (encompassing time of MAP nadir) and baseline average (15 minutes just prior to dosing). (E and F) BP (E) and HR (F) after a single subcutaneous dose of BMN111 (17.5 nmol/kg) to a conscious monkey. Significant hypotension develops rapidly after administration, but begins to resolve within an hour. bpm, beats per minute.

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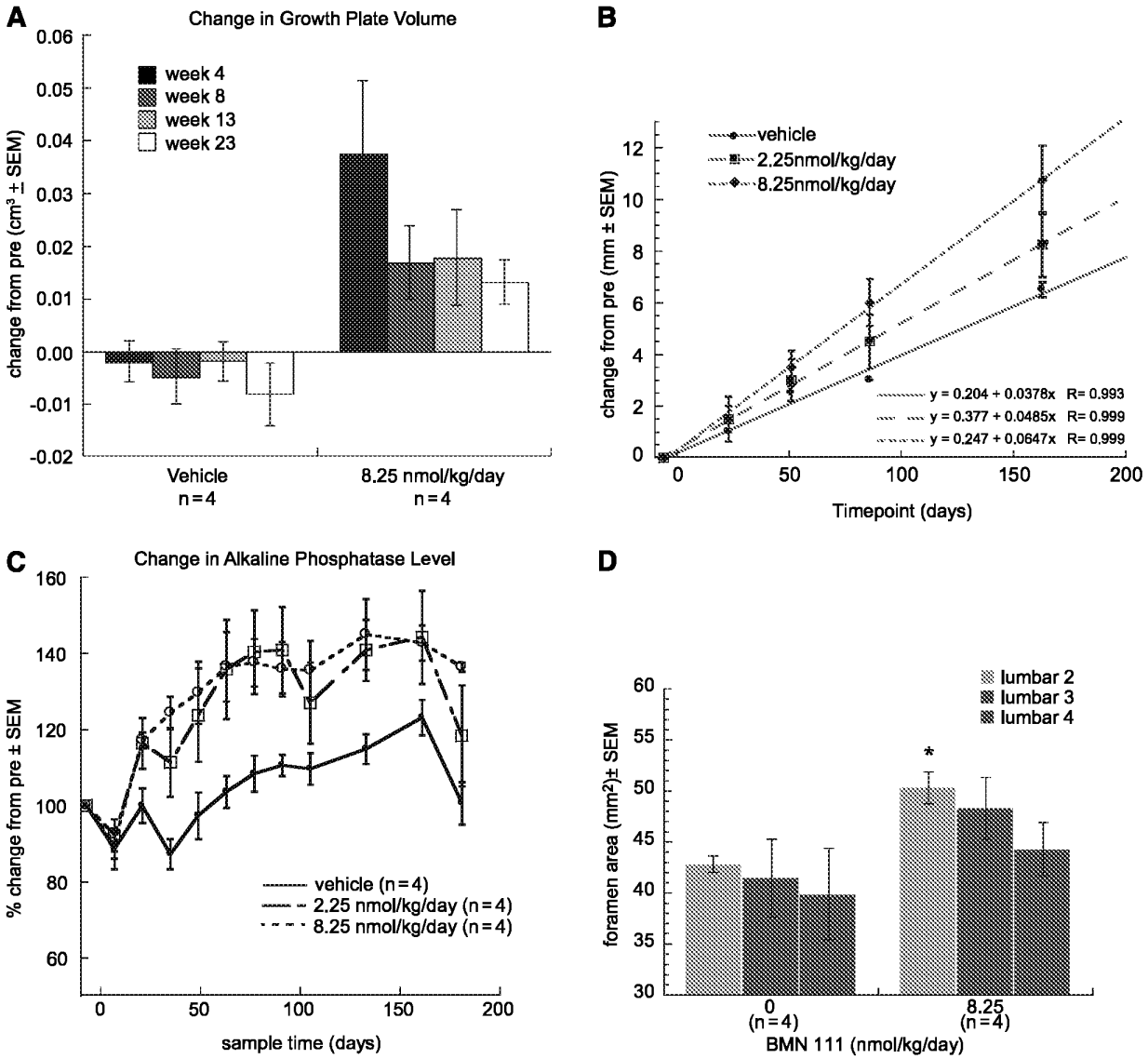


**Fig. 9.** Change in growth plate volume, tibial length, serum alkaline phosphatase levels, and lumbar vertebral foramen in cynomolgus monkeys treated with BMN 111. (A) Change in right proximal tibial growth plate volume with high-dose BMN 111 treatment, measured by MRI ($P$ = N.S. versus vehicle at all time points; $n$ = 4/group). (B) Radiographic evaluation of cynomolgus tibias at several time points in animals treated with BMN 111. Dose-dependent change in rate of growth of tibial length. Right tibial lengths (in millimeters) were measured manually on posterior–anterior projections with dedicated image analysis software ($P$ = N.S. versus vehicle at all time points ($n$ = 4/group). (C) Increase in serum alkaline phosphatase with BMN 111 treatment. Known as markers of bone growth or deposition, changes in both total and bone-specific alkaline phosphatase (data not shown) were not statistically significant over prestudy values ($n$ = 4/group). (D) Area of lumbar vertebral foramen of cynomolgus monkey assessed by microcomputed tomography. In vertebrae L2, L3, and L4, treatment with BMN111 at the high dose resulted in a trend toward greater area of vertebral foramen compared with vehicle controls. For L2, the increase was statistically significant (*$P$ = 0.03 versus vehicle; $n$ = 4/group). N.S., not significant.

native CNP in vitro activity. NEP resistance translated into improved serum half-lives in wild-type rat or murine studies ($t_{1/2}$ = approximately 15 minutes for CNP variants versus 2–5 minutes for native CNP); however, the improved in vivo stability does not exclude the possibility that CNP variants are susceptible to other proteolytic pathways in addition to the known NPR clearance pathway (NPR C) present in the vasculature. BMN 111 was selected for ACH murine studies and larger animal studies based on its superior bone growth-promoting attributes in the wild-type murine studies. The lowest dosage tested in the wild-type murine screening study was 20 nmol/kg per day and this appeared to be well above the minimal effective dose. This dose also appeared to correct most growth deficits in the *Fgfr3*$^{ACH/+}$ mouse model. Importantly, we recently reported that BMN 111 stimulated bone growth in mouse models containing a stronger activating mutation of *Fgfr3* (*Fgfr3*$^{Y367C/+}$), a mutation that results in thanatophoric dysplasia type I (TDI) in humans (Lorget et al., 2012)

To test its effectiveness in larger animals, levels that had minimal effects on hemodynamic parameters were chosen and three cohorts of cynomolgus monkeys were dosed. Dose-dependent growth was observed in this 6-month study. The high-dose group showed measurable increases in growth plate expansion, rate of endochondral bone growth, and trends in

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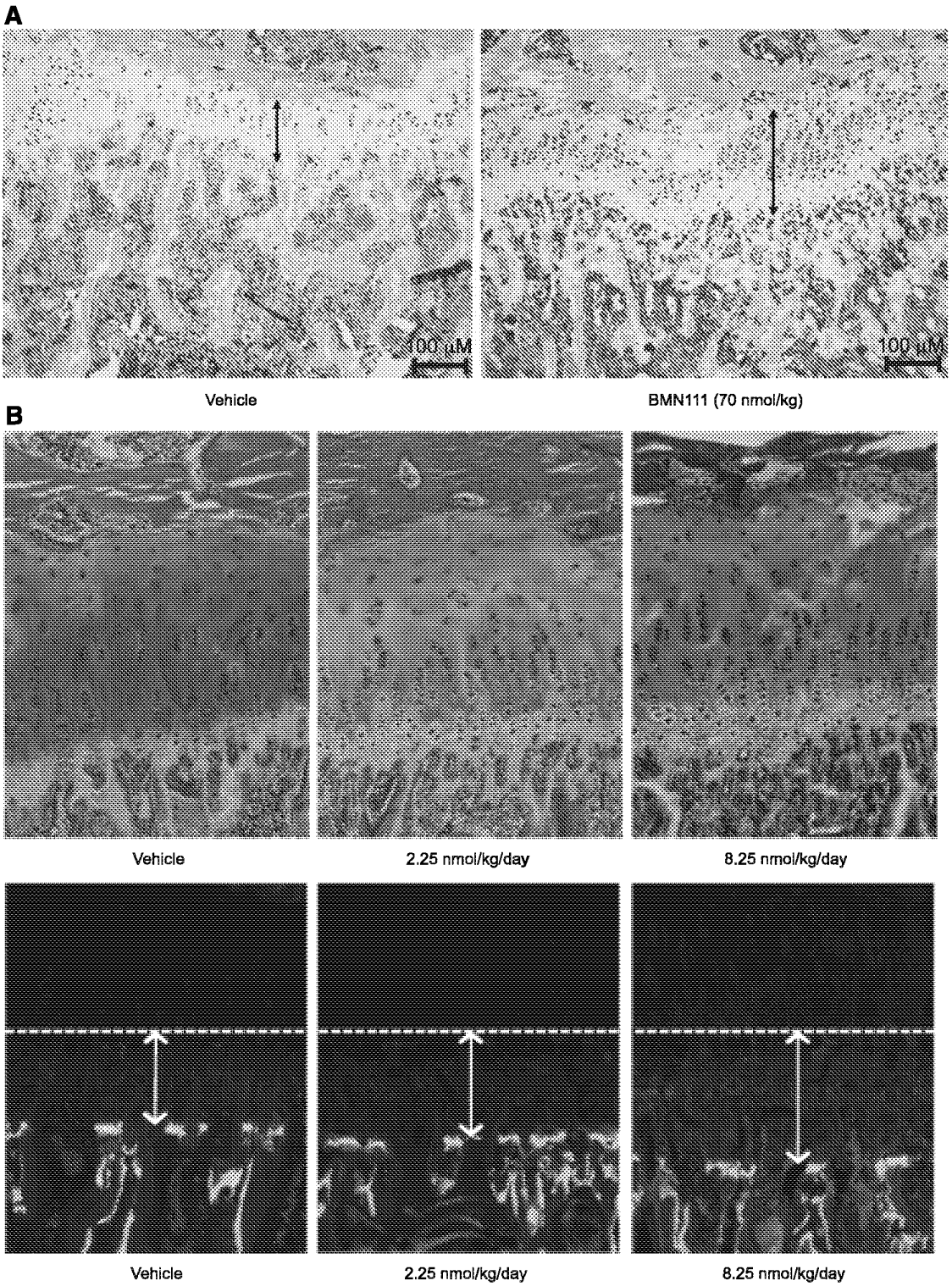


**Fig. 10.** Cynomolgus monkey and wild-type mice growth plate histology after 6 months of treatment with BMN 111. (A) Distal femoral growth plates of mice treated with vehicle or BMN 111 (trichrome stained). Growth plate expansion was observed in mice treated with 20 and 70 nmol/kg BMN 111 (showing 70 nmol/kg). (B) The upper panel shows Goldner trichrome staining of growth plate (purple) and bone (green). The lower panel is the calcein label under UV (green) showing longitudinal growth rate in the last 14 days of treatment. The distal edge of growth plate is delineated with a dashed line, and longitudinal bone growth in 14 days prior to necropsy is represented with arrows ($n$ = 4/group, showing one representative image from each group). Original magnification, 10× in A.

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TABLE 3
Growth plate parameters and longitudinal growth rates
Data are presented as the mean ± S.D. with $P$ values in parentheses. $P$ values for cynomolgus monkeys were obtained using analysis of variance with Tukey post hoc analysis versus vehicle, and $P$ values for wild-type mice and *Fgfr3*$^{Ach/+}$ mice were obtained using analysis of variance with post hoc Dunnett's test.

| Parameter | Cynomolgus Monkey | | | Wild-Type Mice | | | *Fgfr3*$^{Ach/+}$ Mice | | |
|---|---|---|---|---|---|---|---|---|---|
| | Vehicle ($n$ = 4) | 2.25 nmol/kg per day ($n$ = 4) | 8.25 nmol/kg per day ($n$ = 4) | Vehicle ($n$ = 10) | 70 nmol/kg per day ($n$ = 10) | Vehicle ($n$ = 8) | 5 nmol/kg per day ($n$ = 5) | 20 nmol/kg per day ($n$ = 5) | 70 nmol/kg per day ($n$ = 4) |
| Longitudinal growth rate ($\mu$m/day) | 26 ± 7 | 26 ± 5 (N.S.) | 40 ± 9 (<0.05) | | | | | | |
| Growth plate thickness ($\mu$m) | 555 ± 61 | 594 ± 64 (N.S.) | 682 ± 48 (<0.05) | 159.4 ± 17.0 | 200.7 ± 14.4 (<0.001) | | 134.0 ± 22.5 (N.S.) | 142.2 ± 20.2 (N.S.) | 163.2 ± 24 |
| *Fgfr3*$^{Ach/+}$ mice | | | | | | 137.3 ± 12.7 | | | (N.S.) |
| Wild-type mice | | | | | | 125.2 ± 15.1 | | | (<0.05) |
| Proliferating zone thickness ($\mu$m) | 125 ± 10 | 139 ± 89 (N.S.) | 196 ± 14 (<0.001) | | | | | | |
| Proliferating cells/column ($n$) | 13 ± 2 | 11 ± 2 (N.S.) | 11 ± 1.6 (N.S.) | | | | | | |
| Hypertrophic zone thickness ($\mu$m) | 72 ± 26 | 89 ± 23 (N.S.) | 128 ± 56 (<0.05) | | | | | | |
| Hypertrophic cell volume ($\mu$m$^2$) | 232 ± 30 | 258 ± 56 (N.S.) | 286 ± 34 (N.S.) | | | | | | |

expansion of the vertebral foramen. Although this study was not powered for significance, some statistically significant trends were observed; for example, growth plate thickness in the high-dose group, particularly in the proliferating and hypertrophic zones, was statistically larger than in vehicle-treated animals ($P < 0.001$ and $P < 0.05$, respectively), which is consistent with observations that suggest that FGFR3 inhibits both the proliferation and terminal differentiation of growth plate chondrocytes and the synthesis of extracellular matrix by these cells (Laederich and Horton, 2010). Double fluorochrome labeling of newly formed mineralized bone demonstrated that bone formation was increased in the high-dose group in accordance with the increased endochondral activity that caused growth plate expansion. Moreover, the achievement of this bone growth in the last 14 days of the study demonstrated the continued effectiveness of BMN 111 after chronic treatment, and suggested that the growth plate width, which receded after 4 weeks of treatment, was not associated with a reduction of BMN 111 activity. BMN 111–treated animals showed equivalent trabecular architecture parameters compared with vehicle-treated animals, suggesting that BMN 111 treatment did not significantly affect osteoclast activity, if at all.

Several other groups have reported potential therapeutic strategies that modulate the aberrant FGFR3 pathway. Garcia et al., 2013 demonstrated that a soluble fibroblast growth factor receptor-3 (sFGFR3) could act as a decoy receptor to prevent fibroblast growth factor (FGF) from binding to and signaling through the FGFR3. In vitro binding studies with fixed concentrations of FGF2, FGF9, and FGF18 demonstrated that sFGFR3 was required in 100-fold excess to reduce the concentration of these FGFs by one-half. Nevertheless, they were able to show stimulation of bone growth in wild-type and *Fgfr3*$^{ACH/+}$ murine studies. The long-term effects of continuous FGF depletion remain to be determined, but would be expected to impair wound repair and other developmental processes (Lynch et al., 1989; Kurtz et al., 2004). One question that comes to mind with this therapeutic strategy is whether sufficient amounts of this approximately 70-kDa sFGFR3 protein could diffuse through the highly negatively charged extracellular matrix of a larger human growth plate to compete for FGFs expressed locally as paracrine factors. Moreover, there is still no scientific consensus that FGF receptors require ligand for dimerization (He et al., 2011; Placone and Hristova, 2012).

In another report, Jin et al. (2012) discovered a 12–amino acid peptide through phage display, P3, which could bind to the extracellular domain of FGFR3 and partially block FGF2-mediated ERK1/2 phosphorylation. When pregnant *Fgfr3*$^{Neo\text{-}K644E/+}$ mice [phenotypically normal thanatophoric dysplasia type II (TDII) carriers] were given daily peritoneal injections of P3 (100 $\mu$g/kg body weight) at E16.5 until birth, all TDII pups (*Fgfr3*$^{K644E/+}$) survived, whereas all vehicle control TDII pups died. The TDII mice that survived had increased thoracic cavities, which rescued the postnatal lethality phenotype; however, the rescued mice still had smaller bodies and dome-shaped skulls compared with their wild-type littermates. P3 as a postnatal therapy for ACH, perhaps a more acceptable therapeutic regimen, was not tested in this study.

Matsushita et al. (2013) identified meclozine, an antihistamine used for motion sickness, as an antagonist of the

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TABLE 4

Trabecular architecture parameters: histomorphometric analysis of the left proximal tibial trabecular bone of cynomolgus monkeys treated with BMN 111 or vehicle

Data are presented as the mean ± S.D. ($n$ = 4). There were no significant differences (analysis of variance) between groups for all parameters.

| Parameter | Vehicle | 2.25 nmol/kg per day | 8.25 nmol/kg per day |
|---|---|---|---|
| Bone volume/tissue volume (%) | 22 ± 5 | 27 ± 7 | 29 ± 6 |
| Osteoid/bone surface (%) | 33 ± 9 | 33 ± 6 | 33 ± 9 |
| Trabecular thickness ($\mu$m) | 133 ± 17 | 158 ± 24 | 132 ± 11 |
| Trabecular number ($mm^{-1}$) | 1.6 ± 0.4 | 1.7 ± 0.3 | 2.2 ± 0.3 |
| Trabecular spacing ($\mu$m) | 501 ± 137 | 452 ± 124 | 339 ± 83 |
| Osteoblasts/bone surface ($n$) | 22 ± 2.4 | 23 ± 4 | 25 ± 3.8 |
| Osteoclasts/bone surface ($n$) | 1.8 ± 0.5 | 1.4 ± 0.5 | 1.3 ± 0.7 |
| Osteoid thickness ($\mu$m) | 8.2 ± 2.1 | 9 ± 1.3 | 9.4 ± 1.4 |
| Mineral apposition rate per day ($\mu$m/day) | 1.6 ± 0.1 | 1.9 ± 0.3 | 1.7 ± 0.4 |
| Bone formation rate/bone volume | 0.013 ± 0.002 | 0.015 ± 0.003 | 0.011 ± 0.002 |

FGFR3 pathway. They demonstrated that meclozine was able to attenuate FGF2-mediated ERK phosphorylation in rat chondrosarcoma cells, facilitate chondrocytic differentiation of ATDC5 cells expressing ACH or TDII mutant FGFR3, and promote tibial growth in FGF2-suppressed tissue explant studies. In explant studies, they compared CNP (0.2 $\mu$M) to meclozine (20 $\mu$M). Interestingly, meclozine demonstrated no statistically significant enhancement of tibial growth in the absence of FGF2, unlike CNP (Yasoda et al., 1998). Furthermore, meclozine was not tested in any of the available in vivo murine models for its ability to stimulate or correct growth. Thus, questions remain as to whether this is a viable therapeutic option.

In a recent article, Yamashita et al. (2014) demonstrated that statins could correct the degraded cartilage in both chondrogenically differentiated TDI and ACH induced pluripotent stem cells. Interestingly, mRNA expression levels of FGFR3 were increased by lovastatin, but protein levels by immunoblot decreased, which led the authors to postulate that statins increase the degradation rate of FGFR3 in chondrogenically differentiated TDI induced pluripotent stem cells. In an 11-day ACH murine study (days 3–14), mice receiving daily injections of rosuvastatin demonstrated an increase in distal long-bone growth rate comparable to wild-type mice receiving vehicle. The effect beyond 14 days on final growth (6–8 weeks) was not assessed in this study. The mechanism is unclear but could be due to altering membrane dynamics, which may not be a good strategy given the frequency of known side effects of statins as well as the potential developmental consequences (Evans and Rees, 2002; Maji et al., 2013).

We believe that BMN 111 is a promising therapeutic option for children with ACH with open growth plates for a number of reasons. First, BMN 111, an NEP-resistant CNP variant, is a natural antagonist of the FGFR3 pathway, corrects the phenotype in *Fgfr3*$^{ACH/+}$ mice, and attenuates the phenotype in stronger activating mutations of FGFR3 (TDI; Y367C/+) when given daily subcutaneously (Lorget et al., 2012). Second, CNP and its receptor are expressed in the growth plate. Third, the amino acid content is basic (pI = approximately 10) and the peptide is small, which enable subcutaneous administered BMN 111 to target and diffuse through the anionic extracellular matrix barrier of the growth plate. Finally, unlike other small molecule strategies, BMN 111 will only target cells that express its cognate receptor, NPR B, which should mitigate many of the side effects seen with these other approaches. It should be noted here that NPR B is not limited to the growth plate, but humans lacking NPR B have a dwarfism without any other apparent disease (Bartels et al., 2004). Overactive NPR B produces tall stature, scoliosis, and great toe macrodactyly, but apparently nothing else (Toydemir et al., 2006).

An additional unique feature of CNP is that it increases proteoglycan synthesis independent of the FGF receptor/ERK pathway (Krejci et al., 2005; Waldman et al., 2008), which may be partly responsible for the anabolic bone growth effects observed in wild-type mice and normal cynomolgus monkeys. Recent evidence suggests that agonists of the decoy receptor NPR C, such as CNP, may also be contributing to these anabolic effects (Peake et al., 2013). On the basis of these findings and our data in wild-type mice and normal cynomolgus monkeys, it is conceivable that BMN 111 could be used to treat other FGFR3-related skeletal dysplasias, such as hypochondroplasia, and perhaps idiopathic short stature, in which no clear causal mechanism has been ascribed. BMN 111 is currently being investigated in children with ACH (ClinicalTrials.gov identifier NCT02055157).

In conclusion, through a series of in vitro and in vivo rodent studies, we identified five CNP variants comprising three types (PEGylated, chimeric, and natural amino acid extensions) that were resistant to NEP by virtue of size, retained native in vitro potency, and demonstrated prolonged half-lives in rats and mice. One CNP variant, BMN 111, was selected for further study based on potency and similarity to native CNP. When administered subcutaneously to normal mice, normal growing monkeys, or ACH mice, BMN 111 treatment resulted in growth of the axial and appendicular skeletons. In the 6-month daily dose study in juvenile monkeys, BMN 111 (administered at doses that did not cause an unacceptable hemodynamic effect) resulted in expansion of the proximal tibial growth plates, with widening of the hypertrophic zone, increased length and rate of limb growth, and increased area of the foramen of lumbar vertebrae. Concomitant increase in both total and bone-specific alkaline phosphatase levels may provide a biomarker of early BMN 111 activity. Transient, asymptomatic dose-dependent hemodynamic responses were observed in mice and monkeys at doses higher than needed to produce skeletal growth. These experiments indicate that growth in both normal and ACH juvenile animals is governed, at least in part, through the NPR B cGMP signaling pathway, and that BMN 111 affects this pathway. BMN 111 is being investigated as a potential therapeutic for pediatric patients with ACH.

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### Authorship Contributions

*Participated in research design:* Wendt, Dvorak-Ewell, Bullens, Lorget, Bell, Castillo, Aoyagi-Scharber, Krejci, Wilcox, Rimoin, Bunting.

*Conducted experiments:* Wendt, Dvorak-Ewell, Bullens, Lorget, Bell, Castillo, Aoyagi-Scharber, Krejci, Bunting.

*Performed data analysis:* Wendt, Dvorak-Ewell, Bullens, Lorget, Bell, Peng, Castillo, Aoyagi-Scharber, O'Neill, Krejci, Wilcox, Bunting.

*Wrote or contributed to the writing of the manuscript:* Wendt, Dvorak-Ewell, Bullens, Bell.

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**Address correspondence to:** Daniel J. Wendt, Department of Analytical Chemistry, BioMarin Pharmaceutical Inc., 105 Digital Drive, Novato, CA 94949. E-mail: dwendt@bmrn.com

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

Application No.: 15/646,822 Confirmation No.: 7727

Filed: July 11, 2017 Art Unit: 3991

For: Variants of C-Type Natriuretic Peptide Examiner: Bruce Campell

**DECLARATION UNDER §1.132 OF DANIEL WENDT**

I, Daniel J. Wendt, hereby state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced application (hereinafter, "the patent application"). I was involved in the research team at BioMarin that identified the claimed subject matter. A copy of my curriculum vitae is attached as Exhibit 1.

2. I make this declaration to provide facts known to me concerning subject matter in the patent application and a related patent, US 8,377,884, bearing a publication date of February 19, 2013 (hereinafter "Wendt"). I am an inventor on the Wendt patent along with co-inventors Mika Aoyagi-Scharber, Sean Bell, Dong Wei, Joshua R. Bliesath, and Emil D. Kakkis. I am familiar with the contributions made by myself and the co-inventors of Wendt including the data and ideas and work described in Wendt.

3. I understand that Wendt was cited by the U.S. Patent and Trademark Office in an Office Action dated November 26, 2019 as prior art that may be relevant to the patentability of the invention claimed in the patent application. For example, the patent examiner cites disclosure in the Wendt publication relating to variants of C-type natriuretic (CNP) peptides that could be used to treat skeletal dysplasias, such as achondroplasia. To the extent that the experimental work described in Wendt discloses or suggests the CNP variants recited in the pending claims of the patent application, the relevant disclosure in the patents is disclosure(s) of the invention of myself and the research group under the direction of BioMarin Pharmaceuticals Inc. and the invention is subject to assignment to BioMarin Pharmaceutical Inc.

4. The experimental work described in the present application was also conducted by myself and others in the research group at BioMarin Pharmaceutical and was conceived, designed, and performed under the direction of BioMarin Pharmaceuticals Inc. and subject to assignment to BioMarin Pharmaceutical Inc.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date 26 Mai 2020

Daniel J. Wendt

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:
Daniel Wendt et al.

Application No.: 15/646,822 | Confirmation No.: 7727

Filed: July 11,2017 | Art Unit: 3991

For: Variants of C-Type Natriuretic Peptide | Examiner: Bruce Campell

**DECLARATION UNDER 37 C.F.R. § 1.132**

I, Daniel J. Wendt, do hereby declare and state as follows:

1. I am a co-inventor of the subject matter described and presently claimed in the above-identified patent application. A copy of my curriculum vitae is attached as Exhibit 1.

2. I have been employed at BioMarin Pharmaceuticals Inc. for 21 years, and have held the position of Principal Scientist from 2012 to 2020. I was involved in the research team at BioMarin that identified the claimed subject matter. I was the CMC lead responsible writing up the characterization section in our successful IND application for treatment of achondroplasia with CNP.

3. I have read and am familiar with the above-mentioned application as filed in the U.S. Patent and Trademark Office ("USPTO") on July 11, 2017, the Office Action mailed by the USPTO on November 26, 2019, the cited publications, and the pending claims of the application.

4. I understand that the Examiner believes that the CNP variants recited in the claims would be obvious in view of cited publications Nakao '425, Nakao '434, Golembo, Potter, James and others cited in the Action.

5. C-type natriuretic peptide (CNP) is a recombinant human peptide under development as treatment for skeletal dysplasias, such as achondroplasia. Achondroplasia is

an autosomal recessive disorder characterized by a mutation in the FGFR3 gene. CNP is translated as a 126 amino acid peptide that is cleaved to two natural forms, CNP22 and CNP53. Under normal conditions, CNP22 is quickly metabolized from the system. See application page 2, lines 3-26, and page 3, lines 31-32.

6. Prior to the present application, it was disclosed in Nakao and Golembo that certain forms of achondroplasia were caused by a mutation in the FGFR3 gene. The application explains that CNP/NPR-B signaling play a part in the FGFR3 signaling pathway to promote bone growth (specification, page 3, lines 20-25). Nakao and Golembo described that CNP22 may be useful to treat achondroplasia given its role in the FGFR3 stimulatory pathway, but neither Nakao nor Golembo tested CNP22 in the treatment of achondroplasia.

7. CNP22 was known to have a short half-life in vivo and was rapidly degraded by the neural endopeptidase (NEP) enzyme (Potter et al., Endocrine Rev. 27:47-72, 2006). Yeung had shown that CNP53, a larger cleavage product of the CNP precursor, was able to promote cGMP activity, and Golembo had shown that CNP17, which comprises the functional ring structure of the CNP peptides, was also able to promote cGMP stimulation. However, there was nothing known in the field at the time of filing as to the effects of any of these peptides in vivo, and no information about what effects any other CNP variants would have on cGMP production or in vivo activity. Prior to our work, no group (i.e., not Nakao or Golembo) had tested the activity of a CNP in vivo because it was too difficult to test CNP22 in vivo due to its rapid degradation. To test CNP22 in vivo would have required daily, continuous infusion to introduce sufficient amounts of peptide to determine efficacy and such an experiment was not performed.

8. The present invention was the first discovery that CNP peptides having lengths that varied between CNP22 or CNP53 were able to be made and shown to have stability in vitro. Prior to the experiments that led up to this filing, it would not have been predicted whether any of the CNP variants would be able to withstand the natural NEP cleavage and be able to stimulate cGMP production in cells. I, along, with my co-inventors, made systematic changes to the CNP22/CNP53 peptides in order to try and improve the half-life of the peptide, and tested the variants for desired activity such as resistance to NEP cleavage and extended half-life. We made multiple types of alterations, such as addition of PEG of various sizes/weights to CNP22

and other variants, addition of amino acids to the CNP22 peptide, and alterations to wild-type CNP sequences.

9. It was not known at the time whether addition of PEG to CNP22 or other variants would improve half-life, if interim-length CNP peptides would improve half-life, if alterations in sequence would improve half-life, or if the new CNP variants would be cleaved by NEP to CNP22, which would then be rapidly degraded. It was found that some of the CNP22-PEG variants and other variants did exhibit reduced cleavage by NEP, but not all PEG variants had this activity (see Table 2 and Table 4 of the specification). Surprisingly, we found that not only were CNP variants having lengths between CNP22 and CNP53 capable of being stably produced, but we also found that many of these variants were not cleaved down to CNP22 and in fact showed improved NEP resistance and were able to stimulate cGMP production, even without the addition of a PEG moiety (specification, Tables 2-3). This was not expected given the previous disclosures in the field relating to the quick degradation of CNP by enzymatic cleavage (specification, page 3, lines 31-32, and Potter et al., Endocrine Rev. 27:47-72, 2006).

10. Further investigation of CNP variant activity demonstrated that CNP22 was useful to stimulate bone growth in an animal model of achondroplasia (see e.g., Figures 30-33), which was the first time this had been demonstrated. We also showed that many of the CNP variants that exhibited NEP resistance and cGMP production were also effective at increasing long-bone growth in vivo (see e.g., Figures 30-33, Figure 51, Figure 69). Additionally, many of these new variants were more effective than CNP22 in vivo due to the increased NEP resistance that resulted in an increased half-life in vivo (Figures 44-45 and Figures 71-73 of the specification). This therapeutic efficacy also would not have been expected in view of the disclosures of Nakao, Golembo and others in the field at the time of filing.

11. Subsequent research by our group published after the application filing date (Wendt et al., 2015) (Exhibit C) demonstrated that additional CNP variants, either having PEG moieties, having amino acid lengths between CNP22-CNP53 peptide, and having alterations to wild-type CNP sequences, were shown to have NEP resistance and cGMP stimulation activity (measured as potency) (Exhibit C, Table 1). It is expected that those CNP variants demonstrated to have increased NEP resistance and cGMP production in both the specification and in Wendt et al., 2015, such as Pro-Gly-CNP37, Gly-CNP37, CNP27, CNP40–CNP33 and

CNP30 (Table 1), and variants having similar sequences to those having these properties, will have similar half-lives in vivo.

12. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application, and any patent issuing thereon.

Date: 26 Mar 2020

Daniel J. Wendt

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

# NOTICE OF ALLOWANCE AND FEE(S) DUE

90849 7590 06/02/2020

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER |
|---|
| CAMPELL, BRUCE R |

| ART UNIT | PAPER NUMBER |
|---|---|
| 3991 | |

DATE MAILED: 06/02/2020

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

TITLE OF INVENTION: Variants of C-Type Natriuretic Peptide

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1000 | $0.00 | $0.00 | $1000 | 09/02/2020 |

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.**

## PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

90849 7590 06/02/2020
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

**Certificate of Mailing or Transmission**

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

| (Typed or printed name) |
|---|
| (Signature) |
| (Date) |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

TITLE OF INVENTION: Variants of C-Type Natriuretic Peptide

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1000 | $0.00 | $0.00 | $1000 | 09/02/2020 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| CAMPELL, BRUCE R | 3991 | 514-012400 |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 06/02/2020

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| CAMPELL, BRUCE R | |
| ART UNIT | PAPER NUMBER |
| 3991 | |

DATE MAILED: 06/02/2020

## Determination of Patent Term Extension or Adjustment under 35 U.S.C. 154 (b)

A reissue patent is for "the unexpired part of the term of the original patent." See 35 U.S.C. 251. Accordingly, the above-identified reissue application is not eligible for Patent Term Extension or Adjustment under 35 U.S.C. 154(b).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

| Notice of Allowability | Application No. 15/646,822 | Applicant(s) Wendt et al. | |
|---|---|---|---|
| | Examiner BRUCE R CAMPELL | Art Unit 3991 | AIA (FITF) Status No |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--*

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to amendment filed 3/26/20.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 15-76 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐ All  b) ☐ Some  *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

**Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 10/10/19.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Bruce Campell/ /Sharon Tumer/
Patent Reexamination Specialists, Central Reexam Unit 3991

### *Notice of Pre-AIA or AIA Status*

The present application is being examined under the pre-AIA first to invent provisions.

### *Claim Interpretation*

New claims 38, 39, 47, 48, 52, 53, 57, 58, 66, 67, 75 and 76 recite that the CNP variant is at least 95% identical to SEQ ID No. 60 or 75. 35 U.S.C. 112, fourth paragraph states, "A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers." Furthermore, "a dependent claim is directed to a combination including everything recited in the base claim and what is recited in the dependent claim. It is this combination that must be compared with the prior art, exactly as if it were presented as one independent claim." MPEP 608.01(n)(G)(III). Therefore the claims referenced above are construed as limited to sequences recited in the Markush group of the corresponding independent claim which are at least 95% identical to SEQ ID No. 60 or 75.

### *Reasons for Allowance*

The following is an examiner's statement of reasons for allowance:

Applicant submitted two declarations under 37 CFR 1.132 by inventor Daniel Wendt. The first declaration disqualifies U.S. Patent 8,377,884 as prior art under 35 U.S.C. 102(e). The second declaration explains that the CNP peptides recited in the claims are not specifically disclosed in the prior art and have the unexpected property of being more resistant to degradation by neural endopeptidase than the naturally occurring CNP22 (shown most clearly in Table 1 of the Wendt publication submitted with the declaration). In vivo testing suggests that these peptides may provide a therapy for achondroplasia. Therefore the recited CNP peptides, and conjugates thereof, are not obvious over the prior art.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

/BRUCE R CAMPELL/
Patent Reexam Specialist, Art Unit 3991

/SHARON TURNER/
Patent Reexam Specialist, Art Unit 3991

/TIMOTHY M SPEER/
Supervisory Patent Examiner, Art Unit 3991

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | BRUCE R CAMPELL | 3991 |

| CPC | | | | | | |
|---|---|---|---|---|---|---|
| Symbol | | | | | Type | Version |
| C07K | / | 14 | / | 58 | F | 2013-01-01 |
| A61K | / | 47 | / | 60 | I | 2017-08-01 |
| A61K | / | 9 | / | 08 | I | 2013-01-01 |
| A61K | / | 38 | / | 22 | I | 2013-01-01 |
| A61K | / | 38 | / | 2242 | I | 2013-01-01 |
| A61K | / | 47 | / | 10 | I | 2013-01-01 |
| A61K | / | 47 | / | 20 | I | 2013-01-01 |
| A61K | / | 47 | / | 22 | I | 2013-01-01 |
| A61K | / | 47 | / | 26 | I | 2013-01-01 |
| A61K | / | 38 | / | 00 | A | 2013-01-01 |
| G01N | / | 2333 | / | 58 | A | 2013-01-01 |
| G01N | / | 2800 | / | 10 | A | 2013-01-01 |
| G01N | / | 2800 | / | 105 | A | 2013-01-01 |
| G01N | / | 2800 | / | 385 | A | 2013-01-01 |
| G01N | / | 2800 | / | 52 | A | 2013-01-01 |

| CPC Combination Sets | | | | | | | |
|---|---|---|---|---|---|---|---|
| Symbol | | | | | Type | Set | Ranking | Version |
| | / | | / | | | | | |

| NONE | | Total Claims Allowed: | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 62 | |
| /BRUCE R CAMPELL/<br>Primary Examiner, Art Unit 3991 | 14 May 2020 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 15 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20191015

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | **Examiner** | **Art Unit** |
| | BRUCE R CAMPELL | 3991 |

| INTERNATIONAL CLASSIFICATION | | | | |
|---|---|---|---|---|
| **CLAIMED** | | | | |
| A61K | / | 38 | / | 22 |
| A61K | / | 38 | / | 00 |
| A61K | / | 38 | / | 16 |
| C12P | / | 21 | / | 04 |

| NON-CLAIMED | | | | |
|---|---|---|---|---|
| | / | | / | |

| US ORIGINAL CLASSIFICATION | |
|---|---|
| **CLASS** | **SUBCLASS** |
| | |

| CROSS REFERENCES(S) | | | | | | |
|---|---|---|---|---|---|---|
| **CLASS** | **SUBCLASS (ONE SUBCLASS PER BLOCK)** | | | | | |
| | | | | | | |

| NONE | | **Total Claims Allowed:** | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 62 | |
| /BRUCE R CAMPELL/<br>Primary Examiner, Art Unit 3991 | 14 May 2020 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 15 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20191015

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | **Examiner** | **Art Unit** |
| | BRUCE R CAMPELL | 3991 |

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

**CLAIMS**

| Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 11 | 21 | 21 | 31 | 31 | 41 | 41 | 51 | 51 | 61 | 61 | 71 | 71 |
| | 2 | | 12 | 22 | 22 | 32 | 32 | 42 | 42 | 52 | 52 | 62 | 62 | 72 | 72 |
| | 3 | | 13 | 23 | 23 | 33 | 33 | 43 | 43 | 53 | 53 | 63 | 63 | 73 | 73 |
| | 4 | | 14 | 24 | 24 | 34 | 34 | 44 | 44 | 54 | 54 | 64 | 64 | 74 | 74 |
| | 5 | 15 | 15 | 25 | 25 | 35 | 35 | 45 | 45 | 55 | 55 | 65 | 65 | 75 | 75 |
| | 6 | 16 | 16 | 26 | 26 | 36 | 36 | 46 | 46 | 56 | 56 | 66 | 66 | 76 | 76 |
| | 7 | 17 | 17 | 27 | 27 | 37 | 37 | 47 | 47 | 57 | 57 | 67 | 67 | | |
| | 8 | 18 | 18 | 28 | 28 | 38 | 38 | 48 | 48 | 58 | 58 | 68 | 68 | | |
| | 9 | 19 | 19 | 29 | 29 | 39 | 39 | 49 | 49 | 59 | 59 | 69 | 69 | | |
| | 10 | 20 | 20 | 30 | 30 | 40 | 40 | 50 | 50 | 60 | 60 | 70 | 70 | | |

| | | | |
|---|---|---|---|
| NONE<br>(Assistant Examiner) | (Date) | **Total Claims Allowed:**<br>62 | |
| /BRUCE R CAMPELL/<br>Primary Examiner, Art Unit 3991<br>(Primary Examiner) | 14 May 2020<br>(Date) | O.G. Print Claim(s)<br>15 | O.G. Print Figure<br>NONE |

U.S. Patent and Trademark Office Part of Paper No.: 20191015

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | BRUCE R CAMPELL | 3991 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
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| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Reviewed prosecution history in parent application 13/466,672 | 6/6/2018 | /J.R./ |
| Reviewed prosecution in RE 46,707 E | 6/6/2018 | /J.R./ |
| Google Search Terms: C-type natriuretic peptide bone review " fibroblast growth factor receptor" | 6/12/2018 | /J.R./ |
| Inventor Search | 6/18/2018 | /J.R./ |
| updated Inventor Search | 04/03/2019 | /J.R,/ |
| reviewed 13/466,672 and 14/604,262 | 10/15/2019 | /BC/ |
| updated inventor search | 05/14/2020 | /BC/ |
| reviewed sequence searches in 13/466,672 and 12/784,117 | 05/21/2020 | /BC/ |

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/646,822 | Wendt et al. |
| | Examiner | Art Unit |
| | BRUCE R CAMPELL | 3991 |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| A61K | 38/2242 | 10/15/2019 | /BC/ |

| United States Patent And Trademark Office | **Application No.** | **Art Unit** |
|---|---|---|
| **Reissue Terminal Disclaimer Review Form** | 15/646,822 | 3991 |
| | **Examiner** | **AIA Status** |
| | BRUCE R CAMPELL | No |

| **Original Patent Number of Patent to be Reissued is: 8,598,121** | **The Maintenance fee status is:**<br>◉ up to date<br>○ not up to date<br>(Consult with SPRS) |
|---|---|

Is there a terminal disclaimer filed and accepted during the prosecution of (i) the current reissue application, (ii) the underlying patent, and/or (iii) reexamination proceeding(s) of the underlying patent?

○ NO
◉ YES (Complete the rest of the form)

**The reissue patent is subject to Terminal Disclaimer(s) that was/were:**

☑ filed and accepted (DISQ or DISQ.E.FILE) during the prosecution of the current reissue application. (Enter terminal disclaimer(s) filing date(s) below).

1. 26 September 2019 2. _____ 3. _____

**The underlying patent of the current reissue application is subject to Terminal DIsclaimer(s) that was/were:**

☐ accepted (DISQ or DISQ.E.FILE) and of record in the prosecution of the underlying patent and/or reexamination procceeding(s) of the underlying patent. (Enter application/control no(s) and terminal disclaimer(s) filing date(s) below).

1. _____ _____ 2. _____ _____ 3. _____ _____

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.R.C/

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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

*(Use as many sheets as necessary)*

Sheet 1 of 2

| *Complete if Known* | |
|---|---|
| Application Number | 15/646,822-Conf. #7727 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

## U. S. PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Document Number Number-Kind Code[2] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear |
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| Examiner Initials* | Cite No.[1] | Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
|---|---|---|---|---|---|---|
| | | WO-2010/033217-A1 | 03-25-2010 | Nektar Therapeutics | | |
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| | | | | | | |

| Examiner Signature | /BRUCE R CAMPELL/ | Date Considered | 10/15/2019 |
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.R.C/

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| Sheet | 2 | of | 2 |
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| Art Unit | 3991 |
| Examiner Name | B. Campell |
| Attorney Docket Number | 30610/44556C1-R2 |

**NON PATENT LITERATURE DOCUMENTS**

| Examiner Initials* | Cite No.[1] | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T[2] |
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| Examiner Signature | /BRUCE R CAMPELL/ | Date Considered | 10/15/2019 |
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# WEST Search History

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DATE: Tuesday, October 15, 2019

| Hide? | Set Name | Query | Hit Count |
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| *Interference* | | | |
| *DB=PGPB; PLUR=YES; OP=ADJ* | | | |
| ☐ | L4 | L3 and (cnp near polymer) | 0 |
| ☐ | L3 | L2 and cnp | 130 |
| ☐ | L2 | a61k38/2242.cpc. | 214 |

END OF SEARCH HISTORY

# Bibliographic Data

Application No: 15/646,822

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /BRUCE R CAMPELL/ Examiner's Signature | Initials

Title: Variants of C-Type Natriuretic Peptide

| FILING or 371(c) DATE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|
| 07/11/2017 | 514 | 3991 | 30610/44556C1-R2 |
| RULE | | | |

**APPLICANTS**

BioMarin Pharmaceutical Inc., Novato, CA, UNITED STATES

**INVENTORS**

Daniel J. Wendt Novato, CA, UNITED STATES

Shinong Long Novato, CA, UNITED STATES

Sianna Castillo Novato, CA, UNITED STATES

Christopher P. Price Aachen, GERMANY

Mika Aoyagi-Scharber Novato, CA, UNITED STATES

Michel Claude Vellard Novato, CA, UNITED STATES

Augustus O. Okamafe Concord, CA, UNITED STATES

**CONTINUING DATA**

This application is a CON of 14604262 01/23/2015 PAT RE46707

14604262 is a REI of 13466672 05/08/2012 PAT 8598121

13466672 is a CON of 12784117 05/20/2010 PAT 8198242

12784117 has PRO of 61254563 10/23/2009

12784117 has PRO of 61180112 05/20/2009

**FOREIGN APPLICATIONS**

**IF REQUIRED, FOREIGN LICENSE GRANTED****

07/12/2017

**STATE OR COUNTRY**

UNITED STATES

**ADDRESS**

Marshall, Gerstein & Borun LLP (Biomarin)

233 South Wacker Drive

6300 Willis Tower
Chicago, IL 60606
UNITED STATES

**FILING FEE RECEIVED**

$4,300

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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

*(Use as many sheets as necessary)*

Sheet 1 of 2

| *Complete if Known* | |
|---|---|
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| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | J. F. Railey |
| Attorney Docket Number | 30610/44556C1-R2 |

## U. S. PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Document Number<br>Number-Kind Code[2] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear |
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## FOREIGN PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Foreign Patent Document<br>Country Code[3]-Number[4]-Kind Code[5] *(if known)* | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
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| Examiner Signature | | Date Considered | |
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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| Sheet | 2 | of | 2 |
|---|---|---|---|

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| **Application Number** | 15/646,822-Conf. #7727 |
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| **First Named Inventor** | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | J. F. Railey |
| Attorney Docket Number | 30610/44556C1-R2 |

**NON PATENT LITERATURE DOCUMENTS**

| Examiner Initials* | Cite No.[1] | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T[2] |
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# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Audrey Nagelberg |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Miscellaneous:** | | | | |
| SUBMISSION- INFORMATION DISCLOSURE STMT | 1806 | 1 | 240 | 240 |
| | | **Total in USD ($)** | | **240** |

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 39677762 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville/Audrey Nagelberg |
| **Filer Authorized By:** | Katherine Lynn Neville |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 10-JUN-2020 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 12:11:27 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

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| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $240 |
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| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Foreign Reference | WO2009046861A1.PDF | 11063376<br>4741da02980f774afa47d07537206f9015c5b187 | no | 103 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Foreign Reference | WO03079979A2.PDF | 3827770<br>f193c08e97166800d40f712d7f5fe89d0e0ee688 | no | 58 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Transmittal Letter | Information_Disclosure_Statement.PDF | 18394<br>cff3a11d2a4566c63217e400a456a6b62b5e922e | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Information Disclosure Statement (IDS) Form (SB08) | IDS_Citation_by_Applicant_2_References.PDF | 34541<br>1ce32552d543fa36fa2a5666784e7e51305d07c3 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 5 | Fee Worksheet (SB06) | fee-info.pdf | 30702<br>ad5ec4a50249665ade6c6e2f098f01c233729180 | no | 2 |
| **Warnings:** | | | | | |
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**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

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*A61K 38/22* (2006.01) *A61P 25/28* (2006.01)
*A61K 38/04* (2006.01) *A61P 31/00* (2006.01)
*A61P 3/00* (2006.01) *A61P 35/00* (2006.01)
*A61P 9/00* (2006.01) *A61P 37/00* (2006.01)
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9 September 2008 (09.09.2008)

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(71) Applicant *(for all designated States except US)*: **MONDOBIOTECH LABORATORIES AG** [LI/LI]; Herrengasse 21, FL-9490 Vaduz (LI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BEVEC, Dorian** [DE/DE]; Kriegerstrasse 62, 82110 Germering (DE). **CAVALLI, Fabio** [CH/CH]; Via Pasquée 23, CH-6925 Gentilino (CH). **CAVALLI, Vera** [CH/CH]; Via Pasquée, 23, CH-6925 Gentilino (CH). **BACHER, Gerald** [DE/DE]; Masurenweg 5, 82110 Germering (DE).

(74) Agent: **ARTH, Hans-Lothar**; ABK, Jasminweg 9, 14052 Berlin (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

**Published:**
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(54) **Title:** USE OF CNP-22, ALONE1 OR IN COMBINATION WITH PHYSALEMIN, AS A THERAPEUTIC AGENT

(57) **Abstract:** The present invention is directed to the use of the peptide compound Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-lle-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH as a therapeutic agent for the prophylaxis and/or treatment of cancer, autoimmune diseases, fibrotic diseases, inflammatory diseases, neurodegenerative diseases, infectious diseases, lung diseases, heart and vascular diseases and metabolic diseases. Moreover the present invention relates to pharmaceutical compositions preferably in form of a lyophilisate or liquide buffer solution or artificial mother milk formulation or mother milk substitute containing the peptide Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-lle-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH optionally together with at least one pharmaceutically acceptable carrier, cryoprotectant, lyoprotectant, excipient and/or diluent.

WO 2009/046861 A1

# USE OF CNP-22 , ALONE1 OR IN COMBINATION WITH PHYSALEMIN, AS A THERAPEUTIC AGENT

## Specification

The present invention is directed to the use of the peptide compound Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH (CNP (1-22)) as a therapeutic agent for the prophylaxis and/or treatment of cancer, an autoimmune disease, an infectious disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease.

### Background of the invention

The identification of a therapeutic compound effective for the prophylaxis and/or treatment of a disease can be based on the activity of the compound in a biological assay. A biological assay that mimics a disease causative mechanism can be used to test the therapeutic activity of a candidate peptide.

The causative mechanism of many diseases is the over activity of a biological pathway. A peptide that can reduce the activity of the biological pathway can be effective in the prophylaxis and/or treatment of the disease caused by the over activity of the biological pathway. Similarly the causative mechanism of many diseases is the over production of a biological molecule. A peptide that can reduce the production of the biological molecule or block the activity of the over produced biological molecule can be effective in the prophylaxis and/or treatment of the disease caused by the over production of the biological molecule.

Conversely, the causative mechanism of many diseases is the under activity of a biological pathway. A peptide that can increase the activity of the biological pathway can be effective in the prophylaxis and/or treatment of the disease caused by the under activity of the biological pathway. Also similarly the causative mechanism of many diseases is the under production of a biological molecule. A peptide that can increase the production of the biological molecule or mimic the biological activity of the under produced biological molecule can be effective in the prophylaxis and/or treatment of the disease caused by the under production of the biological molecule.

It is the object of the present invention to provide a compound for the prophylaxis and/or treatment of cancer, autoimmune diseases, fibrotic diseases, inflammatory diseases, neurodegenerative diseases, infectious diseases, lung diseases, heart and vascular diseases and metabolic diseases.

The object of the present invention is solved by the teaching of the independent claims. Further advantageous features, aspects and details of the invention are evident from the dependent claims, the description, and the examples of the present application.

**Description of the invention**

The present invention relates to the use of the peptide Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH (CNP (1-22)), its use as a therapeutic in medicine and for the prophylaxis and/or treatment of cancer, autoimmune diseases, fibrotic diseases, inflammatory diseases, neurodegenerative diseases, infectious diseases, lung diseases, heart and vascular diseases and metabolic diseases. Also disclosed are pharmaceutical formulations preferably in form of a lyophilisate or liquid buffer solution or artificial mother milk formulation containing the inventive peptide. The peptide is especially useful for prophylaxis and/or treatment of AIDS, AIDS related diseases, HIV, asthma, Omenn's syndrome, idiopathic pulmonary fibrosis, and atopic disorders in genetically susceptible individuals and other diseases which are described in the following.

Moreover the present invention relates to a peptide combination of the above mentioned peptide with the peptide compound Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ as well as to pharmaceutical compositions containing said peptide combination.

**Cancer, tumors, proliferative diseases, malignancies and their metastases**

The term "cancer" as used herein refers also to tumors, proliferative diseases, malignancies and their metastases. Examples for cancer diseases are adenocarcinoma, choroidal melanoma, acute leukemia, acoustic neurinoma, ampullary carcinoma, anal carcinoma, astrocytoma, basal cell carcinoma, pancreatic cancer, desmoid tumor, bladder cancer, bronchial carcinoma, non-small cell lung cancer (NSCLC), breast cancer, Burkitt's lymphoma, corpus cancer, CUP-syndrome (carcinoma of unknown primary), colorectal cancer, small intestine cancer, small intestinal tumors, ovarian cancer, endometrial carcinoma, ependymoma, epithelial cancer types, Ewing's tumors, gastrointestinal tumors, gastric cancer, gallbladder cancer, gall bladder carcinomas, uterine cancer, cervical cancer, cervix, glioblastomas, gynecologic tumors, ear, nose and throat tumors, hematologic neoplasias, hairy cell leukemia, urethral cancer, skin cancer, skin testis cancer, brain tumors (gliomas), brain metastases, testicle cancer, hypophysis tumor, carcinoids, Kaposi's sarcoma, laryngeal cancer, germ cell tumor, bone cancer, colorectal carcinoma, head and neck tumors (tumors of the ear, nose and throat area), colon carcinoma, craniopharyngiomas, oral

cancer (cancer in the mouth area and on lips), cancer of the central nervous system, liver cancer, liver metastases, leukemia, eyelid tumor, lung cancer, lymph node cancer (Hodgkin's/Non-Hodgkin's), lymphomas, stomach cancer, malignant melanoma, malignant neoplasia, malignant tumors gastrointestinal tract, breast carcinoma, rectal cancer, medulloblastomas, melanoma, meningiomas, Hodgkin's disease, mycosis fungoides, nasal cancer, neurinoma, neuroblastoma, kidney cancer, renal cell carcinomas, non-Hodgkin's lymphomas, oligodendroglioma, esophageal carcinoma, osteolytic carcinomas and osteoplastic carcinomas, osteosarcomas, ovarial carcinoma, pancreatic carcinoma, penile cancer, plasmocytoma, squamous cell carcinoma of the head and neck (SCCHN), prostate cancer, pharyngeal cancer, rectal carcinoma, retinoblastoma, vaginal cancer, thyroid carcinoma, Schneeberger disease, esophageal cancer, spinalioms, T-cell lymphoma (mycosis fungoides), thymoma, tube carcinoma, eye tumors, urethral cancer, urologic tumors, urothelial carcinoma, vulva cancer, wart appearance, soft tissue tumors, soft tissue sarcoma, Wilm's tumor, cervical carcinoma and tongue cancer.

The peptides and peptide combination of the present invention were tested using the assays described in Examples 1-7, 9-17 for their effect as active therapeutic agents in the prophylaxis and/or treatment of cancer, proliferative diseases, tumors and their metastases.

**Infectious disease**

The immune system in higher vertebrates represents the first line of defense against various antigens that can enter the vertebrate body, including microorganisms such as bacteria, fungi and viruses that are the causative agents of a variety of diseases.

Despite large immunization programs, viral infections, such as influenza virus, human immunodeficiency virus ("HIV"), herpes simplex virus ("HSV", type 1 or 2), human papilloma virus ("HPV", type 16 or 18), human cytomegalovirus ("HCMV") or human hepatitis B or C virus ("HBV", Type B; "HCV", type C) infections, remain a serious source of morbidity and mortality throughout the world and a significant cause of illness and death among people with immune-deficiency associated with aging or different clinical conditions. Although antiviral chemotherapy with compounds such as amantadine and rimantadine have been shown to reduce the duration of symptoms of clinical infections (i.e., influenza infection), major side effects and the emergence of drug-resistant variants have been described. New classes of antiviral agents designed to target particular viral proteins such as influenza neuraminidase are being developed. However, the ability of viruses to mutate the target proteins represents an obstacle for effective treatment with molecules which selectively inhibit

the function of specific viral polypeptides. Thus, there is need for new therapeutic strategies to prevent and treat viral infections.

Additionally, there is a need for new therapies for the prevention and treatment of bacterial infections, especially bacterial infections caused by multiple drug resistant bacteria. Currently, bacterial infections are treated with various antibiotics. Although antibiotics have and can be effective in the treatment of various bacterial infections, there are a number of limitations to the effectiveness and safety of antibiotics. For example, some individuals have an allergic reaction to certain antibiotics and other individuals suffer from serious side effects. Moreover, continued use of antibiotics for the treatment of bacterial infections contributes to formation of antibiotic-resistant strains of bacteria.

Another aspect of the present invention is directed to the use of the peptide or the peptide combination for prophylaxis and/or treatment of infectious diseases including opportunistic infections.

Examples of infectious diseases are AIDS, alveolar hydatid disease (AHD, echinococcosis), amebiasis (Entamoeba histolytica infection), Angiostrongylus infection, anisakiasis, anthrax, babesiosis (Babesia infection), Balantidium infection (balantidiasis), Baylisascaris infection (raccoon roundworm), bilharzia (schistosomiasis), Blastocystis hominis infection (blastomycosis), boreliosis, botulism, Brainerd diarrhea, brucellosis, bovine spongiform encephalopathy (BSE), candidiasis, capillariasis (Capillaria infection), chronic fatigue syndrome (CFS), Chagas disease (American trypanosomiasis), chickenpox (Varicella-Zoster virus), Chlamydia pneumoniae infection, cholera, Creutzfeldt-Jakob disease (CJD), clonorchiasis (Clonorchis infection), cutaneous larva migrans (CLM) (hookworm infection), coccidioidomycosis, conjunctivitis, Coxsackievirus A16 (hand, foot and mouth disease), cryptococcosis, Cryptosporidium infection (cryptosporidiosis), Culex mosquito (West Nile virus vector), cyclosporiasis (Cyclospora infection), cysticercosis (neurocysticercosis), Cytomegalovirus infection, Dengue / Dengue fever, Dipylidium infection (dog and cat flea tapeworm), Ebola virus hemorrhagic fever, encephalitis, Entamoeba coli infection, Entamoeba dispar infection, Entamoeba hartmanni infection, Entamoeba histolytica infection (amebiasis), Entamoeba polecki infection, enterobiasis (pinworm infection), enterovirus infection (non-polio), Epstein-Barr virus infection, Escherichia coli infection, foodborne infection, foot and mouth disease, fungal dermatitis, gastroenteritis, group A streptococcal disease, group B streptococcal disease, Hansen's disease (leprosy), Hantavirus pulmonary syndrome, head lice infestation (pediculosis), Helicobacter

pylori infection, hematologic disease, Hendra virus infection, hepatitis (HCV, HBV), herpes zoster (shingles), HIV Infection, human ehrlichiosis, human parainfluenza virus infection, influenza, isosporiasis (Isospora infection), Lassa fever, leishmaniasis, Kala-azar (Kala-azar, Leishmania Infection), lice (body lice, head lice, pubic lice), Lyme disease, malaria, Marburg hemorrhagic fever, measles, meningitis, mosquito-borne diseases, Mycobacterium avium complex (MAC) infection, Naegleria infection, nosocomial infections, nonpathogenic intestinal ameobae infection, onchocerciasis (river blindness), opisthorciasis (Opisthorcis infection), parvovirus infection, plague, Pneumocystis carinii pneumonia (PCP), polio, Q fever, rabies, respiratory syncytial virus (RSV) Infection, rheumatic fever, Rift Valley fever, river blindness (onchocerciasis), rotavirus infection, roundworm infection, salmonellosis, salmonella enteritidis, scabies, shigellosis, shingles, sleeping sickness, smallpox, streptococcal Infection, tapeworm infection (Taenia infection), tetanus, toxic shock syndrome, tuberculosis, ulcers (peptic ulcer disease), valley fever, Vibrio parahaemolyticus infection, Vibrio vulnificus infection, viral hemorrhagic fever, warts, waterborne infectious diseases, West Nile virus infection (West Nile encephalitis), whooping cough, yellow fever.

Another aspect of the present invention is directed to the use of the peptide or the peptide combination for prophylaxis and/or treatment of prion diseases.

Prions are infectious agents which do not have a nucleic acid genome. It seems that a protein alone is the infectious agent. A prion has been defined as "small proteinaceous infectious particle which resists inactivation by procedures that modify nucleic acids". The discovery that proteins alone can transmit an infectious disease came as a considerable surprise to the scientific community. Prion diseases are often called “transmissible spongiform encephalopathies”, because of the post mortem appearance of the brain with large vacuoles in the cortex and cerebellum. Probably most mammalian species develop these diseases. Prion diseases are a group of neurodegenerative disorders of humans and animals and the prion diseases can manifest as sporadic, genetic or infectious disorders. Examples of prion diseases acquired by exogenous infection are bovine spongiform encephalitis (BSE) of cattle and the new variant of Creutzfeld-Jakob disease (vCJD) caused by BSE as well as scrapie of animals. Examples of human prion diseases include kuru, sporadic Creutzfeldt-Jakob disease (sCJD), familial CJD (fCJD), iatrogenic CJD (iCJD), Gerstmann-Sträussler-Scheinker (GSS) disease, fatal familial insomnia (FFI), and especially the new variant CJD (nvCJD or vCJD).

The name "prion" is used to describe the causative agents which underlie the transmissible spongiform encephalopathies. A prion is proposed to be a novel infectious particle that differs from viruses and viroids. It is composed solely of one unique protein that resists most inactivation procedures such as heat, radiation, and proteases. The latter characteristic has led to the term protease-resistant isoform of the prion protein. The protease-resistant isoform has been proposed to slowly catalyze the conversion of the normal prion protein into the abnormal form.

The term "isoform" in the context of prions means two proteins with exactly the same amino acid sequence that can fold into molecules with dramatically different tertiary structures. The normal cellular isoform of the prion protein ($PrP^C$) has a high α-helix content, a low β-sheet content, and is sensitive to protease digestion. The abnormal, disease-causing isoform ($PrP^{Sc}$) has a lower α-helix content, a much higher β-sheet content, and is much more resistant to protease digestion.

As used herein the term "prion diseases" refers to transmissible spongiform encephalopathies. Examples for prion diseases comprise scrapie (sheep, goat), transmissible mink encephalopathy (TME; mink), chronic wasting disease (CWD; muledeer, deer, elk), bovine spongiform encephalopathy (BSE; cows, cattles), Creutzfeld-Jacob Disease (CJD), variant CJD (vCJD), sporadic Creutzfeldt-Jakob disease (sCJD), familial CJD (fCJD), iatrogenic CJD (iCJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and kuru. Preferred are BSE, vCJD, and CJD.

The peptide and the peptide combination of the present invention were tested using the assays described in Examples 1-7 for their effect as active therapeutic agents in the prophylaxis and/or treatment of infectious diseases and disorders.

**Autoimmune disease**

Autoimmune disease refers to any of a group of diseases or disorders in which tissue injury is associated with a humoral and/or cell-mediated immune response to body constituents or, in a broader sense, an immune response to self. The pathological immune response may be systemic or organ specific. That is, for example, the immune response directed to self may affect joints, skin, myelin sheath that protects neurons, kidney, liver, pancreas, thyroid, adrenals, and ovaries.

In fact, the list of autoimmune diseases is composed of more than eighty disorders. A few autoimmune diseases such as vitiligo, in which patches of skin lose

pigmentation, are merely annoying. Most others are debilitating, often progressive with time and eventually fatal. Systemic lupus erythematosus (SLE), for example, is a chronic disease in which 10-15% of patients die within a decade of diagnosis, in all but a few autoimmune diseases, the sex ratio skews towards women. For example, in SLE the ratio of female to male patients is nine to one. In one particular case, Hashimoto's disease in which the immune system attacks the thyroid gland, the ratio is fifty to one.

It has long been known that immune complex formation plays a role in the etiology and progression of autoimmune disease. For example, inflammation in patients with arthritis has long been considered to involve phagocytosis by leukocytes of complexes of antigen, antibody and complement-immune complexes. However, only now it is being recognized that inflammation caused by immune complexes in the joints (arthritis), the kidneys (glomerulonephritis), and blood vessels (vasculitis) is a major cause of morbidity in autoimmune diseases. Increased immune complex formation correlates with the presence of antibodies directed to self or so-called autoantibodies, and the presence of the latter can also contribute to tissue inflammation either as part of an immune complex or unbound to antigen (free antibody). In some autoimmune diseases, the presence of free autoantibody contributes significantly to disease pathology. This has been clearly demonstrated for example in SLE (anti-DNA antibodies), immune thrombocytopenia (antibody response directed to platelets), and to a lesser extent rheumatoid arthritis (IgG reactive rheumatoid factor). The important role of immune complexes and free autoantibodies is further demonstrated by the fact that successful treatment of certain autoimmune diseases has been achieved by the removal of immune complexes and free antibody by means of specific immunoadsorption procedures. For example, the use of an apheresis procedure in which immune complexes and antibodies are removed by passage of a patient's blood through an immunoaffinity column was approved by the U.S. FDA in 1987 for immune thrombocytopenia (ITP) and in 1999 for rheumatoid arthritis. However, currently there is no approved method for the treatment of autoimmune diseases which facilitates the elimination of immune complexes and autoantibodies by administration of a drug.

Another aspect of the etiology and progression of autoimmune disease is the role of proinflammatory cytokines. Under normal circumstances, proinflammatory cytokines such as tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin-1 (IL-1) play a protective role in the response to infection and cellular stress. However, the pathological consequences which result from chronic and/or excessive production of TNF$\alpha$ and IL-1 are believed to underlie the progression of many autoimmune diseases such as

rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, and psoriasis. Other proinflammatory cytokines include interleukin-6, interleukin-8, interleukin-17, and granulocyte-macrophage colony stimulating factor.

Naturally occurring CD4+CD25+ regulatory T cells (Tregs) play a critical role in the control of periphery tolerance to self-antigens. Interestingly, they also control immune responses to allergens and transplant antigens. Recent studies in animal models have shown that adoptive transfer of CD4+CD25+ Tregs can prevent or even cure allergic and autoimmune diseases, and appear to induce transplantation tolerance. Thus, adoptive cell therapy using patient-specific CD4+CD25+ Tregs has emerged as an individualized medicine for the treatment of inflammatory disease including allergy, autoimmune disease and transplant rejection. Furthermore, strategies to activate and expand antigen-specific CD4+CD25+ Tregs in vivo using pharmacological agents may represent a novel avenue for drug development.

The interaction of leukocytes with the vessel endothelium to facilitate the extravasation into the tissue represents a key process of the body's defense mechanisms. Excessive recruitment of leukocytes into the inflamed tissue in chronic diseases like autoimmune disorders could be prevented by interfering with the mechanisms of leukocyte extravasation. Significant progress in elucidating the molecular basis of the trafficking of leukocytes from the blood stream to the extravascular tissue has been achieved that enables new strategies for therapeutic approaches. The multistep process of leukocyte rolling, firm adhesion and transmigration through the endothelial wall is facilitated by a dynamic interplay of adhesion receptors on both leukocytes and on endothelial cells as well as chemokines. In preclinical studies using various animal models, promising results have been obtained demonstrating that blocking of adhesion receptors of the selectin and integrin families improved the inflammation process in models of ulcerative colitis, autoimmune encephalomyelitis or contact hypersensitivity. In addition to the targeting of adhesion receptors by antibodies, small molecules that mimic epitopes of adhesion receptor ligands have been developed and successfully applied in animal models. Clinical studies revealed a limited response using antibodies to selectins or leukocyte function-associated antigen 1 (LFA-1) integrins compared with animal models. However, using humanized antibodies to the alpha 4-integrin subunit significant efficacy has been demonstrated in autoimmune diseases like psoriasis, multiple sclerosis and inflammatory bowel disease.

Examples of autoimmune diseases of the eyes are idiopathic opticus-neuritis, ophthalmia sympathica, anterior uveitis and other uveitis forms, retina degeneration, and Mooren's ulcer.

Examples of autoimmune diseases of the skin are bullous pemphigoides, chronic urticaria (autoimmune subtype), dermatitis herpetiformis (morbus Duhring), epidermolysis bullosa aquisita (EBA), acquired angioedema, herpes gestationes, hypocomplementemic urticarial vasculitis syndrome (HUVS), linear IgA-dermatosis, and pemphigus.

Examples of hematological autoimmune diseases are autoimmune hemolytic anemia, autoimmune neutropenia, Evans syndrome, inhibitor hemophilia, idiopathic thrombocytopenial purpura (ITP) and pernicious anemia.

Examples of gynecological autoimmune diseases are habitual abortion and infertility.

Examples of autoimmune diseases of the heart are congenital heart block, idiopathic dilatative cardiomyopathy, peripartum-cardiomyopathy, postcardiotomy syndrome, and postinfarct syndrome (Dressler syndrome).

Examples of autoimmune diseases of the ear, nose and throat are chronic sensorineural hearing loss and morbus Meniére.

Examples of autoimmune diseases of the colon are autoimmune enteropathy, colitis ulcerosa, indeterminant colitis, Crohn's disease and gluten-sensitive enteropathy.

Examples of autoimmune endocrinological autoimmune disorders are autoimmune polyglandulary syndrome type 1, autoimmune polyglandulary syndrome type 2, diabetes mellitus type 1 (IDDM), Hashimoto-thyroiditis, insulin-autoimmune-syndrome (IAS), idiopathic diabetes insipidus, idiopathic hypoparathyroidism, idiopathic Addison's disease and Graves-Basedow disease.

Examples of autoimmune diseases of the liver are autoimmune hepatitis (AIH type 1, 2 and 3), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis.

Example of autoimmune diseases of the lung is Goodpasture's syndrome.

An example of an autoimmune disease of the stomach is chronic atrophic (type A) gastritis.

Examples of neurological autoimmune disorders are Guillain-Barré syndrome, IgM gammopathy-associated neuropathy, Lambert-Eaton syndrome, Miller-Fisher syndrome, multiple sclerosis, multifocal motoric neuropathy, myasthenia gravis, paraneoplastic neurological syndrome, Rasmussen's encephalitis, and stiff-man syndrome.

Examples of autoimmune diseases of the kidney are anti-TBM-nephritis, Goodpasture's syndrome/anti-GBM-nephritis, IgA-nephropathy, interstitial nephritis, and membrane proliferative glomerulonephritides.

Further diseases that may be caused by an autoimmune reaction are Behcet disease, chronic fatigue immune dysfunction syndrome (CFIDS), Cogan syndrome I, endometriosis, HELLP syndrome, Bechterew's disease, polymyalgia rheumatica, psoriasis, sarcoidosis and vitiligo.

During the last decade, new biotherapies have been developed for the treatment of systemic autoimmune diseases. The targets of these new treatments are all the steps of the immune response. These new therapies are: B lymphocyte (BL) inhibitors such as anti-CD20 monoclonal antibody, B lymphocyte stimulator (BLyS) antagonists and tolerogens of pathogenic-antibody secreting LB; inhibitors of the costimulation between antigen-presenting cells and T lymphocyte (TL) like monoclonal anti-CD40 ligand antibody or CTLA4-Ig (abatecept); TL antagonists which can inhibit the proliferation of autoreactive T cells; cytokine antagonists; chemokine and adhesin antagonists which inhibit trafficking of immunocompetent cells to target organs. These new approaches are based on a better understanding of the autoimmune response.

The peptide and the peptide combination of the present invention were tested using the assays described in Examples 14 - 15 for their effect as active therapeutic agents in the prophylaxis and/or treatment of autoimmune diseases and disorders.

**Fibrotic disease**

Fibrosis or fibrosis associated disorder affects the liver, epidermis, endodermis, muscle, tendon, cartilage, heart, pancreas, lung, uterus, nervous system, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract, or stomach. In a further embodiment, the fibrosis or fibrosis associated disorder is interstitial lung fibrosis. In another embodiment the fibrosis or fibrosis associated disorder is the

result of an infection with schistosoma. In another embodiment the fibrosis or fibrosis associated disorder is the result of wound healing.

Fibrosis is generally characterized by the pathologic or excessive accumulation of collagenous connective tissue. Fibrotic diseases and disorders include, but are not limited to, collagen disease, interstitial lung disease, human fibrotic lung disease (e.g., obliterative bronchiolitis, idiopathic pulmonary fibrosis, pulmonary fibrosis from a known etiology, tumor stroma in lung disease, systemic sclerosis affecting the lungs, Hermansky-Pudlak syndrome, coal worker's pneumoconiosis, asbestosis, silicosis, chronic pulmonary hypertension, AIDS associated pulmonary hypertension, sarcoidosis, and the like), fibrotic vascular disease, tubulointerstitial and glomerular fibrosis, myocardial fibrosis, arterial sclerosis, atherosclerosis, varicose veins, coronary infarcts, cerebral infarcts, myocardial fibrosis, musculoskeletal fibrosis, post-surgical adhesions, human kidney disease (e.g., nephritic syndrome, Alport's syndrome, HIV associated nephropathy, polycystic kidney disease, Fabry's disease, diabetic nephropathy, chronic glomerulonephritis, nephritis associated with systemic lupus, and the like), cutis keloid formation, progressive systemic sclerosis (PSS), primary sclerosing cholangitis (PSC), liver fibrosis, liver cirrhosis, renal fibrosis, pulmonary fibrosis, cystic fibrosis, chronic graft versus host disease, scleroderma (local and systemic), Grave's opthalmopathy, diabetic retinopathy, glaucoma, Peyronie's disease, penis fibrosis, urethrostenosis after a test using a cystoscope, inner accretion after surgery, scarring, myelofibrosis, idiopathic retroperitoneal fibrosis, peritoneal fibrosis from a known etiology, drug induced ergotism, fibrosis incident to benign or malignant cancer, fibrosis incident to microbial infection (e.g., viral, bacterial, parasitic, fungal, etc.), Alzheimer's disease, fibrosis incident to inflammatory bowel disease (including stricture formation in Crohn's disease and microscopic colitis), fibrosis induced by chemical or environmental insult (e.g., cancer chemotherapy, pesticides, radiation/cancer radiotherapy), and the like.

Diseases associated with fibrosis include lupus, graft versus host disease, scleroderma, systemic sclerosis, scleroderma-like disorders, sine scleroderma, calcinosis, Raynaud's esophageal dysfunction, sclerodactyly, telangiectasiae, hypersensitivity pneumonitis, collagen vascular disease, asthma, pulmonary arterial hypertension, glomerulonephritis, chronic obstructive pulmonary disease, fibrosis following myocardial infarction, central nervous system fibrosis following a stroke or neuro-degenerative diseases (e.g. Alzheimer´s disease), proliferative vitreoretinopathy (PVR) and arthritis, silicosis, asbestos induced pulmonary fibrosis, acute lung injury and acute respiratory distress syndrome (including bacterial

pneumonia induced, trauma induced, viral pneumonia induced, tuberculosis, ventilator induced, non-pulmonary sepsis induced, and aspiration induced).

*Increased number of activated myofibroblasts in fibrotic diseases*

The emergence and disappearance of the myofibroblast appears to correlate with the initiation of active fibrosis and its resolution, respectively. In addition, the myofibroblast has many phenotypic features, which embody much of the pathologic alterations in fibrotic tissue, e.g. lung tissue. These features would seem to argue for an important role for the myofibroblast in the pathogenesis of fibrosis, e.g. lung fibrosis. Furthermore, the persistence of the myofibroblast may herald progressive disease, and, conversely, its disappearance may be an indicator of resolution. This in turn suggests that future therapeutic strategies targeting the myofibroblast would be productive.

Patients usually exhibit evidence of active fibrosis with increased numbers of activated fibroblasts, many of which have the phenotypic characteristics of myofibroblasts. At these sites, increased amounts of extracellular matrix deposition are evident with effacement of the normal alveolar architecture. Animal model studies show the myofibroblast to be the primary source of type I collagen gene expression in active fibrotic sites. *In vitro* studies show differentiation of these cells from fibroblasts under the influence of certain cytokines but indicate their susceptibility to nitric oxide mediated apoptosis. In addition to promoting myofibroblast differentiation, transforming growth factor-ß1 (TGF-ß1) provides protection against apoptosis. Thus, this well-known fibrogenic cytokine is important both for the emergence of the myofibroblast and its survival against apoptotic stimuli. This is consistent with the critical importance of this cytokine in diverse models of fibrosis in various tissues. In view of these properties, the persistence or prolonged survival of the myofibroblast may be the key to understanding why certain forms of lung injury may result in progressive disease, terminating in end stage disease.

Although pulmonary fibrosis has diverse etiologies, there is a common feature characteristic of this process, namely, the abnormal deposition of extracellular matrix that effaces the normal lung tissue architecture. A key cellular source of this matrix is the mesenchymal cell population that occupies much of the fibrotic lesion during the active period of fibrosis. This population is heterogeneous with respect to a number of key phenotypes. One of these phenotypes is the myofibroblast, which is commonly identified by its expression in α-smooth muscle actin and by features that are intermediate between the bona fide smooth muscle cell and the fibroblast. The *de novo* appearance of myofibroblasts at sites of wound healing and tissue

repair/fibrosis is associated with the period of active fibrosis and is considered to be involved in wound contraction. Furthermore, the localization of myofibroblasts at sites undergoing active extracellular matrix deposition suggests an important role for these cells in the genesis of the fibrotic lesion.

*Increased TGF-$\beta_1$ family levels in fibrotic diseases*

The transforming growth factor-$\beta_1$ (TGF-$\beta_1$) family of proteins has the most potent stimulatory effect on extracellular matrix deposition of any cytokines so far examined. In animal models of pulmonary fibrosis enhanced TGF-$\beta_1$ gene expression is temporally and spatially related to increased collagen gene expression and protein deposition. TGF-$\beta_1$ antibodies reduce collagen deposition in murine bleomycin-induced lung fibrosis and human fibrotic lung tissue shows enhanced TGF-$\beta_1$ gene and protein expression. Several lines of evidence suggest that TGF-β is a central regulator of pulmonary fibrosis. Several animal models over expressing TGF-β showed extensive progressive fibrosis but limited inflammation, indicating that TGF-β may play a predominant role in the progression of pulmonary fibrosis. Therapeutic efforts are therefore focusing on inhibition of TGF-β activity, for instance by anti-TGF-β1-antibodies, or modulators of TGF-β1 such as pirfenidone. Pirfenidone inhibits TGF-β1 gene expression *in vivo* resulting in inhibition of TGF-β1-mediated collagen synthesis and appears to slow progression of IPF in patients. Other novel, promising antifibrotic agents include relaxin (inhibits TGF-β-mediated overexpression of collagen and increases collagenases), suramin (inhibits growth factors), prostaglandin E2 (inhibits collagen production) and lovastatin (blocks formation of granulation tissue by induction of fibroblast apoptosis).

Diseases involving the lung associated with increased levels of TGF-ß include chronic lung disease of prematurity, idiopathic pulmonary fibrosis, rapid progressive pulmonary fibrosis, giant-cell interstitial pneumonia, acute rejection after lung transplantation, cytomegalovirus pneumonitis after lung transplantation, bronchiolitis obliterans, asbestosis, coal worker's pneumoconiosis, silicosis, histiocytosis, sarcoidosis, eosinophilic granuloma, scleroderma, systemic lupus erythematosus, lymphangioleiomyomatosis, central fibrosis in pulmonary adenocarcinoma, cystic fibrosis, chronic obstructive lung disease, and asthma.

*Increased TNF-α levels in fibrotic diseases*

An important role of tumor necrosis factor-α (TNF-α) in interstitial fibrosis has been established using transgenic mice, which either overexpress or display a deficiency of this cytokine. Mice transgenically modified to overexpress TNF-α develop lung fibrosis. In contrast, mice null for TNF-α show marked resistance to bleomycin

induced fibrosis. TNF-α can stimulate fibroblast replication and collagen synthesis in vitro, and pulmonary TNF-α gene expression rises after administration of bleomycin in mice. Soluble TNF-α receptors reduce lung fibrosis in murine models and pulmonary overexpression of TNF-α in transgenic mice is characterized by lung fibrosis. In patients with CFA or asbestosis, bronchoalveolar lavage fluid-derived macrophages release increased amounts of TNF-α compared with controls.

Increased TNF-α may induce fibrosis or fibrosis-associated conditions affecting any tissue including, for example, fibrosis of an internal organ, a cutaneous or dermal fibrosing disorder, and fibrotic conditions of the eye. Fibrosis of internal organs (e.g., liver, lung, kidney, heart blood vessels, gastrointestinal tract) occurs in disorders such as pulmonary fibrosis, idiopathic fibrosis, autoimmune fibrosis, myelofibrosis, liver cirrhosis, veno-occlusive disease, mesangial proliferative glomerulonephritis, crescentic glomerulonephritis, diabetic nephropathy, renal interstitial fibrosis, renal fibrosis in subjects receiving cyclosporin, allograft rejection, HTV associated nephropathy. Other fibrosis-associated disorders include systemic sclerosis, eosinophilia-myalgia syndrome, and fibrosis-associated CNS disorders such as intraocular fibrosis. Dermal fibrosing disorders include, for example, scleroderma, morphea, keloids, hypertrophic scars, familial cutaneous collagenoma, and connective tissue nevi of the collagen type. Fibrotic conditions of the eye include conditions such as diabetic retinopathy, post-surgical scarring (for example, after glaucoma filtering surgery and after crossed-eyes (strabismus) surgery), and proliferative vitreoretinopathy. Additional fibrotic conditions that may be treated by the methods of the present invention may result, for example, from rheumatoid arthritis, diseases associated with prolonged joint pain and deteriorated joints; progressive systemic sclerosis, polymyositis, dermatomyositis, eosinophilic fascitis, morphea, Raynaud's syndrome, and nasal polyposis.

*Increased matrix metalloproteases levels in fibrotic diseases*

The abnormal extracellular matrix (ECM) remodeling observed in the lungs of patients with interstitial pulmonary fibrosis (IPF) is due, at least in part, to an imbalance between matrix metalloproteases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). Normal lung fibroblasts do not make MMP-9 in vitro, whereas fibroblasts from IPF lungs strongly express MMP-9. In addition, fibroblasts from patients with IPF express increased levels of all TIMPs. In this setting, TIMPs may play a role in apoptosis in some cell populations. In vitro studies of alveolar macrophages obtained from untreated patients with idiopathic pulmonary fibrosis showed marked increase in MMP-9 secretion compared to macrophages collected from healthy individuals. In animals models of bleomycin-induced pulmonary fibrosis

MMPs have been shown to be elevated in bronchoalveolar lavage (BAL) fluid. Indeed, a synthetic inhibitor of MMP, Batimastat, has been shown to significantly reduce bleomycin-induced lung fibrosis, again pointing to the importance of MMPs in the development of this fibrotic disease in the lung. A number of studies have shown that the actions of MMPs can result in the release of growth factors and cytokines. These profibrotic factors require proteolytic processing for their activation or release from extracellular matrix or carrier proteins before they can exert their activity. In fact, the proteolytic activity processing of several key factors involved in the pathogenesis of pulmonary fibrosis such as insulin-like growth factor (IGF), TGF-$\beta_1$ and TNF-α occur through the actions of MMPs, thereby activating or releasing them from inhibitory protein-protein interactions. For example, IGFs in vivo are sequestered by six high affinity IGF binding proteins (IGFBPs1-6), preventing their ability to interact with IGF receptors. Studies examining adults and children IPF and interstitial lung disease show that beside IPF, IGFBP-3 and IFPB-2 levels are increased in IPF BAL fluid. MMPs have recently been shown to regulate the cleavage of IGF binding proteins, thereby liberating the complexed ligand to affect IGF actions in target cells. Observations have also shown that the gelatinases, MMP-9 and MMP-2 may be involved in proteolytic activation of latent TGF-β complexes. Furthermore, the MMP inhibitor Batimastat reduces MMP-9 activity in BAL fluid, which was associated with decreased amount of TGF-β and TNF-α.

Pulmonary fibrosis can be an all too common consequence of an acute inflammatory response of the lung to a host of inciting events. Chronic lung injury due to fibrotic changes can result from an identifiable inflammatory event or an insidious, unknown event. The inflammatory process can include infiltration of various inflammatory cell types, such as neutrophils and macrophages, the secretion of inflammatory cytokines and chemokines and the secretion of matrix remodeling proteinases.

*Increased CCL18 levels in fibrotic diseases*

The expression and regulation of cysteine-cysteine (CC) chemokine ligand 18 (CCL18), a marker of alternative activation, by human alveolar macrophages (AMs) is increased in patients with pulmonary fibrosis and correlates negatively with pulmonary function test parameters. Thus, CCL18 is an ideal diagnostic marker for pulmonary fibrosis.

The peptide and the peptide combination of the present invention were tested using the assays described in Examples 14 - 15 for their effect as active therapeutic agents in the prophylaxis and/or treatment of fibrotic diseases and disorders.

**Inflammatory disease**

Inflammation is the final common pathway of various insults, such as infection, trauma, and allergies to the human body. It is characterized by activation of the immune system with recruitment of inflammatory cells, production of pro-inflammatory cells and production of pro-inflammatory cytokines. Most inflammatory diseases and disorders are characterized by abnormal accumulation of inflammatory cells including monocytes/macrophages, granulocytes, plasma cells, lymphocytes and platelets. Along with tissue endothelial cells and fibroblasts, these inflammatory cells release a complex array of lipids, growth factors, cytokines and destructive enzymes that cause local tissue damage.

One form of inflammatory response is neutrophilic inflammation which is characterized by infiltration of the inflamed tissue by neutrophil polymorphonuclear leukocytes (PMN), which are a major component of the host defense. Tissue infection by extracellular bacteria represents the prototype of this inflammatory response. On the other hand, various non-infectious diseases are characterized by extravascular recruitment of neutrophils. This group of inflammatory diseases includes chronic obstructive pulmonary disease, adult respiratory distress syndrome, some types of immune-complex alveolitis, cystic fibrosis, bronchitis, bronchiectasis, emphysema, glomerulonephritis, rheumatoid arthritis, gouty arthritis, ulcerative colitis, certain dermatoses such as psoriasis and vasculitis. In these conditions neutrophils are thought to play a crucial role in the development of tissue injury which, when persistent, can lead to the irreversible destruction of the normal tissue architecture with consequent organ dysfunction. Tissue damage is primarily caused by the activation of neutrophils followed by their release of proteinases and increased production of oxygen species.

Chronic obstructive pulmonary disease (COPD) is described by the progressive development of airflow limitation that is not fully reversible. Most patients with COPD have three pathological conditions; bronchitis, emphysema and mucus plugging. This disease is characterized by a slowly progressive and irreversible decrease in forced expiratory volume in the first second of expiration (FEVi), with relative preservation of forced vital capacity (FVC). In both asthma and COPD there is significant, but distinct, remodeling of airways. Most of the airflow obstruction is due to two major components, alveolar destruction (emphysema) and small airways obstruction (chronic obstructive bronchitis). COPD is mainly characterized by profound mucus cell hyperplasia. Neutrophil infiltration of the patient's lungs is a primary characteristic of COPD. Elevated levels of proinflammatory cytokines, like TNF-α, and especially chemokines like interleukin-8 (IL-8) and growth-regulated

oncogene-α (GRO-α) play a very important role in pathogenesis of this disease. Platelet thromboxane synthesis is also enhanced in patients with COPD. Most of the tissue damage is caused by activation of neutrophils followed by their release of metalloproteinases, and increased production of oxygen species.

TNF-α has several biologic activities that are important in homeostasis as well as in pathophysiological conditions. The main sources of TNF-α are monocytes-macrophages, T-lymphocytes and mast cells. The finding that anti-TNF-α antibodies (cA2) are effective in the treatment of patients suffering from rheumatoid arthritis (RA) intensified the interest to find new TNF-α inhibitors as possible potent medicaments for RA. Rheumatoid arthritis is an autoimmune chronic inflammatory disease characterized by irreversible pathological changes of the joints. In addition to RA, TNF-α antagonists are also applicable to several other pathological conditions and diseases such as spondylitis, osteoarthritis, gout and other arthritic conditions, sepsis, septic shock, toxic shock syndrome, atopic dermatitis, contact dermatitis, psoriasis, glomerulonephritis, lupus erythematosus, scleroderma, asthma, cachexia, chronic obstructive lung disease, congestive heart failure, insulin resistance, lung (pulmonary) fibrosis, multiple sclerosis, Crohn's disease, ulcerative colitis, viral infections and AIDS.

The term "immunoinflammatory disorder" encompasses a variety of conditions, including autoimmune diseases, proliferative skin diseases, and inflammatory dermatoses. Immunoinflammatory disorders result in the destruction of healthy tissue by an inflammatory process, dysregulation of the immune system, and unwanted proliferation of cells. Examples of immunoinflammatory disorders are acne vulgaris; acute respiratory distress syndrome; Addison's disease; allergic rhinitis; allergic intraocular inflammatory diseases, antineutrophil cytoplasmic antibody (ANCA)-associated small-vessel vasculitis; ankylosing spondylitis; arthritis, asthma; atherosclerosis; atopic dermatitis; autoimmune hepatitis; autoimmune hemolytic anemia; autoimmune hepatitis; Behcet's disease; Bell's palsy; bullous pemphigoid; cerebral ischemia; chronic obstructive pulmonary disease; cirrhosis; Cogan's syndrome; contact dermatitis; COPD; Crohn's disease; Cushing's syndrome; dermatomyositis; diabetes mellitus; discoid lupus erythematosus; eosinophilic fasciitis; erythema nodosum; exfoliative dermatitis; fibromyalgia; focal glomerulosclerosis; focal segmental glomerulosclerosis; giant cell arteritis; gout; gouty arthritis; graft versus host disease; hand eczema; Henoch-Schonlein purpura; herpes gestationis; hirsutism; idiopathic cerato-scleritis; idiopathic pulmonary fibrosis; idiopathic thrombocytopenic purpura; immune thrombocytopenic purpura inflammatory bowel or gastrointestinal disorders, inflammatory dermatoses; lichen

planus; lupus nephritis; lymphomatous tracheobronchitis; macular edema; multiple sclerosis; myasthenia gravis; myositis; nonspecific fibrosing lung disease; osteoarthritis; pancreatitis; pemphigoid gestationis; pemphigus vulgaris; periodontitis; polyarteritis nodosa; polymyalgia rheumatica; pruritus scroti; pruritis/inflammation, psoriasis; psoriatic arthritis; pulmonary histoplasmosis; rheumatoid arthritis; relapsing polychondritis; rosacea caused by sarcoidosis; rosacea caused by scleroderma; rosacea caused by Sweet's syndrome; rosacea caused by systemic lupus erythematosus; rosacea caused by urticaria; rosacea caused by zoster-associated pain; sarcoidosis; scleroderma; segmental glomerulosclerosis; septic shock syndrome; shoulder tendinitis or bursitis; Sjogren's syndrome; Still's disease; stroke-induced brain cell death; Sweet's disease; systemic lupus erythematosus; systemic sclerosis; Takayasu's arteritis; temporal arteritis; toxic epidermal necrolysis; transplant-rejection and transplant-rejection-related syndromes; tuberculosis; type-1 diabetes; ulcerative colitis; uveitis; vasculitis; and Wegener's granulomatosis.

As used herein, "non-dermal inflammatory disorders" include, for example, rheumatoid arthritis, inflammatory bowel disease, asthma, and chronic obstructive pulmonary disease. By "dermal inflammatory disorders" or "inflammatory dermatoses" is meant an inflammatory disorder selected from psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis, erythrodermic psoriasis, acute febrile neutrophilic dermatosis, eczema, asteatotic eczema, dyshidrotic eczema, vesicular palmoplantar eczema, acne vulgaris, atopic dermatitis, contact dermatitis, allergic contact dermatitis, dermatomyositis, exfoliative dermatitis, hand eczema, pompholyx, rosacea, rosacea caused by sarcoidosis, rosacea caused by scleroderma, rosacea caused by Sweet's syndrome, rosacea caused by systemic lupus erythematosus, rosacea caused by urticaria, rosacea caused by zoster-associated pain, Sweet's disease, neutrophilic hidradenitis, sterile pustulosis, drug eruptions, seborrheic dermatitis, pityriasis rosea, cutaneous kikuchi disease, pruritic urticarial papules and plaques of pregnancy, Stevens-Johnson syndrome and toxic epidermal necrolysis, tattoo reactions, Wells syndrome (eosinophilic cellulitis), reactive arthritis (Reiter's syndrome), bowel-associated dermatosis-arthritis syndrome, rheumatoid neutrophilic dermatosis, neutrophilic eccrine hidradenitis, neutrophilic dermatosis of the dorsal hands, balanitis circumscripta plasmacellularis, balanoposthitis, Behcet's disease, erythema annulare centrifugum, erythema dyschromicum perstans, erythema multiforme, granuloma annulare, hand dermatitis, lichen nitidus, lichen planus, lichen sclerosus et atrophicus, lichen simplex chronicus, lichen spinulosus, nummular dermatitis, pyoderma gangrenosum, sarcoidosis, subcorneal pustular dermatosis, urticaria, and transient acantholytic dermatosis.

By "proliferative skin disease" is meant a benign or malignant disease that is characterized by accelerated cell division in the epidermis or dermis. Examples of proliferative skin diseases are psoriasis, atopic dermatitis, nonspecific dermatitis, primary irritant contact dermatitis, allergic contact dermatitis, basal and squamous cell carcinomas of the skin, lamellar ichthyosis, epidermolytic hyperkeratosis, premalignant keratosis, acne, and seborrheic dermatitis. As will be appreciated by one skilled in the art, a particular disease, disorder, or condition may be characterized as being both a proliferative skin disease and an inflammatory dermatosis. An example of such a disease is psoriasis.

Symptoms and signs of inflammation associated with specific conditions include:

• rheumatoid arthritis:- pain, swelling, warmth and tenderness of the involved joints; generalized and morning stiffness;

• insulin-dependent diabetes mellitus-insulitis; this condition can lead to a variety of complications with an inflammatory component, including:- retinopathy, neuropathy, nephropathy; coronary artery disease, peripheral vascular disease, and cerebrovascular disease;

• autoimmune thyroiditis:- weakness, constipation, shortness of breath, puffiness of the face, hands and feet, peripheral edema, bradycardia;

• multiple sclerosis:- spasticity, blurry vision, vertigo, limb weakness, paresthesias;

• uveoretinitis:- decreased night vision, loss of peripheral vision;

• lupus erythematosus:- joint pain, rash, photosensitivity, fever, muscle pain, puffiness of the hands and feet, abnormal urinalysis (hematuria, cylinduria, proteinuria), glomerulonephritis, cognitive dysfunction, vessel thrombosis, pericarditis;

• scleroderma:- Raynaud's disease; swelling of the hands, arms, legs and face; skin thickening; pain, swelling and stiffness of the fingers and knees, gastrointestinal dysfunction, restrictive lung disease; pericarditis; renal failure;

• other arthritic conditions having an inflammatory component such as rheumatoid spondylitis, osteoarthritis, septic arthritis and polyarthritis:- fever, pain, swelling, tenderness;

• other inflammatory brain disorders, such as meningitis, Alzheimer's disease, AIDS dementia encephalitis:- photophobia, cognitive dysfunction, memory loss;

• other inflammatory eye inflammations, such as retinitis:- decreased visual acuity;

• inflammatory skin disorders, such as , eczema, other dermatites (e.g., atopic, contact), psoriasis, burns induced by UV radiation (sun rays and similar UV sources):- erythema, pain, scaling, swelling, tenderness;

• inflammatory bowel disease, such as Crohn's disease, ulcerative colitis:- pain, diarrhea, constipation, rectal bleeding, fever, arthritis;

• asthma:- shortness of breath, wheezing;
• other allergy disorders, such as allergic rhinitis:- sneezing, itching, runny nose
• conditions associated with acute trauma such as cerebral injury following stroke-sensory loss, motor loss, cognitive loss;
• heart tissue injury due to myocardial ischemia:- pain, shortness of breath;
• lung injury such as that which occurs in adult respiratory distress syndrome:- shortness of breath, hyperventilation, decreased oxygenation, pulmonary infiltrates;
• inflammation accompanying infection, such as sepsis, septic shock, toxic shock syndrome:- fever, respiratory failure, tachycardia, hypotension, leukocytosis;
• other inflammatory conditions associated with particular organs or tissues, such as:
(i) nephritis (e.g., glomeralonephritis):-oliguria, abnormal urinalysis;
(ii) inflamed appendix:- fever, pain, tenderness, leukocytosis;
(iii) gout:- pain, tenderness, swelling and erythema of the involved joint, elevated serum and/or urinary uric acid;
(iv) inflamed gall bladder:- abdominal pain and tenderness, fever, nausea, leukocytosis;
(v) congestive heart failure:- shortness of breath, rales, peripheral edema;
(vi) Type II diabetes:- end organ complications including cardiovascular, ocular, renal, and peripheral vascular disease;
(vii) lung (pulmonary) fibrosis:- hyperventilation, shortness of breath, decreased oxygenation;
(viii) vascular disease, such as atherosclerosis and restenosis:- pain, loss of sensation, diminished pulses, loss of function; and
(ix) alloimmunity leading to transplant rejection:- pain, tenderness, fever.

A human peptide is "active" in an inflammatory disease if the inhibition is > 50% in one of the assays described below. Inhibition (as percentage) was calculated using the following formula: % inhibition = (1 - concentration of cytokines in sample/concentration of cytokines in positive control) x 100. The positive control refers to stimulated samples, not treated with substances.

**Th1/Th2 cytokine profiles**

T lymphocytes are a major source of cytokines. These cells bear antigen specific receptors on their cell surface to allow recognition of foreign pathogens. They can also recognize normal tissue during episodes of autoimmune diseases. There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4 and CD8. T lymphocytes expressing CD4 are also known as helper T cells, and these are regarded as being the most prolific cytokine

producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines.

Th1-type cytokines tend to produce the pro-inflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. Interferon gamma is the main Th1 cytokine. Excessive pro-inflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this. The Th2-type cytokines include Interleukin 4, Interleukin 5, and Interleukin 13, which are associated with the promotion of IgE and eosinophilic responses in atopy, and also Interleukin-10, which has more of an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action. The optimal scenario would therefore seem to be that humans should produce a well balanced Th1 and Th2 response, suited to the immune challenge.

Allergy is regarded as a Th2 weighted imbalance. Pregnancy and early postnatal life are also viewed as Th2 phenomena (to reduce the risk of miscarriage, a strong Th2 response is necessary to modify the Th1 cellular response in utero). The fetus can switch on an immune response early in pregnancy, and because pregnancy is chiefly a Th2 situation, babies tend to be born with Th2 biased immune responses. These can be switched off rapidly postnatally under the influence of microbiological exposure or can be enhanced by early exposure to allergens.

Th1 cells produce interferon-gamma, Interleukin 2, and tumor necrosis factor-beta, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. By contrast, Th2 cells produce cytokines which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. Th1 cells mainly develop following infections by intracellular bacteria and some viruses, whereas Th2 cells predominate in response to infestations by gastrointestinal nematodes. Polarized Th1 and Th2 cells not only exhibit different functional properties, but also show the preferential expression of some activation markers and distinct transcription factors. Several mechanisms may influence the Th cell differentiation, which include the cytokine profile of "natural immunity" evoked by different offending agents, the nature of the peptide ligand, as well as the activity of some costimulatory molecules and microenvironmentally secreted hormones, in the context of the individual genetic background. In addition to playing different roles in protection, polarized Th1-type and Th2-type responses are also responsible for different types of immunopathological reactions. Th1 cells are involved in the pathogenesis of organ-specific autoimmune disorders like Crohn's disease is one, Helicobacter pylori-induced peptic ulcer, acute kidney allograft rejection, unexplained recurrent abortions, tuberculosis, myocarditis, multiple

sclerosis, scleroderma, Type 1 diabetes, rheumatoid arthritis (RA), sarcoidosis, autoimmune thyroiditis and uveitis. In contrast, allergen-specific Th2 responses are responsible for atopic disorders in genetically susceptible individuals like asthma. Moreover, Th2 responses against still unknown antigens predominate in Omenn's syndrome, idiopathic pulmonary fibrosis, and progressive systemic sclerosis. Finally, the prevalence of Th2 responses may play some role in a more rapid evolution of human immunodeficiency virus infection to the full-blown disease.

It is a further object of current application to find peptides which produce a well balanced Th1 and Th2 response in situations of Th1 predominance, in the situation of Th2 predominance, or even at situations where both, Th1 and Th2 cells are too much activated, as exemplified after severe infections.

Table 1: Infections in humans associated with autoimmune diseases

| Disease | Infection |
|---|---|
| Multiple sclerosis | Epstein-Barr virus (EBV), measles virus |
| Lyme arthritis | *Borrelia burgdorferi* |
| Type I diabetes | Coxsackie virus B4, rubella virus, cytomegalovirus (CMV), mumps virus |
| Rheumatoid arthritis | *Escherichia coli*, mycobacteria, EBV, hepatitis C virus (HCV) |
| Lupus erythematosis | EBV |
| Myocarditis | CB3, CMV, chlamydia |
| Rheumatic fever/myocarditis | Streptococci |
| Chagas' disease/myocarditis | *Trypanosoma cruzi* |
| Myasthenia gravis | Herpes simplex virus, HCV |

The peptide or the peptide combination of the present invention were tested using the assays described in Examples 1-7, 9-17 for their effect as active therapeutic agents in the prophylaxis and/or treatment of inflammatory diseases and disorders.

**Neurodegenerative disease**

The present invention also relates generally to the fields of neurology and psychiatry and to methods of protecting the cells of a mammalian central nervous system from damage or injury.

Injuries or trauma of various kinds to the central nervous system (CNS) or the peripheral nervous system (PNS) can produce profound and long-lasting neurological and/or psychiatric symptoms and disorders. One form that this can take is the

progressive death of neurons or other cells of the central nervous system (CNS), i.e., neurodegeneration or neuronal degeneration.

Neuronal degeneration as a result of, for example; Alzheimer's disease, multiple sclerosis, cerebral-vascular accidents (CVAs)/stroke, traumatic brain injury, spinal cord injuries, degeneration of the optic nerve, e.g., ischemic optic neuropathy or retinal degeneration and other central nervous system disorders is an enormous medical and public health problem by virtue of both its high incidence and the frequency of long-term sequelae. Animal studies and clinical trials have shown that amino acid transmitters (especially glutamate), oxidative stress and inflammatory reactions contribute strongly to cell death in these conditions. Upon injury or upon ischemic insult, damaged neurons release massive amounts of the neurotransmitter glutamate, which is excitotoxic to the surrounding neurons. Glutamate is a negatively charged amino acid that is an excitatory synaptic transmitter in the mammalian nervous system. Although the concentration of glutamate can reach the millimolar range in nerve terminals its extracellular concentration is maintained at a low level to prevent neurotoxicity. It has been noted that glutamate can be toxic to neurons if presented at a high concentration. The term "excitotoxicity" has been used to describe the cytotoxic effect that glutamate (and other such excitatory amino acids) can have on neurons when applied at high dosages.

Patients with injury or damage of any kind to the central (CNS) or peripheral (PNS) nervous system including the retina may benefit from neuroprotective methods. This nervous system injury may take the form of an abrupt insult or an acute injury to the nervous system as in, for example, acute neurodegenerative disorders including, but not limited to; acute injury, hypoxia-ischemia or the combination thereof resulting in neuronal cell death or compromise. Acute injury includes, but is not limited to, traumatic brain injury (TBI) including, closed, blunt or penetrating brain trauma, focal brain trauma, diffuse brain damage, spinal cord injury, intracranial or intravertebral lesions (including, but not limited to, contusion, penetration, shear, compression or laceration lesions of the spinal cord or whiplash shaken infant syndrome).

In addition, deprivation of oxygen or blood supply in general can cause acute injury as in hypoxia and/or ischemia including, but not limited to, cerebrovascular insufficiency, cerebral ischemia or cerebral infarction (including cerebral ischemia or infarctions originating from embolic occlusion and thrombosis, retinal ischemia (diabetic or otherwise), glaucoma, retinal degeneration, multiple sclerosis, toxic and ischemic optic neuropathy, reperfusion following acute ischemia, perinatal hypoxic-

ischemic injury, cardiac arrest or intracranial hemorrhage of any type (including, but not limited to, epidural, subdural, subarachnoid or intracerebral hemorrhage).

Trauma or injury to tissues of the nervous system may also take the form of more chronic and progressive neurodegenerative disorders, such as those associated with progressive neuronal cell death or compromise over a period of time including, but not limited to, Alzheimer's disease, Pick's disease, diffuse Lewy body disease, progressive supranuclear palsy (Steel-Richardson syndrome), multisystem degeneration (Shy-Drager syndrome), chronic epileptic conditions associated with neurodegeneration, motor neuron diseases (amyotrophic lateral sclerosis), multiple sclerosis, degenerative ataxias, cortical basal degeneration, ALS-Parkinson's-dementia complex of Guam, subacute sclerosing panencephalitis, Huntington's disease, Parkinson's disease, synucleinopathies (including multiple system atrophy), primary progressive aphasia, striatonigral degeneration, Machado-Joseph disease or spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, bulbar and pseudobulbar palsy, spinal and spinobulbar muscular atrophy (Kennedy's disease), primary lateral sclerosis, familial spastic paraplegia, Werdnig-Hoffmann disease, Kugelberg-Welander disease, Tay-Sach's disease, Sandhoff disease, familial spastic disease, Wohlfart-Kugelberg-Welander disease, spastic paraparesis, progressive multifocal leukoencephalopathy, familial dysautonomia (Riley-Day syndrome) or prion diseases (including, but not limited to Creutzfeld-Jakob disease, Gerstmann-Strussler-Scheinker disease, Kuru disease or fatal familial insomnia).

In addition, trauma and progressive injury to the nervous system can take place in various psychiatric disorders, including but not limited to, progressive, deteriorating forms of bipolar disorder or schizoaffective disorder or schizophrenia, impulse control disorders, obsessive compulsive disorder (OCD), behavioral changes in temporal lobe epilepsy and personality disorders.

In one preferred embodiment the compounds of the invention would be used to provide neuroprotection in disorders involving trauma and progressive injury to the nervous system in various psychiatric disorders. These disorders would be selected from the group consisting of; schizoaffective disorder, schizophrenia, impulse control disorders, obsessive compulsive disorder (OCD) and personality disorders.

In addition, trauma and injury make take the form of disorders associated with overt and extensive memory loss including, but not limited to, neurodegenerative disorders associated with age-related dementia, vascular dementia, diffuse white matter disease (Binswanger's disease), dementia of endocrine or metabolic origin, dementia

of head trauma and diffuse brain damage, dementia pugilistica or frontal lobe dementia, including but not limited to Pick's Disease.

Other disorders associated with neuronal injury include, but are not limited to, disorders associated with chemical, toxic, infectious and radiation injury of the nervous system including the retina, injury during fetal development, prematurity at time of birth, anoxic-ischemia, injury from hepatic, glycemic, uremic, electrolyte and endocrine origin, injury of psychiatric origin (including, but not limited to, psychopathology, depression or anxiety), injury from peripheral diseases and plexopathies (including plexus palsies) or injury from neuropathy (including neuropathy selected from multifocal, sensory, motor, sensory-motor, autonomic, sensory-autonomic or demyelinating neuropathies (including, but not limited to Guillain-Barre syndrome or chronic inflammatory demyelinating polyradiculoneuropathy) or those neuropathies originating from infections, inflammation, immune disorders, drug abuse, pharmacological treatments, toxins, trauma (including, but not limited to compression, crush, laceration or segmentation traumas), metabolic disorders (including, but not limited to, endocrine or paraneoplastic), Charcot-Marie-Tooth disease (including, but not limited to, type 1a, 1b, 2, 4a or 1-X linked), Friedreich's ataxia, metachromatic leukodystrophy, Refsum's disease, adrenomyeloneuropathy, ataxia-telangiectasia, Djerine-Sottas (including, but not limited to, types A or B), Lambert-Eaton syndrome or disorders of the cranial nerves).

Further indications are cognitive disorders. The term "cognitive disorder" shall refer to anxiety disorders, delirium, dementia, amnestic disorders, dissociative disorders, eating disorders, mood disorders, schizophrenia, psychotic disorders, sexual and gender identity disorders, sleep disorders, somatoform disorders, acute stress disorder, obsessive-compulsive disorder, panic disorder, posttraumatic stress disorder, specific phobia, social phobia, substance withdrawal delirium, Alzheimer's disease, Creutzfeldt-Jakob disease, head trauma, Huntington's disease, HIV disease, Parkinson's disease, Pick's disease, learning disorders, motor skills disorders, developmental coordination disorder, communication disorders, phonological disorder, pervasive developmental disorders, Asperger's disorder, autistic disorder, childhood disintegrative disorder, Rett's disorder, pervasive developmental disorder, attention-deficit/hyperactivity disorder (ADHD), conduct disorder, oppositional defiant disorder, pica, rumination disorder, tic disorders, chronic motor or vocal tic disorder, Tourette's disorder, elimination disorders, encopresis, enuresis, selective mutism, separation anxiety disorder, dissociative amnesia, depersonalization disorder, dissociative fugue, dissociative identity disorder, anorexia nervosa, bulimia nervosa,

bipolar disorders, schizophreniform disorder, schizoaffective disorder, delusional disorder, psychotic disorder, shared psychotic disorder, delusions, hallucinations, substance-induced psychotic disorder, orgasmic disorders, sexual pain disorders, dyspareunia, vaginismus, sexual dysfunction, paraphilias, dyssomnias, breathing-related sleep disorder, circadian rhythm sleep disorder, hypersomnia, insomnia, narcolepsy, dyssomnia, parasomnias, nightmare disorder, sleep terror disorder, sleepwalking disorder, parasomnia, body dysmorphic disorder, conversion disorder, hypochondriasis, pain disorder, somatization disorder, alcohol related disorders, amphetamine related disorders, caffeine related disorders, cannabis related disorders, cocaine related disorders, hallucinogen related disorders, inhalant related disorders, nicotine related disorders, opioid related disorders, phencyclidine-related disorder, abuse, persisting amnestic disorder, intoxication, withdrawal.

The term "bipolar and clinical disorders" shall refer to adjustment disorders, anxiety disorders, delirium, dementia, amnestic and other cognitive disorders, disorders usually first diagnosed in infancy (e.g. ), childhood, or adolescence, dissociative disorders (e.g. dissociative amnesia, depersonalization disorder, dissociative fugue and dissociative identity disorder), eating disorders, factitious disorders, impulse-control disorders, mental disorders due to a general medical condition, mood disorders, other conditions that may be a focus of clinical attention, personality disorders, schizophrenia and other psychotic disorders, sexual and gender identity disorders, sleep disorders, somatoform disorders, substance-related disorders, generalized anxiety disorder (e.g. acute stress disorder, posttraumatic stress disorder), panic disorder, phobia, agoraphobia, obsessive-compulsive disorder, stress, acute stress disorder, anxiety neurosis, nervousness, phobia, posttraumatic stress disorder, posttraumatic stress disorder (PTSD), abuse, obsessive-compulsive disorder (OCD), manic depressive psychosis, specific phobias, social phobia, adjustment disorder with anxious features.

Examples for disorders usually first diagnosed in infancy, childhood, or adolescence are: mental retardation, learning disorders, mathematics disorder, reading disorder, disorder of written expression, motor skills disorders, developmental coordination disorder, communication disorders, expressive language disorder, phonological disorder, mixed receptive-expressive language disorder, stuttering, pervasive developmental disorders, Asperger's disorder, autistic disorder, childhood disintegrative disorder, Rett's disorder, pervasive developmental disorder, attention-deficit/hyperactivity disorder (ADHD), conduct disorder, oppositional defiant disorder, feeding disorder of infancy or early childhood, pica, rumination disorder, tic disorders, chronic motor or vocal tic disorder, Tourette's syndrome, elimination disorders,

encopresis, enuresis, selective mutism, separation anxiety disorder, reactive attachment disorder of infancy or early childhood, stereotypic movement disorder.

Examples for substance-related disorders are: alcohol related disorders, amphetamine related disorders, caffeine related disorders, cannabis related disorders, cocaine related disorders, hallucinogen related disorders, inhalant related disorders, nicotine related disorders, opioid related disorders, psychotic disorder, psychotic disorder, phencyclidine-related disorder, abuse, persisting amnestic disorder, anxiety disorder, persisting dementia, dependence, intoxication, intoxication delirium, mood disorder, psychotic disorder, withdrawal, withdrawal delirium, sexual dysfunction, sleep disorder.

The term "neuroprotection" as used herein shall mean; inhibiting, preventing, ameliorating or reducing the severity of the dysfunction, degeneration or death of nerve cells, axons or their supporting cells in the central or peripheral nervous system of a mammal, including a human. This includes the treatment or prophylaxis of a neurodegenerative disease; protection against excitotoxicity or ameliorating the cytotoxic effect of a compound (for example, a excitatory amino acid such as glutamate; a toxin; or a prophylactic or therapeutic compound that exerts an immediate or delayed cytotoxic side effect including but not limited to the immediate or delayed induction of apoptosis) in a patient in need thereof.

The term "a patient in need of treatment with a neuroprotective drug " as used herein will refer to any patient who currently has or may develop any of the above syndromes or disorders, or any disorder in which the patient's present clinical condition or prognosis could benefit from providing neuroprotection to prevent the development, extension, worsening or increased resistance to treatment of any neurological or psychiatric disorder.

The term "treating" or "treatment" as used herein, refers to any indicia of success in the prevention or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology, or condition more tolerable to the patient; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; or improving a subject's physical or mental well-being. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination, neurological examination, and/or psychiatric evaluations.

In some embodiments this invention provides methods of neuroprotection. In certain embodiments, these methods comprise administering a therapeutically effective amount of the peptide combination of the invention to a patient who has not yet developed overt, clinical signs or symptoms of injury or damage to the cells of the nervous system but who may be in a high risk group for the development of neuronal damage because of injury or trauma to the nervous system or because of some known predisposition either biochemical or genetic or the finding of a verified biomarker of one or more of these disorders.

Thus, in some embodiments, the methods and compositions of the present invention are directed toward neuroprotection in a subject who is at risk of developing neuronal damage but who has not yet developed clinical evidence. This patient may simply be at "greater risk" as determined by the recognition of any factor in a subject's, or their families, medical history, physical exam or testing that is indicative of a greater than average risk for developing neuronal damage. Therefore, this determination that a patient may be at a "greater risk" by any available means can be used to determine whether the patient should be treated with the methods of the present invention.

Accordingly, in an exemplary embodiment, subjects who may benefit from treatment by the methods and the peptide or the peptide combination of this invention can be identified using accepted screening methods to determine risk factors for neuronal damage. These screening methods include, for example, conventional work-ups to determine risk factors including but not limited to: for example, head trauma, either closed or penetrating, CNS infections, bacterial or viral, cerebrovascular disease including but not limited to stroke, brain tumors, brain edema, cysticercosis, porphyria, metabolic encephalopathy, drug withdrawal including but not limited to sedative-hypnotic or alcohol withdrawal, abnormal perinatal history including anoxia at birth or birth injury of any kind, cerebral palsy, learning disabilities, hyperactivity, history of febrile convulsions as a child, history of status epilepticus, family history of epilepsy or any seizure related disorder, inflammatory disease of the brain including lupis, drug intoxication either direct or by placental transfer, including but not limited to cocaine poisoning, parental consanguinity, and treatment with medications that are toxic to the nervous system including psychotropic medications.

The determination of which patients may benefit from treatment with a neuroprotective drug in patients who have no clinical signs or symptoms may be based on a variety of "surrogate markers" or "biomarkers".

As used herein, the terms "surrogate marker" and "biomarker" are used interchangeably and refer to any anatomical, biochemical, structural, electrical, genetic or chemical indicator or marker that can be reliably correlated with the present existence or future development of neuronal damage. In some instances, brain-imaging techniques, such as computer tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET), can be used to determine whether a subject is at risk for neuronal damage. Suitable biomarkers for the methods of this invention include, but are not limited to: the determination by MRI, CT or other imaging techniques, of sclerosis, atrophy or volume loss in the hippocampus or overt mesial temporal sclerosis (MTS) or similar relevant anatomical pathology; the detection in the patient's blood, serum or tissues of a molecular species such as a protein or other biochemical biomarker, e.g., elevated levels of ciliary neurotrophic factor (CNTF) or elevated serum levels of a neuronal degradation product; or other evidence from surrogate markers or biomarkers that the patient is in need of treatment with a neuroprotective drug.

It is expected that many more such biomarkers utilizing a wide variety of detection techniques will be developed in the future. It is intended that any such marker or indicator of the existence or possible future development of neuronal damage, as the latter term is used herein, may be used in the methods of this invention for determining the need for treatment with the compounds and methods of this invention.

A determination that a subject has, or may be at risk for developing, neuronal damage would also include, for example, a medical evaluation that includes a thorough history, a physical examination, and a series of relevant bloods tests. It can also include an electroencephalogram (EEG), CT, MRI or PET scan. A determination of an increased risk of developing neuronal damage or injury may also be made by means of genetic testing, including gene expression profiling or proteomic techniques. For psychiatric disorders that may be stabilized or improved by a neuroprotective drug, e.g., bipolar disorder, schizoaffective disorder, schizophrenia, impulse control disorders, etc. the above tests may also include a present state exam and a detailed history of the course of the patients symptoms such as mood disorder symptoms and psychotic symptoms over time and in relation to other treatments the patient may have received over time, e.g., a life chart. These and other specialized and routine methods allow the clinician to select patients in need of therapy using the methods and formulations of this invention. In some embodiments of the present invention peptides suitable for use in the practice of this invention will be administered either singly or concomitantly with at least one or more

other compounds or therapeutic agents, e.g., with other neuroprotective drugs or antiepileptic drugs, anticonvulsant drugs. In these embodiments, the present invention provides methods to treat or prevent neuronal injury in a patient. The method includes the step of; administering to a patient in need of treatment, an effective amount of one of the peptides disclosed herein in combination with an effective amount of one or more other compounds or therapeutic agents that have the ability to provide neuroprotection or to treat or prevent seizures or epileptogenesis or the ability to augment the neuroprotective effects of the compounds of the invention.

As used herein the term "combination administration" of a compound, therapeutic agent or known drug with the peptide combination of the present invention means administration of the drug and the one or more compounds at such time that both the known drug and the peptide combination will have a therapeutic effect. In some cases this therapeutic effect will be synergistic. Such concomitant administration can involve concurrent (i.e. at the same time), prior, or subsequent administration of the drug with respect to the administration of the peptide combination of the present invention. A person of ordinary skill in the art would have no difficulty determining the appropriate timing, sequence and dosages of administration for particular drugs and peptides of the present invention.

The said one or more other compounds or therapeutic agents may be selected from compounds that have one or more of the following properties: antioxidant activity; NMDA receptor antagonist activity, augmentation of endogenous GABA inhibition; NO synthase inhibitor activity; iron binding ability, e.g., an iron chelator; calcium binding ability, e.g., a Ca (II) chelator; zinc binding ability, e.g., a Zn (II) chelator; the ability to effectively block sodium or calcium ion channels, or to open potassium or chloride ion channels in the CNS of a patient.

The peptide or the peptide combination of the present invention were tested using the assays described in Examples 1-7, 9-17 for their effect as active therapeutic agents in the prophylaxis and/or treatment of neurodegenerative diseases and disorders.

**Heart and vascular disease**

Heart disease is a general term used to describe many different heart conditions. For example, coronary artery disease, which is the most common heart disease, is characterized by constriction or narrowing of the arteries supplying the heart with oxygen-rich blood, and can lead to myocardial infarction, which is the death of a

portion of the heart muscle. Heart failure is a condition resulting from the inability of the heart to pump an adequate amount of blood through the body. Heart failure is not a sudden, abrupt stop of heart activity but, rather, typically develops slowly over many years, as the heart gradually loses its ability to pump blood efficiently. Risk factors for heart failure include coronary artery disease, hypertension, valvular heart disease, cardiomyopathy, disease of the heart muscle, obesity, diabetes, and/or a family history of heart failure.

Examples of cardiovascular diseases and disorders are: aneurysm, stable angina, unstable angina, angina pectoris, angioneurotic edema, aortic valve stenosis, aortic aneurysm, arrhythmia, arrhythmogenic right ventricular dysplasia, arteriosclerosis, arteriovenous malformations, atrial fibrillation, Behcet syndrome, bradycardia, cardiac tamponade, cardiomegaly, congestive cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, carotid stenosis, cerebral hemorrhage, Churg-Strauss syndrome, diabetes, Ebstein's Anomaly, Eisenmenger complex, cholesterol embolism, bacterial endocarditis, fibromuscular dysplasia, congenital heart defects, heart diseases, congestive heart failure, heart valve diseases, heart attack, epidural hematoma, hematoma, subdural, Hippel-Lindau disease, hyperemia, hypertension, pulmonary hypertension, cardiac hypertrophy, left ventricular hypertrophy, right ventricular hypertrophy, hypoplastic left heart syndrome, hypotension, intermittent claudication, ischemic heart disease, Klippel-Trenaunay-Weber syndrome, lateral medullary syndrome, long QT syndrome mitral valve prolapse, moyamoya disease, mucocutaneous lymph node syndrome, myocardial infarction, myocardial ischemia, myocarditis, pericarditis, peripheral vascular diseases, phlebitis, polyarteritis nodosa, pulmonary atresia, Raynaud disease, Sneddon syndrome, superior vena cava syndrome, syndrome X, tachycardia, Takayasu's arteritis, hereditary hemorrhagic telangiectasia, telangiectasis, temporal arteritis, tetralogy of Fallot, thromboangiitis obliterans, thrombosis, thromboembolism, tricuspid atresia, varicose veins, vascular diseases, vasculitis, vasospasm, ventricular fibrillation, Williams syndrome, peripheral vascular disease, varicose veins and leg ulcers, deep vein thrombosis, Wolff-Parkinson-White syndrome.

Vascular diseases are often the result of decreased perfusion in the vascular system or physical or biochemical injury to the blood vessel.

Peripheral vascular disease (PVD) is defined as a disease of blood vessels often encountered as narrowing of the vessels of the limbs. There are two main types of these disorders, functional disease which doesn't involve defects in the blood vessels but rather arises from stimuli such as cold, stress, or smoking, and organic disease

which arises from structural defects in the vasculature such as atherosclerotic lesions, local inflammation, or traumatic injury. This can lead to occlusion of the vessel, aberrant blood flow, and ultimately to tissue ischemia.

One of the more clinically significant forms of PVD is peripheral artery disease (PAD). PAD is often treated by angioplasty and implantation of a stent or by artery bypass surgery. Clinical presentation depends on the location of the occluded vessel. For example, narrowing of the artery that supplies blood to the intestine can result in severe postprandial pain in the lower abdomen resulting from the inability of the occluded vessel to meet the increased oxygen demand arising from digestive and absorptive processes. In severe forms the ischemia can lead to intestinal necrosis. Similarly, PAD in the leg can lead to intermittent pain, usually in the calf, that comes and goes with activity. This disorder is known as intermittent claudication (IC) and can progress to persistent pain while resting, ischemic ulceration, and even amputation.

Peripheral vascular disease is also manifested in atherosclerotic stenosis of the renal artery, which can lead to renal ischemia and kidney dysfunction.

One disease in which vascular diseases and their complications are very common is diabetes mellitus. Diabetes mellitus causes a variety of physiological and anatomical irregularities, the most prominent of which is the inability of the body to utilize glucose normally, which results in hyperglycemia. Chronic diabetes can lead to complications of the vascular system which include atherosclerosis, abnormalities involving large and medium size blood vessels (macroangiopathy) and abnormalities involving small blood vessels (microangiopathy) such as arterioles and capillaries.

Patients with diabetes mellitus are at increased risk of developing one or more foot ulcers as a result of established long-term complications of the disease, which include impaired nerve function (neuropathy) and/or ischemia. Local tissue ischemia is a key contributing factor to diabetic foot ulceration.

In addition to large vessel disease, patients with diabetes suffer further threat to their skin perfusion in at least two additional ways. First, by involvement of the non-conduit arteries, which are detrimentally affected by the process of atherosclerosis, and secondly, and perhaps more importantly, by impairment of the microcirculatory control mechanisms (small vessel disease). Normally, when a body part suffers some form of trauma, the body part will, as part of the body's healing mechanism, experience an increased blood flow. When small vessel disease and ischemia are

both present, as in the case of many diabetics, this natural increased blood flow response is significantly reduced. This fact, together with the tendency of diabetics to form blood clots (thrombosis) in the microcirculatory system during low levels of blood flow, is believed to be an important factor in ulcer pathogenesis.

Neuropathy is a general term which describes a disease process which leads to the dysfunction of the nervous system, and is one of the major complications of diabetes mellitus, with no well-established therapies for either its symptomatic treatment or for prevention of progressive decline in nerve function.

The thickening and leakage of capillaries caused by diabetes primarily affect the eyes (retinopathy) and kidneys (nephropathy). The thickening and leakage of capillaries caused by diabetes are also associated with skin disorders and disorders of the nervous system (neuropathy).

The eye diseases associated with diabetes are nonproliferative diabetic retinopathy, proliferative diabetic retinopathy, diabetic maculopathy, glaucoma, cataracts and the like.

Other diseases, although not known to be related to diabetes are similar in their physiological effects on the peripheral vascular system. Such diseases include Raynaud syndrome, CREST syndrome, autoimmune diseases such as erythematosis, rheumatoid disease, and the like.

As used herein, the term "peripheral vascular diseases" comprises any peripheral vascular disease including peripheral and autonomic neuropathies. Examples of "peripheral vascular disease" include peripheral arterial disease, such as chronic arterial occlusion including arteriosclerosis, arteriosclerosis obliterans and thromboangiitis obliterans (Buerger's disease), macroangiopathy, microangiopathy, diabetes mellitus, thrombophlebitis, phlebemphraxis, Raynaud's disease, Raynaud's syndrome, CREST syndrome, health hazard due to vibration, Sudeck's syndrome, intermittent claudication, cold sense in extremities, abnormal sensation in extremities, sensitivity to the cold, Meniere's disease, Meniere's syndrome, numbness, lack of sensation, anesthesia, resting pain, causalgia (burning pain), disturbance of peripheral circulation function, disturbance of nerve function, disturbance of motor function, motor paralysis, diabetic peripheral circulation disorder, lumbar spinal canal stenosis, diabetic neuropathy, shock, autoimmune disease such as erythematosis, rheumatoid disease and rheumatoid arthritis, autonomic neuropathy, diabetic autonomic neuropathy, autonomic imbalance, orthostatic hypotension, erectile

dysfunction, female sexual dysfunction, retrograde ejaculation, cystopathy, neurogenic bladder, defective vaginal lubrication, exercise intolerance, cardiac denervation, heat intolerance, gustatory sweating, diabetic complication, hyperglycemia, hypoglycemia unawareness, hypoglycemia unresponsiveness; glaucoma, neovascular glaucoma, cataract, retinopathy, diabetic retinopathy, diabetic maculopathy, occlusion of retinal artery, obstruction of central artery of retina, occlusion of retinal vein, macular edema, aged macular degeneration, aged disciform macular degeneration, cystoid macular edema, palpebral edema, retinal edema, chorioretinopathy, neovascular maculopathy, uveitis, iritis, retinal vasculitis, endophthalmitis, panophthalmitis, metastatic ophthalmia, choroiditis, retinal pigment epithelitis, conjunctivitis, cyclitis, scleritis, episcleritis, optic neuritis, retrobulbar optic neuritis, keratitis, blepharitis, exudative retinal detachment, corneal ulcer, conjunctival ulcer, chronic nummular keratitis, Thygeson keratitis, progressive Mooren' s ulcer, damage of skin, skin ulcer including foot ulcer, diabetic ulcer, burn ulcer, lower leg ulcer, postoperative ulcer, traumatic ulcer, ulcer after herpes zoster, radiation ulcer, drug induced ulcer, frostbite (cold injury) , chilblain, gangrene and sudden gangrene, angina pectoris/ variant angiitis, coronary arteriosclerosis (chronic ischemic heart disease, asymptomatic ischemic heart disease, arteriosclerotic cardiovascular disease), myocardial infarction, heart failure, congestive heart failure and painless ischemic heart disease, pulmonary edema, hypertension, pulmonary hypertension; portal hypertension, diabetic nephropathy, decubitus, renal failure.

The peptides of the present invention were tested using the assays described in Examples 1-7, 9-17 for their effect as active therapeutic agents in the prophylaxis and/or treatment of heart and vascular diseases and disorders and of diseases and disorders dependent on increased or decreased angiogenesis.

**Rare or Orphan diseases**

Another aspect of the present invention is directed to the use of the peptide compound or the peptide combination as a therapeutic agent for the prophylaxis and/or treatment of the following orphan diseases as well as for the prophylaxis and/or treatment of an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, or a heart and vascular disease in patients suffering from one or more of the following Rare or Orphan Diseases:

ABCD syndrome, AAE, ABSD, ACPS III, ACRP syndrome, ACS, ACTH deficiency, isolated ACTH resistance, ADANE, ADCA, ADCME, ADEM, ADLTE, ADULT syndrome, AEC syndrome, AGM2, AHDS, AIDS wasting syndrome, ALS, ALSG, AMME syndrome, ANOTHER syndrome, AOA1, AOS, APC, Autoimmune

polyendocrinopathy - candidiasis - ectodermal dystrophy syndrome, APUDoma, AR-CMT, ARC syndrome, ARCA, AREDYLD syndrome, ASD, ASPED, ASPWSCR duplication, ATLD, ATR16, ATRUS syndrome, ATS-MR, AVED Aagenaes syndrome, Aarskog like syndrome, Aarskog-Ose-Pande syndrome, Aarskog-Scott syndrome, Aase syndrome, Aase-Smith syndrome, Abdominal aortic aneurysm, Aberrant left pulmonary artery, Abetalipoproteinemia, Ablepharon macrostomia syndrome, Abruzzo-Erickson syndrome, Acalvaria, Acampomelic campomelic dysplasia, Acanthamoeba keratitis, Acanthocytic disorder, Acanthocytosis, Acanthosis nigricans, Acatalasemia, Aceruloplasminemia, Achalasia, Achard Thiers syndrome,Ad Acheiropodia, Achondroplasia, Achromatopsia, Acitretin embryofetopathy, Ackerman syndrome, Acoustic neurinoma, Acquired generalized lipodystrophy, Acquired hypoprothrombinemia, Acquired ichthyosis, Acquired idiopathic sideroblastic anaemia, Acquired lipoatrophic diabates, Acquired prothrombin deficiency, Acrodermatitis enteropathica zinc deficiency type, Acrodysostosis, Acrodysplasia, Acrofacial dysostosis, Acrokeratoderma, Acrokeratoelastoidosis, Acromelanosis, Acromesomelic dwarfism, Acromicric dysplasia, Acroosteolysis dominant type, Acrorenal defect - ectodermal dysplasia - diabetes, Acrorenal syndrome, Actinic porokeratosis disseminated superficial, Actinic porokeratosis, Acute Respiratory Distress Syndrome, Acute basophilic leukaemia, Acute erythroblastic leukaemia, Acute febrile neutrophilic dermatosis, Acute inflammatory demyelinating polyradiculoneuropathy (aidp), Acute interstitial pneumonia, Acute leukaemia of ambiguous lineage, Acute leukaemia of indeterminate lineage, Acute liver failure, Acute lymphoblastic leukaemia, Acute medullary lesions, Acute megacaryoblastic leukaemia, Acute monoblastic leukaemia, Acute motor and sensory axonal neuropathy (AMSAN), Acute motor axonal neuropathy (AMAN), Acute myeloblastic leukaemia, Acute myelodysplasia with myelofibrosis, Acute myelofibrosis, Acute myeloid leukaemia in Down syndrome, Acute myelomonocytic leukaemia, Acute myelosclerosis, Acute non lymphoblastic leukaemia, Acute panmyelosis with myelofibrosis, Acute peripheral arterial occlusion, Acute promyelocytic leukaemia, Acute tubulointerstitial nephritis and uveitis syndrome, Adactylia unilateral, Adamantinoma, Adams nance syndrome, Adams-Oliver syndrome, Addison's disease, Adenine phosphoribosyltransferase deficiency, Adenosine deaminase deficiency, Adenosylcobalamin deficiency, Adenovirus infection in immunocompromised patients, Adenylosuccinase deficiency Adhesive arachnoiditis, Adie syndrome, Adrenal adenoma, Adrenal hyperplasia, Adrenal incidentaloma, Adrenal insufficiency, Adrenocortical carcinoma, Adrenoleukodystrophy, Adrenomyeloneuropathy, Adrenomyodystrophy, Adult Onset Still's disease, Adult T-cell leukaemia/lymphoma, Adult idiopathic neutropenia, Adult neuronal ceroid lipofuscinosis (Kufs disease, CLN4), Adult spinal muscular atrophy,

Afibrinogenemia, African tick typhus, African trypanosomiasis, Agammaglobulinemia, Age-related macular degeneration, Ahn-Lerman-Sagie syndrome, Ahumada-Del Castillo syndrome, Aicardi syndrome, Aicardi-Goutieres syndrome, AIDS, Akaba hayasaka syndrome, Akesson syndrome, Alagille syndrome, Alanine-glyoxylate aminotransferase deficiency (hyperoxaluria type 1), Albers-Schonberg disease, Albright hereditary osteodystophy, Alcock syndrome, Aldolase A deficiency, Aldosterone synthase deficiency, Aldred syndrome, Alexander disease, Algodystrophy, Alkaptonuria, Alkylglycerone phosphate synthase deficiency, Allan-Herndon-Dudley syndrome, Allergic bronchopulmonary aspergillosis, Allgrove syndrome, Alopecia, Alpers syndrome, Alpers-Huttenlocher syndrome, Alpha-thalassemia, Alport syndrome, Alström syndrome, Alternating hemiplegia, Alveolar echinococcosis, Alves dos santos castello syndrome, Alzheimer disease, Amaurosis - hypertrichosis, Ambras syndrome, Amegacaryocytosis, Amelia, Aminoaciduria, Amoebiasis due to Entamoeba histolytica, Ampola syndrome, Amyloid cardiopathy, Amyloid nephropathy, Amyloid polyneuropathy, Amyloidosis, Amylopectinosis, Amyoplasia congenita, Amyotrophic lateral sclerosis, Amyotrophy fat tissue anomaly, Anemia, Anauxetic dysplasia, Ancylostomiasis, Andermann syndrome, Andersen disease, Aneurysmal subarachnoid haemorrhage, Angelman syndrome, Angio-osteohypertrophic syndrome, Angiodysgenetic necrotizing myelopathy, Angioedema, Angiofollicular ganglionic hyperplasia, Angiokeratoma, Angioma and vascular malformation, Angiomatosis systemic cystic seip syndrome, Angioneurotic oedema, Angiostrongyliasis, Anguillulosis, Aniridia, Anisakiasis, Ankylosing spondylarthritis, Ankylostomiasis, Annuloaortic ectasia, Anodontia, Anonychia, Anophthalmia - heart and pulmonary anomalies, Anorchidia, Anorexia nervosa, Anotia, Antenatal Epstein-Barr virus infection, Anterior horn cell disease, Anti-phospholipid syndrome, Antinolo nieto borrego syndrome, Antiplasmin deficiency, Antithrombin deficiency, Antley-Bixler syndrome, Anyane-Yeboa syndrome, Aorta coarctation, Aorta hypoplasia, Aorta-pulmonary artery fistula, Aortic aneurysm syndrome, due to TGFbeta receptors anomalies, Aortic malformation, Aortic valve atresia, Aortic valve dysplasia, Aortic valve stenosis, APECED syndrome, Apert syndrome, Aphasia, Apical ballooning syndrome, Aplasia cutis, Aplastic anaemia, Apnea of infancy (AOI), Apnea of prematurity (AOP), Apo A-I deficiency, Apolipoprotein AI amyloidosis, Apple peel syndrome, Apraxia, Arbovirus fever, Arena syndrome, Areolar atrophy of the macula, Argyria, Argyrophilic grain disease, Arhinia choanal atresia microphthalmia, Arkless-Graham syndrome, Armfield syndrome, Arndt-Gottron disease, Arnold-Chiari malformation, Aromatase deficiency, Arrhinia, Arrhythmogenic right ventricular dysplasia, Arterial calcification, Arterial duct anomalies, Arterial occlusive disease, Arterial tortuosity, Arteriohepatic dysplasia, Arthritis juvenile, Arthrogryposis, Arthroophtalmopathy, Arthropathy, Arts syndrome, Asbestosis, Ascher syndrome,

Aseptic abscesses syndrome, Aseptic osteitis, Asherman's syndrome, Aspartylglucosaminidase deficiency, Asperger syndrome, Aspergillosis, Asphyxiating thoracic dystrophy of the newborn, Astley-Kendall dysplasia, Astrocytoma, Ataxia, Atelencephaly, Atelosteogenesis, Atherosclerosis, Atkin-Flaitz syndrome, Atransferrinemia, Atresia, Atrial cardiomyopathy, Atrial myxoma, Atrial septal defect, Atrichia, Atrioventricular canal complete - fallot tetralogy, Atrophia aerata, Atrophoderma vermiculata, Atypical Mole syndrome, Atypical Werner syndrome, Aughton sloan milad syndrome, Aughton-Hufnagle syndrome, Ausems wittebol post hennekam syndrome, Autism, Autoimmune haemolytic anemia, Autoimmune lymphoproliferative syndrome, Autoimmune pancreatitits, Axenfeld-Rieger syndrome, Ayazi syndrome, B-cell chronic lymphocytic leukaemia, BAFME, BBB syndrome, X-linked, BCD, BEEC, BES, BIDS syndrome, BOD syndrome, BOFS, BOR syndrome, BOS syndrome, BPD, BRESEK syndrome, BRESHECK syndrome, BRIC, BS, BSCL, BTHS, BTK-deficiency, Babesiosis, Bacterial toxic-shock syndrome, Bahemuka brown syndrome, Baird syndrome, Balantidiasis, Ballard syndrome, Baller-Gerold syndrome, Ballooning cardiomyopathy, Balo diseases, Bamforth syndrome, Bangstad syndrome, Banti syndrome, Bannayan-Riley-Ruvalcaba syndrome, Barachydactyly type A4, Baraitser burn fixen syndrome, Baraitser-Brett-Piesowicz syndrome, Barakat syndrome, Barber-Say syndrome, Bardet-Biedl syndrome, Bare lymphocyte syndrome, Barnicoat baraitser syndrome, Barraquer-Simons syndrome, Barrett eosophagus, Barth syndrome, Bartonellosis, Bartsocas-Papas syndrome, Bartter syndrome, Basan syndrome, Bassen-Kornzweig disease, Bassoe syndrome, Battaglia neri syndrome, Batten disease, Baughman syndrome, Bazex syndrome, Bazex-Dupre-Christol syndrome, Bazopoulou kyrkanidou syndrome, Bd syndrome, Beals syndrome, Beals-Hecht syndrome, Bean syndrome, Beare stevenson syndrome, Bechterew syndrome, Beckwith-Wiedemann, Beemer-Ertbruggen syndrome, Behcet disease, Behr syndrome, Behrens-Baumann-Vogel syndrome, Bell's palsy, Bellini-Chiumello-Rimoldi syndrome, Benallegue Lacete syndrome, Bejel, Bencze syndrome, Bennion-Patterson syndrome, Benson's syndrome, Beradinelli-Seip syndrome, Berdon syndrome, Berger disease, Berk tabatznik syndrome, Berlin breakage syndrome, Bernard-soulier syndrome, Berylliosis, Besnier-Boeck-Schaumann disease, Bessel-Hagen disease, Best disease, Beta thalassemia, Bethlem myopathy, Bickel-Fanconi glycogenosis, Bickers-Adams syndrome, Bickerstaff's brainstem encephalitis, Bicuspid aortic valve, Biemond syndrome, Biermer disease, Bietti's crystalline dystrophy, Bile acid synthesis defect, Bile duct cancer, Biliary atresia, Biliary inflammatory disease, Bilineal acute leukaemia, Billard-Toutain-Maheut syndrome, Binder syndrome, Bindewald-Ulmer-Muller syndrome, Binswanger disease, Birt-Hogg-Dube syndrome, Bixler christian gorlin syndrome, Bjornstad syndrome, Blackfan-Diamond anaemia, Blaichman

syndrome, Blake's pouch cyst, Blau syndrome, Blepharophimosis, Blepharoptosis, Blepharospasm, Blethen wenick hawkins syndrome, Bloch-Sulzberger syndrome, Bloom syndrome, Blount disease, Blue Diaper syndrome, Bohring syndrome, Bohring-Opitz syndrome, Boichis syndrome, Bone disease with defective bone mineralisation, Bone disease with increased bone density, Bone marrow failure, Bonneau-Beaumont syndrome, Bonnemann-Meinecke-Reich syndrome, Bonnet-Dechaume-Blanc syndrome, Book syndrome, Boomerang dysplasia, Booth haworth dilling syndrome, Borjeson-Forssman-Lehmann syndrome, Bork syndrome, Bornholm eye disease, Bosley-Salih-Alorainy syndrome, Bosma henkin christiansen syndrome, Bothnia retinal dystrophy, Boucher-Neuhauser syndrome, Bourneville syndrome, Boutonneuse fever, Bouwes Bavinck syndrome, Bowen syndrome, Boyadjiev-Jabs syndrome, Boylan dew syndrome, Brachman-de Lange syndrome, Brachydactyly - arterial hypertension, Brachymesophalangy II and V, Brachyolmia, Braddock carey syndrome, Bradyopsia, Brain inflammatory disease, Brain injury, Brain sclerosis, Brauer syndrome, Braun bayer syndrome, Braun-Tinschert, Breast cancer, Brill-Zinsser disease, Brittle bone disease, Brody myopathy, Bronchial carcinoid tumour, Bronchiectasis, Bronchiolitis obliterans organizing pneumonia, Bronchiolitis obliterans with obstructive pulmonary disease, Bronchogenic cyst, Bronchopulmonary dysplasia, Bronspiegel-Zelnick syndrome, Brooke-Spiegler syndrome, Brown-Vialetto-van Laere syndrome, Bruce winship syndrome, Brucellosis, Bruck syndrome, Brugada syndrome, Brunner-Winter syndrome, Brunzell syndrome, Bruyn scheltens syndrome, Buckley syndrome, Budd-Chiari syndrome, Buerger's disease, Bull-Nixon syndrome, Bulldog syndrome, Bulimia, Bullous systemic lupus erythematosus, Buntinx lormans martin syndrome, Burkitt lymphoma, Burn-McKeown syndrome, Burning Mouth syndrome, Buschke-Fischer-Brauer syndrome, Buschke-Ollendorff syndrome, Buttiens-Fryns syndrome, C syndrome, CACD, CACH syndrome, CADASIL, CAMAK syndrome, CAMFAK syndrome, CAMOS syndrome, CANOMAD syndrome, CAP syndrome, CAPOS syndrome, CAPS (cryopyrin associated periodoc syndrome), CAR syndrome, CATCH 22, CATSHL syndrome, CAVC, CCFDN, CCGE syndrome, CDA type 1, CDG syndrome, CDGIIc, CDP, CDPD, CEDNIK syndrome, CFC syndrome, CHAND syndrome, CREST syndrome, CRMO, CRV, CSD, CSID, CSWSS syndrome, CVID, Cacchi-Ricci disease, Cafe au lait spots syndrome, Caffey disease, Cahmr syndrome, Calcinosis, Calderon gonzalez cantu syndrome, Calpainopathy, Camera lituania cohen syndrome, Campomelia Cumming type, Camptodactyly, Camurati engelmann disease, Canale-Smith syndrome, Canavan disease, Candidiasis, Cantalamessa baldini ambrosi syndrome, Canthus, Carbohydrate metabolism disorder, Cardiogenital syndrome, Cardiomyopathy, Cardioskeletal myopathy, Carey fineman ziter syndrome, Carnevale canun mendoza syndrome, Carnevale-

Hernandez-del Castillo syndrome, Carnevale-Krajewska-Fischetto syndrome, Carney complex, Carney-Stratakis syndrome, Carnosinase deficiency, Carnosinemia, Caroli's disease, Carpal Tunnel syndrome, Carpenter syndrome, Carpenter-Waziri syndrome, Carrington's disease, Carrion disease, Carvajal syndrome, Casamassima-Morton-Nance syndrome, Cassia Stocco dos Santos syndrome, Castleman disease, Castro gago pombo novo syndrome, Catalase deficiency, Cataract, Catel-Manzke syndrome, Cayler syndrome, Celiac disease, Celosomia, Cenani lenz syndactylism, Central neurocytoma, Cephalopolysyndactyly, Ceramidase deficiency, Cerebellar hypoplasia, Cerebral arteriovenous shunt, Cerebral hemorrhage with amyloidosis, Cerebroretinal vasculopathy, Cfc syndrome, Chagas disease, Chanarin disease, Chandler syndrome, Chang-Davidson-Carlson syndrome, Chaotic atrial tachycardia, Char douglas dungan syndrome, Char syndrome, Charge syndrome, Charlevoix disease, Charlie m syndrome, Chediak-Higashi like syndrome, Cheilitis glandularis, Chemke oliver mallek syndrome, Chemodectoma, Cherry-red-spot myoclonus syndrome, Cherubism, Chiari Frommel syndrome, Chitayat haj chahine syndrome, Chitayat moore del bigio syndrome, Chitayat-Meunier-Hodgkinson syndrome, Chitty hall webb syndrome, Chitty-Hall-Baraitser syndrome, Cholera, Cholestasis, Cholesteryl ester storage disease, Choline acetyltransferase (ChAT) deficiency, Chondrocalcinosis, Chondrodysplasia, Chondrodystrophy, Chordoma, Choreoacanthocytosis, Chorioretinal atrophy, Choristoma, Choroidal dystrophy, Choroidal sclerosis, Choroideremia, Christ-Siemens-Touraine syndrome, Christian syndrome, Christian-Rosenberg syndrome, Christianson syndrome, Christianson-Fourie syndrome, Christmas tree syndrome, Chromomycosis, Chronic eosinophilic pneumonia, Chronic fatigue syndrome, Chronic inflammatory demyelinating polyneuropathy, Chronic myeloproliferative disease, Chronic neutrophilic leukaemia, Chronic pain requiring intraspinal analgesia, Chronic pneumonitis of infancy, Chronic osteomyelitis, Chronic spinal muscular atrophy, Chudley rozdilsky syndrome, Chudley-Lowry-Hoar syndrome, Churg-Strauss syndrome, Chylomicron retention disease, Ciliary dysentery, Ciliary dyskinesia-bronchiectasis, Cilliers-Beighton syndrome, Cirrhosis associated cardiac dysfunction, Cirrhotic cardiomyopathy, Clarkson disease, Classical Hodgkin disease, Classical homocystinuria, Claude-Bernard-Horner syndrome, ClaytonSmith-Donnai syndrome, Cleido rhizomelic syndrome, Cleidocranial dysostosis, Cleidocranial dysplasia, Clouston syndrome, Coagulation disorder, Coarctation of aorta, Coats disease, Cobb syndrome, Cocaine poisoning, Cockayne syndrome, Codas syndrome, Coeliac disease, Coenzyme Q cytochrome c reductase deficiency, Coffin syndrome, Coffin-Lowry syndrome, Coffin-Siris syndrome, Cogan syndrome, Cogan-reese syndrome, Cohen hayden syndrome, Cohen lockood wyborney syndrome, Cohen syndrome, Cole carpenter syndrome, Colitis, Collagen anomaly, Collins pope syndrome, Collins sakati

syndrome, Coloboma, Colon cancer, Colonic atresia, Colorado tick encephalitis, Combined pituitary hormone deficiencies, Complement component deficiency, Congenital Lambert-Eaton-like syndrome, Congenital leptin deficiency, Congenital lobar emphysema, Conjunctival disease, Conjunctival vascular anomaly, Conn syndrome, Connective tissue disease, Conradi-Hünermann-Happle syndrome, Constrictive bronchiolitis, Cooks syndrome, Cooley anaemia, Cooper-Jabs syndrome, Cormier rustin munnich syndrome, Corneal dystrophy, Cornelia de Lange syndrome, Corneodermatoosseous syndrome, Corneogoniodysgenesis, Coronaro-cardiac fistula, Coronary arterial malformations, Coronary artery aneurysm, Coronary sinus type ASD, Cortada koussef matsumoto syndrome, Costeff optic atrophy syndrome, Costeff syndrome, Costello syndrome, Cote katsantoni syndrome, Cousin-Walbraum-Cegarra syndrome, Cowchock syndrome, Cowchock-Wapner-Kurtz syndrome, Cowden syndrome, Coxoauricular syndrome, Cramer-Niederdellmann syndrome, Crandall syndrome, Crane heise syndrome, Cranial malformation, Craniopharyngioma, Craniorachischisis, Craniostenosis, Craniosynostosis, Craniotelencephalic dysplasia, Craniotubular syndrome, Creatine deficiency, Creeping disease, Creutzfeldt-Jakob disease, Cri du chat syndrome, Crigler-Najjar syndrome, Crimean-Congo haemorragic fever (CCHF), Crisponi syndrome, Criss-cross heart, Criswick-Schepens syndrome, Crohn disease, Crome syndrome, Cronkhite canada syndrome, Cross syndrome, Crouzon disease, Crow-Fukase syndrome, Cryoglobulinaemia mixed, Cryptococcosis, Cryptogenic organizing pneumonia, Cryptophthalmia, Cryptosporidiosis, Culler-Jones syndrome, Currarino triad, Curry-Hall syndrome, Curry-Jones syndrome, Cushing disease, Cutaneomeningospinal angiomatosis, Cutaneous lupus erythematosus, Cutaneous mastocytoma, Cutaneous mastocytosis, Cutaneous photosensitivity colitis, Cutaneous vasculitis, Cutaneuous myiasis, Cutis laxa, Cutler bass romshe syndrome, Cyclosporosis, Cystathioninuria, Cystic fibrosis, Cystic hamartoma of lung and kidney, Cystic lymphangioma, Cystic renal disease, Cystinosis, Cystinuria, Cytochrome c oxydase deficiency, Cytomegalovirus (CMV) disease in patients with impaired cell mediated immunity deemed at risk, Cytopenia, Czeizel brooser syndrome, Czeizel losonci syndrome, D ercole syndrome, D-2-hydroxyglutaricaciduria, D-glycerate dehydrogenase deficiency (hyperoxaluria type 2), D-glycerate kinase deficiency, D-glycericacidemia, DCMA syndrome, DCMD, DEND syndrome, DI-CMT, DIDMOAD syndrome (Diabetes Insipidus - Diabetes Mellitus - Optic Atrophy - Deafness), DIS, DK phocomelia syndrome, DKC, DOOR syndrome, DORV, DTDP1, DYT6, Da silva syndrome, Dacryocystitis osteopoikilosis, Daentl-Townsend-Siegel syndrome, Dahlberg-Borer-Newcomer syndrome, Daish hardman lamont syndrome, Dancing Eye syndrome, Dandy walker malformation, Daneman davy mancer syndrome, Danon disease, Darier disease, Darier-Gottron

disease, Davenport donlan syndrome, David syndrome, Davies disease, Davis lafer syndrome, De Barsy syndrome, De Hauwere-Leroy-Adriaenssens syndrome, De Santis-Cacchione syndrome, De Smet-Fabry-Fryns syndrome, De Vaal disease, De la Chapelle dysplasia, De morsier syndrome, Deafness - small bowel diverticulosis - neuropathy, Deal barratt dillon syndrome, Degos disease, Dejerine-Sottas syndrome, Dekaban-Arima syndrome, Delayed graft function after organ transplantation, Delleman-Oorthuys syndrome, Dementia associated with a metabolic disease, Dementia associated with a neurodegenerative disease, Dementia associated with an infectious disease, Dementia associated with hepatic and renal failure, Demodicidosis, Dendritic cell sarcoma, Dendritic cell tumor, Dengue, Dennis cohen syndrome, Dennis fairhurst moore syndrome, Dense (delta) granule disease, Dent disease, Dentin dysplasia, Denys-Drash syndrome, Der Kaloustian-Jarudi-Khoury syndrome, Der kaloustian mcintosh silver syndrome, Dercum's disease, Dermatofibrosarcoma protuberans, Dermatologic allergic disease, Dermatostomatitis Stevens Johnson type, Desbuquois syndrome, Desminopathy, Desmoid disease, Desmosterolosis, Devic's disease, Devriendt legius fryns syndrome, Devriendt vandenberghe fryns syndrome, DiGeorge syndrome, Diabetes, Dialysis-related arthropathy, Diaphanospondylodysostosis, Diaphragmatic agenesia, Diaphragmatic spinal muscular atrophy, Diffuse alveolar haemorrhage, Diffuse large B cell lymphoma, Diffuse leiomyomatosis - Alport syndrome X-linked, Diffuse neonatal haemangiomatosis, Dihydropyrimidinuria, Dilated cardiomyopathy with ataxia, Dincsoy-Salih-Patel syndrome, Dinno shearer weisskopf syndrome, Diomedi bernardi placidi syndrome, Dionisi-Vici-Sabetta-Gambarara syndrome, Diphtheria, Diprosopia, Discoid lupus erythematosus, Discrete fibromuscular subaortic stenosis, Distichiasis - congenital heart defects - peripheral vascular anomalies, Distomatosis, Dobrow syndrome, Donath-Landsteiner syndrome, Donnai-Barrow syndrome, Donohue syndrome, Doose syndrome, Dorfman-chanarin disease, Dowling-Degos disease, Dowling-Degos-Kitamura disease, Down syndrome, Doyne honeycomb retinal dystrophy (DHRD), Drachtman weinblatt sitarz syndrome, Drash syndrome, Dravet syndrome, Drummond syndrome, Du Pan syndrome, Duane syndrome, Dubin-Johnson syndrome, Dubowitz syndrome, Duhring brocq disease, Duker-Weiss-Siber syndrome, Dunnigan syndrome, Dupont sellier chochillon syndrome, Dyggve-Melchior-Clausen disease, Dykes-Markes-Harper syndrome, Dyschondrosteosis, Dyschromatosis universalis, Dysferlinopathy, Dysfibrinogenemia, Dyskeratosis, Dysmorphic syndrome with connective tissue involvement, Dysosteosclerosis, Dysostosis, Dysphagia lusoria, Dysplasia, Dysprothrombinemia, Dyssegmental dysplasia glaucoma, Dysspondyloenchondromatosis, Dystoni-like syndrome with paroxysmal disease, Dystonia, EBD, EBJ, EBS, ECP syndrome, EDS III, EEC syndrome, EEM syndrome, EGE, ENT, ERA, ESS1, Eagle-Barret syndrome, Eales

disease, Ebola virus disease, Echinocytic disorder, Ectodermal dysplasia, Ectromelia, Ectropion, Eczema-thrombocytopenia-immunodeficiency syndrome, Edinburgh malformation syndrome, Edward syndrome, Edwards-Patton-Dilly syndrome, Ehlers-Danlos syndrome, Ehrlichiosis, Eiken syndrome, Eisenmenger syndrome, Elastosis perforans serpiginosa, Elejalde syndrome, Elliott ludman teebi syndrome, Elliptocytosis, Ellis Van Creveld syndrome, Ellis yale winter syndrome, Elsching syndrome, Emanuel syndrome, Emery-Dreifuss muscular dystrophy, Emery-Nelson syndrome, Empty Sella syndrome, Encephalitis, Encephalomyelitis, Encephalopathy, Enchondromatosis, Endometriosis, Endotheliitis, Eng strom syndrome, Engel congenital myasthenia, Engelhard yatziv syndrome, Enolase deficiency, Enteric anendocrinosis, Enteropathy, Enterovirus antenatal infection, Entropion, Envenomization, Eosinophilic endocarditis, Eosinophilic pneumonia, Ependymoma, Epidermolysis bullosa, Epilepsy, Epiphyseal dysplasia, Episodic ataxia, Epispadias, Epithelial ovarian cancer, Epithelioma, Epstein-Barr virus infection, Erdheim disease, Erdheim-Chester disease, Eronen-Somer-Gustafsson syndrome, Erythema, Erythermalgia, Erythroblastopenia, Erythrocytosis, Erythroderma, Erythrokeratoderma, Erythromelalgia, Escher hirt syndrome, Escobar syndrome, Esophageal adenocarcinoma, Esophageal atresia, Essential cryoglobulinaemia, Essential iris atrophy, Essential osteolysis, Esthesioneuroblastoma, Estrogen receptor deficiency, Estrogen resistance syndrome, Evans syndrome, Ewing sarcoma, Exner syndrome, Exostoses, Exsudative retinopathy, Extracutaneous mastocytoma, Extrinsic allergic alveolitis, Eye disease, F syndrome, FAP, FAS deficiency, FCS syndrome, FCU, FENIB, FEOM, FFDD type I, FG syndrome, FLOTCH syndrome, FOP, FOSMN syndrome, FPS/AML syndrome, FRAXA syndrome, FRAXE syndrome, FRAXF syndrome, FSH resistance, Fabry disease, Factor VII deficiency, Factor VIII deficiency, Factor X deficiency, Factor XI deficiency, Factor XII deficiency, Factor XIII deficiency, Factors II,VII,IX and X, combined deficiency, Fahr syndrome, Fallot complex, Familial LCAT deficiency, Fanconi anaemia, Fanconi ichthyosis dysmorphism, Fanconi syndrome, Fanconi-Bickel disease, Fara-Chlupackova syndrome, Farber lipogranulomatosis, Farmer's lung disease, Fatal infantile COX deficiency, Faulk-Epstein-Jones syndrome, Favism, Fazio-Londe disease, Fechtner syndrome, Feigenbaum-Bergeron-Richardson syndrome, Feingold syndrome, Felty syndrome, Fenton wilkinson toselano syndrome, Ferlini-Ragno-Calzolari syndrome, Fernhoff-Blackston-Oakley syndrome, Fetal cytomegalovirus syndrome, Fetal edema, Fetal left ventricular aneurysm, Fibrinogen disorder, Fibrochondrogenesis, Fibrodysplasia ossificans progressiva, Fibromatosis, Fibromuscular dysplasia of arteries, Fibromyalgia, Fibronectin glomerulopathy, Fibrosarcoma, Fibrosing mediastinitis, Fibrosis of extraocular muscles, Fiessinger-Leroy-Reiter's syndrome, Figuera

syndrome, Filamin anomaly, Filariasis, Filippi syndrome, Fine-Lubinsky syndrome, Finlay-Markes syndrome, Finucane kurtz scott syndrome, Fitz Hugh Curtis syndrome, Fitzsimmons-Guilbert syndrome, Fitzsimmons-McLachlan-Gilbert syndrome, Fitzsimmons-Walson-Mellor syndrome, Fixed subaortic stenosis, Flegel disease, Floating-Harbor syndrome, Florid cemento-osseous dysplasia, Flynn aird syndrome, Foix chavany marie syndrome, Foix-Alajouanine syndrome, Follicular atrophoderma-basal cell carcinoma, Follicular dendritic cell sarcoma, Follicular dyskeratoma, Follicular ichthyosis, Follicular lymphoma, Fontaine-Farriaux-Blanckaert syndrome, Forbes disease, Forney-Robinson-Pascoe syndrome, Forunculoid myiasis, Fountain syndrome, Fowler-Christmas-Chapple syndrome, Fox Fordyce disease, Fra-X syndrome, Fragile X syndrome, Fragoso cid garcia hernandez syndrome, Franceschetti-Klein syndrome, Francois dyscephalic syndrome, Francois syndrome, Franek bocker kahlen syndrome, Frank-Ter Haar syndrome, Franklin disease, Fraser like syndrome, Fraser syndrome, Frasier syndrome, Freeman-Sheldon syndrome, Freiberg's disease, Freire maia pinheiro opitz syndrome, Frey's syndrome, Frias syndrome, Fried syndrome, Fried-Goldberg-Mundel syndrome, Friedman goodman syndrome, Friedreich ataxia, Froelich's syndrome, Froster-Huch syndrome, Froster-Iskenius-Waterson syndrome, Fructosuria, Frydman-Cohen-Karmon syndrome, Fryns macrocephaly, Fryns-Aftimos syndrome, Fryns-Hofkens-Fabry syndrome, Fuhrmann-Rieger-de Sousa syndrome, Fukuda miyanomae nakata syndrome, Fukuhara syndrome, Fuqua-Berkovitz syndrome, Furlong syndrome, Furukawa takagi nakao syndrome, G syndrome, G6PD deficiency, GABA metabolism disease, GAMT deficiency, GAPO syndrome, GIST, GM1 gangliosidosis, GOSHS, GRACILE syndrome, GRF Tumour, GSD, GTN, GVH, Gaisbock syndrome, Galactokinase deficiency, Galactosemia, Galactosialidosis, Galloway syndrome, Galloway-Mowat syndrome, Gamborg nielsen syndrome, Game-Friedman-Paradice syndrome, Gamstorp episodic adynamy, Ganglioglioma, Garcia torres guarner syndrome, Garcia-Lurie syndrome, Gardner silengo wachtel syndrome, Gardner-Morrison-Abbott syndrome, Garret tripp syndrome, Gastric cancer, Gastroschisis, Gaucher disease, Gaucher-like disease, Geen sandford davison syndrome, Gelineau disease, Gemignani syndrome, Gemss syndrome, Genes syndrome, Genochondromatosis, Gerbode defect, Gerhardt syndrome, German syndrome, Gershonibaruch-Leibo syndrome, Gerstmann-Straussler-Scheinker syndrome, Ghosal syndrome, Gianotti Crosti syndrome, Giant cell arteritis, Giant platelet syndrome, Gilbert syndrome, Gilles de la Tourette syndrome, Gillespie syndrome, Gitelman syndrome, Glanzmann thrombasthenia, Glass bone disease, Glass-Chapman-Hockley syndrome, Glaucoma, Glioblastoma, Glomerular disease, Glomerulonephritis, Glomerulopathy with fibronectin deposits (GFND), Gloomy syndrome, Glucagonoma, Glucocorticoid resistance, Glycogen storage disease, Gms syndrome, Goiter-deafness syndrome,

Golabi-Rosen syndrome, Goldberg syndrome, Goldberg-Maxwell syndrome, Goldberg-Shprintzen megacolon syndrome, Goldblatt viljoen syndrome, Goldblatt wallis syndrome, Goldenhar syndrome, Goldmann-Favre syndrome, Goldstein hutt syndrome, Goldston syndrome, Gollop syndrome, Gollop wolfgang complex, Goltz syndrome, Goltz-Gorlin syndrome, Gombo syndrome, Gonzales del angel syndrome, Goodman syndrome, Goodpasture syndrome, Goossens-Devriendt syndrome, Gordon syndrom, Gorham syndrome, Gorham-Stout disease, Gorlin syndrome, Gorlin-Chaudhry-Moss syndrome, Graft rejection after lung transplantation, Graft versus host disease, Graham boyle troxell syndrome, Graham-Cox syndrome, Grand-Kaine-Fulling syndrome, Grange occlusive arterial syndrome, Grant syndrome, Granulocytic sarcoma, Granulomatous allergic angiitis, Granulomatous inflammatory arthritis, dermatitis, and uveitis, Granulomatous mastitis, Graves' disease, Gray platelet syndrome, Greenberg dysplasia, Greig syndrome, Greither's disease, Griscelli disease, Grix blankenship peterson syndrome, Groll hirschowitz syndrome, Gronblad-Strandberg-Touraine syndrome, Grosse syndrome, Grover's disease, Growth hormone deficiency, Grubben de cock borghgraef syndrome, Gräsbeck-Imerslund disease, Guam disease, Guanidinoacetate methyltransferase deficiency, Guibaud-Vainsel syndrome, Guillain-Barré syndrome, Guizar-Vasquez-Luengas syndrome, GuizarVazquez-Sanchez-Manzano syndrome, Gunal seber basaran syndrome, Gurrieri-Sammito-Bellussi syndrome, Gusher syndrome, Gynandroblastoma, Günther disease, HAD deficiency, HAE, HAIRAN syndrome, HANAC syndrome, HARD syndrome (Hydrocephalus - agyria - retinal dysplasia), HCDD, HCL, HDL metabolism disorder, HEM, HEP, HERNS syndrome, HHE syndrome, HHT, HHV-8, HID syndrome, HIGM1, HIT, HMSN 5, HMSNP, HNPCC, HNSCC, HPA-1 deficiency, HPE, HSAN 1, HSD deficiency, HSV encephalitis, HSV keratitis, HUS, HVR, Haas-Robinson syndrome, Haddad syndrome, Haematologic cancers, Haemochromatosis, Haemoglobin disorders, Haemolysis, Haemolytic anaemia, Haemolytic uremic syndrome, Haemorrhagic fever, Haemorrhagiparous thrombocytic dystrophy, Hageman factor deficiency, Hagemoser weinstein bresnick syndrome, Hailey-Hailey disease, Haim-Munk syndrome, Hairy cell leukaemia, Hajdu-Cheney syndrome, Hal-Berg-Rudolph syndrome, Halal syndrome, Halal-Setton-Wang syndrome, Hallermam streiff like syndrome, Hallermann-Streiff-Francois syndrome, Hallervorden-Spatz disease, Hamanishi ueba tsuji syndrome, Hamano tsukamoto syndrome, Hamman-Rich syndrome, Hanhart syndrome, Hand Foot Mouth syndrome, Hand-Shuller-Christian disease, Hanot syndrome, Hantavirus pulmonary syndrome, Hapnes boman skeie syndrome, Happy puppet syndrome, Harboyan syndrome, Hardcastle syndrome, Harding ataxia, Harrod syndrome, Harrod-Keele syndrome, Hartnup disorder, Hartsfield bixler demyer syndrome, Hashimoto struma, Hashimoto-Pritzker syndrome, Haspeslagh-Fryns-Muelenaere

syndrome, Hawkinsinuria, Hay wells syndrome, Heart block progressive, Heart-hand syndrome, Heavy chain deposition disease, Hec syndrome, Hecht scott syndrome, Heckenlively syndrome, Heide syndrome, Heimler syndrome, Heiner syndrome (cow's milk hypersensitivity), Helmerhorst heaton crossen syndrome, Hemangioma-thrombocytopenia syndrome, Hemangiopericytoma, Hematopoietic hypoplasia, Hemeralopia, Hemi 3 syndrome, Hemiconvulsion-Hemiplegia-Epilepsy syndrome, Hemifacial hyperplasia strabismus, Hemihypertrophy intestinal web corneal opacity, Hemimelia, Hemitruncus, Hemochromatosis, Hemoglobin C disease, Hemoglobin E disease, Hemoglobin H disease, Hemolytic anaemia, Hemophilia, Hemorrhagiparous thrombocytic dystrophy, Hennekam koss de geest syndrome, Hennekam syndrome, Hennekam-Beemer syndrome, Henoch-Schoenlein purpura, Hepatic cystic hamartoma, Hepatic fibrosis, Hepatic cancer, Hepatic venoocclusive disease, Hepatitis B re-infection following liver transplantation, Hepatitis, Hepatoblastoma, Hepatocellular adenoma, Hepatocellular carcinoma, Hepatoerythropoeitic porphyria, Hepatoportal sclerosis, Hereditary coproporphyria, Hereditary endotheliopathy - retinopathy - nephropathy - stroke, Hereditary lymphoedema type I, Hereditary motor and sensory neuropathy, Hereditary vascular retinopathie - Raynaud phenomenon - migraine, Hermansky-Pudlak syndrome, Hernandez fragoso syndrome, Hernandez-Aguirre Negrete syndrome, Herpes virus infection, Herrmann opitz arthrogryposis syndrome, Hers disease, Hersh-Podruch-Weisskopf syndrome, Herva disease, Heterotaxia, Heterozygous OSMED, Hillig syndrome, Hinman syndrome, Hinson-Pepys disease, Hipo syndrome, Hirayama disease, Hirschsprung disease, Hirsutism, His bundle tachycardia, Histidine metabolism disorder, Histidinuria renal tubular defect, Histiocytic and dendritic cell tumour, Histiocytic sarcoma, Histiocytoid cardiomyopathy, Histiocytosis X, Histoplasmosis, Hittner hirsch kreh syndrome, Hmc syndrome, Hodgkin lymphoma, Hoepffner dreyer reimers syndrome, Hoffman's syndrome, Holmes benacerraf syndrome, Holmes collins syndrome, Holmes-Gang syndrome, Holoacardius amorphus, Holoprosencephaly, Holt-Oram syndrome, Holzgreve wagner rehder syndrome, Homocarnosinosis, Homocystinuria, Homogentisic acid oxydase deficiency, Hoon hall syndrome, Horner syndrome, Horton disease, Houlston ironton temple syndrome, House allergic alveolitis, Howard young syndrome, Howell-Evans syndrome, Hoyeraal-Hreidarsson syndrome, Humeroradial synostosis, Humeroradioulnar synostosis, Humerospinal dysostosis, Hunter carpenter mc donald syndrome, Hunter jurenka thompson syndrome, Hunter syndrome, Hunter-Rudd-Hoffmann syndrome, Hunter-Thompson-Reed syndrome, Huntington disease, Huriez syndrome, Hurler syndrome, Hurler-Scheie syndrome, Hutchinson-Gilford syndrome, Hutteroth spranger syndrome, Hyaline membrane disease, Hyaluronidase deficiency, Hydatidosis, Hyde-Forster-Mccarthy-Berry syndrome, Hygroma cysticum, Hyperaldosteronism, Hyperargininemia,

Hyperbilirubinemia, Hypercalciuria idiopathic, Hypercholesterolemia, Hyperchylomicronemia, Hypercortisolism, Hyperexplexia, Hyperglycinemia, Hyperimidodipeptiduria, Hyperinsulinism, Hyperkeratosis, Hyperlipidaemia, Hyperlipoproteinemia, Hyperlysinemia, Hypermethioninemia, Hyperornithinemia, Hyperostosis, Hyperoxaluria, Hyperparathyroidism, Hyperphalangism dysmorphy bronchomalacia, Hyperphenylalaninemic embryopathy, Hyperpipecolatemia, Hypersensitivity pneumonitis, Hypertelorism, Hyperthermia, Hyperthyroidism, Hypertrichosis, Hypertrophic neuropathy, Hypertrophic or verrucous lupus erythematosus, Hypertrophic subaortic stenosis, Hypobetalipoproteinemia, Hypobetalipoproteinemia, Hypochondroplasia, Hypocomplementaemic leucocytoclasic vasculitis, Hypodontia, Hypofibrinogenemia, Hypokalemic alkalosis, Hypokeratosis, Hypomyelination, Hypoparathyroidism, Hypopituitarism, Hypoplastic left heart syndrome, Hypoplastic right heart syndrome, Hypospadias, Hypothalamic hamartoblastoma syndrome, Hypothyroidism, Hypotrichosis, Hypoxanthine guanine phosphoribosyltransferase (HPRT) complete deficiency, I-cell disease, IBIDS syndrome, ICCA syndrome, ICE syndrome, ICF syndrome, ICOS deficiency, IDI, IED, IFAP syndrome, IGDA, IGF-1 deficiency, IGHD, IMAGe syndrome, INAD, INCL, IOMID syndrome, IOSCA, IPEX, IPSID, IRAK4 deficiency, ISOD, ITP, IVC stenosis, Ichthyiosis, Idaho syndrome, Idiopathic dystonia DYT1, Idiopathic granulomatous mastitis, Idiopathic hypereosinophilic syndrome, Idiopathic infantile arterial calcification, Idiopathic infection caused by BCG or atypical mycobacteria, Idiopathic interstitial pneumonia, Idiopathic juvenile osteoporosis, Idiopathic myelofibrosis, Idiopathic obliterative arteriopathy, Idiopathic orthostatic hypotension, Idiopathic pulmonary fibrosis, Idiopathic thrombocytopenic purpura, Ieshima-Koeda-Inagaki syndrome, Illum syndrome, Ilyina amoashy grygory syndrome, Imaizumi kuroki syndrome, Immune thrombocytopaenia, Immunodeficiency, Immunoproliferative small intestinal disease, Infant respiratory distress syndrome, Insulin-resistance syndrome, Insulinoma, Interdigitating dendritic cell sarcoma, Intermediate DEND syndrome, Intermediate spinal muscular atrophy, Internal carotid agenesis, Interstitial cystitis, Interstitial granulomatous dermatitis with arthritis, Interstitial pneumonia, Interventricular septum aneurysm, Intestinal atresia multiple, Intestinal epithelial dysplasia, Intestinal hypomagnesemia with secondary hypocalcemia, Intestinal lipodystrophy, Intestinal lipophagic granulomatosis, Intestinal lymphangiectasia, Intestinal pseudoobstruction, Intracerebral haemorrhage, Intracranial aneurysms, Intracranial arterioveinous malformation, Inverse Marcus-Gunn phenomenon, Iridocorneal endothelial syndrome, Iridogoniodysgenesis, Irons-Bhan syndrome, Irritable bowel syndrome, Isaac's syndrome, Isaacs mertens syndrome, Ischaemic brain injury, Ischemia/perfusion injury associated with solid organ transplantation procedure, Ischio-vertebral dysplasia, Iso-Kikuchi syndrome,

Isosporiasis, Isotretinoin syndrome, Isotretinoin-like syndrome, Isovaleric acidemia, Itin syndrome, Ito hypomelanosis, Ivemark syndrome, JAE, JWS, Jackson-Barr syndrome, Jackson-Weiss syndrome, Jacobs syndrome, Jacobsen syndrome, Jaffe campanacci syndrome, Jaffe-Lichtenstein disease, Jagell holmgren hofer syndrome, Jalili syndrome, Jancar syndrome, Japanese encephalitis, Jarcho-Levin syndrome, Jaw-Winking syndrome, Jensen syndrome, Jequier-Kozlowski syndrome, Jervell and Lange-Nielsen syndrome, Jeune syndrome, Job syndrome, Johanson-Blizzard syndrome, Johnson syndrome, Johnson-McMillin syndrome, Johnson-Munson syndrome, Johnston-Aarons-Schelley syndrome, Jones syndrome, Jorgenson lenz syndrome, Joubert syndrome, Joubert-Boltshauser syndrome, Juberg hayward syndrome, Juberg-Marsidi syndrome, Judge misch wright syndrome, Jumping Frenchman of Maine, Jung wolff back stahl syndrome, Juvenile chronic myelomonocytic leukaemia, Juvenile gastrointestinal polyposis, Juvenile glaucoma, Juvenile hemochromatosis, Juvenile hyaline fibromatosis, Juvenile idiopathic arthritis, Juvenile macular degeneration, Juvenile myelomonocytic leukaemia, Juvenile polyposis syndrome (JPS), Juvenile temporal arteritis, KBG syndrome, KBG-like syndrome, KID syndrome, Kabuki syndrome, Kaeser syndrome, Kahler's disease, Kaler garrity stern syndrome, Kallin syndrome, Kallmann syndrome, Kalyanaraman syndrome, Kanzaki disease, Kaplan-Plauchu-Fitch syndrome, Kaplowitz-Bodurtha syndrome, Kaposi's sarcoma, Kaposiform hemangioendothelioma, Kapur-Toriello syndrome, Karandikar-Maria-Kamble syndrome, Karsch neugebauer syndrome, Kartagener syndrome, Kasabach-Merritt syndrome, Kashani-Strom-Utley syndrome, Kasznica carlson coppedge syndrome, Katsantoni papadakou lagoyanni syndrome, Kaufman-Mckusick syndrome, Kawasaki disease, Kawashima syndrome, Kawashima-Tsuji syndrome, Kearns-Sayre syndrome, Kelley-Seegmiller syndrome, Kelly-Kirson-Wyatt syndrome, Kennedy disease, Kennedy-Teebi syndrome, Kennerknecht syndrome, Kenny syndrome, Kenny-Caffey syndrome, Kenya tick-bite fever, Keratinisation disorder associated with genetic eye disease, Keratitis, Keratoacanthoma, Keratoconus, Keratoderma, Keratosis, Kerion celsi, Kersey syndrome, Ketoacidosis, Ketoaciduria, Ketolysis disorder, Keutel syndrome, KGB syndrome, Khalifa-Graham syndrome, Kienbock disease, Kikuchi disease, Kikuchi-Fujimoto disease, Kimura disease, King-Denborough syndrome, Kinsbourne syndrome, Klatskin tumour, Klein-Waardenburg syndrome, Kleine-Levin syndrome, Kleiner holmes syndrome, Klinefelter syndrome, Klippel-Feil malformation, Klippel-Trenaunay syndrome, Kluver-Bucy syndrome, Kniest dysplasia, Knobloch layer syndrome, Kocher-Debre-Semelaigne syndrome, Kohler's disease, Kohlschuttér-Tonz syndrome, Kok disease, Komar syndrome, Konigsmark knox hussels syndrome, Kopysc barczyk krol syndrome, Kosenow syndrome, Kostmann syndrome, Kosztolanyi syndrome, Koussef nichols syndrome, Kousseff syndrome,

Kowarski syndrome, Kozlowski brown hardwick syndrome, Kozlowski massen syndrome, Kozlowski ouvrier syndrome, Kozlowski tsuruta syndrome, Kozlowski-Krajewska syndrome, Krabbe disease, Krasnow-Qazi syndrome, Krauss herman holmes syndrome, Kudo tamura fuse syndrome, Kugelberg-Welander disease, Kumar-Levick syndrome, Kunze riehm syndrome, Kurczynski-Casperson syndrome, Kuskokwim disease, Kuzniecky syndrome, Kynureninase deficiency, Kyphomelic dysplasia, Kyphosis brachyphalangy optic atrophy, Küssmaul-Maier disease, L1 syndrome, L-2-hydroxyglutaricaciduria, LADD syndrome, LBSL, LBWC syndrome, LCAD, LCAT deficiency, LCCS, LCDD, LCH, LCHAD deficiency, LDD, LEOPARD syndrome, LGMD, LHCDD, LIG4 syndrome, LMS, LORD, LPI, Laband syndrome, Lachiewicz sibley syndrome, Lactate dehydrogenase deficiency, Lactic acidosis, Lactotroph adenoma, Ladda zonana ramer syndrome, Lafora disease, Laing distal myopathy, Lambdoid synostosis, Lambert syndrome, Lambert-Eaton myasthenic syndrome, Lamellar ichthyosis, Laminopathy, Landau-Kleffner syndrome (LKS), Landing disease, Landouzy-Dejerine myopathy, Langer-Giedion syndrome, Langerhans cell granulomatosis, Langerhans cell histiocytosis, Langerhans cell sarcoma, Laparoschisis, Laplane fontaine lagardere syndrome, Laron syndrome, Larsen syndrome, Larsen-like syndrome, Laryngeal abductor paralysis, Laryngo onycho cutaneous syndrome, Laryngo-tracheo-esophageal cleft pulmonary hypoplasia, Lassa fever, Lassueur-Graham-Little syndrome, Late infantile neuronal ceroid lipofuscinosis, Late onset sepsis in premature infants, Lathosterolosis, Laubry-pezzi syndrome, Launois-Bensaude adenolipomatosis, Laurence-Moon syndrome, Laurin-Sandrow syndrome, Lawrence syndrome, Lawrence-Seip syndrome, Laxova-Opitz syndrome, Le Merrer syndrome, Le marec bracq picaud syndrome, Leao-da Silva syndrome, Learman syndrome, Leber 'plus' disease, Leber congenital amaurosis, Leber miliary aneurysm, Left renal vein entrapment syndrome, Left ventricular hypertrabeculation, Left ventricular noncompaction, Legg-Calve-Perthes disease, Legionellosis, Legionnaires' disease, Leichtman-Wood-Rohn syndrome, Leifer lai buyse syndrome, Leigh disease, Leiner disease, Leiomyomatosis of esophagus cataract hematuria, Leiomyomatosis, Leiomyosarcoma, Leipala kaitila syndrome, Leishmaniasis, Leisti-Hollister-Rimoin syndrome, Lemierre syndrome, Lenegre disease, Lennox-Gastaut syndrome, Leprechaunism, Leprosy, Leptospirosis, Leri pleonosteosis, Leri-Weill syndrome, Lesch-Nyhan syndrome, Lethal arthrogryposis with anterior horn cell disease (LAAHD), Lethal chondrodysplasia moerman type, Lethal congenital contracture syndrome, Lethal osteosclerotic bone dysplasia, Letterer-Siwe disease, Leucinosis, Leukaemia, Leukocyte adhesion deficiency (LAD), Leukodystrophy, Leukoencephalopathy, Leukonychia totalis, Leukotriene C4 (LTC4) synthase deficiency, Levic stefanovic nikolic syndrome, Levine-Critchley syndrome, Levocardia, Levy-Hollister syndrome,

Levy-Yeboa syndrome, Lewis-Pashayan syndrome, Lewis-Sumner syndrome, Lewy body dementia, Leydig cell hypoplasia, Lhermitte-Duclos disease, Li-Fraumeni syndrome, Lichen, Lichstenstein syndrome, Liddle syndrome, Lindsay-Burn syndrome, Linear hamartoma syndrome, Linitis plastica, Lip-pit syndrome, Lipid storage disease, Lipodystrophy, Lipodystrophy-HIV related, Lipoedema, Lipoid proteinosis, Lipomatosis, Lipoprotein metabolism disease, Liposarcoma, Lisker-Garcia-Ramos syndrome, Lissencephaly, Listeriosis, Little syndrome, Lobar atrophy of brain, Lobstein disease, Lobster-claw deformity, Localized Castleman disease, Localized scleroderma, Locked-in syndrome, Loeffler's endocarditis, Loeys-Dietz syndrome, Loffredo cennamo cecio syndrome, Logic syndrome, Loiasis, Long QT syndrome, Longman-Tolmie syndrome, Loose anagen syndrome, Lopes gorlin syndrome, Lopes marques de faria syndrome, Lopez-Hernandez syndrome, Lou-Gehrig disease, Louis-Bar syndrome, Lowe kohn cohen syndrome, Lowe oculocerebrorenal syndrome, Lowe syndrome, Lower mesodermal defects, Lown-Ganong-Levine syndrome, Lowry syndrome, Lowry-MacLean syndrome, Lowry-Yong syndrome, Lubani-Al Saleh-Teebi syndrome, Lubinsky syndrome, Lubs-Arena Syndrome, Lucey driscoll syndrome, Lucky gelehrter syndrome, Lujan-Fryns syndrome, Lunatomalacia, Lundberg syndrome, Lung agenesis heart defect thumb anomalies, Lung cancer small cell, Lung fibrosis, Lupus erythematosus, Lurie kletsky syndrome, Luteinizing hormone releasing hormone deficiency with ataxia, Lutz-Richner-Landolt syndrome, Lyell syndrome, Lyme borreliosis, Lyme disease, Lymphangioleiomyomatosis, Lymphangioma, Lymphatic filariasis, Lymphatic malformation, Lymphedema, Lymphocyte apoptosis anomaly, Lymphocyte-depleted classical hodgkin lymphoma, Lymphocyte-rich classical hodgkin lymphoma, Lymphocytic colitis, Lymphoid interstitial pneumonia, Lymphomatoid granulomatosis, Lymphoproliferative disease associated with primary immune disease, Lynch lee murday syndrome, Lynch syndrome, Lyngstadaas syndrome, Lysosomal disease, Lytico-bodig disease, M-CMTC, M/SCHAD, MAD, MADSAM, MAE, MALT lymphoma, MASA syndrome, MCA, MCAD deficiency, MCOPS1, MDC1A, MEB (Muscle-Eye-Brain) syndrome, MEHMO syndrome, MELAS, MEN 1, MEN 2, MERRF syndrome, MGA type I, MHBD deficiency, MIDD, MIRAS, MMEP syndrome, MMND, MNGIE syndrome, MOBA syndrome, MOCOD, MODY syndrome, MORM syndrome, MPPH syndrome, MPS, MRGH, MRKH syndrome, MRXS7, MSA, MTHFR deficiency, MVA syndrome, MYH9, Mac Duffie's syndrome, Mac dermot winter syndrome, Maccario mena syndrome, Macdermot-Patton-Williams syndrome, Machado-Joseph disease, Macias flores garcia cruz rivera syndrome, Mackay shek carr syndrome, Macroglossia, Macrophage or histiocytic tumour, Macrophagic activation syndrome, Macrophagic myofasciitis, Macrothrombocytopenia with leukocyte inclusions, Macular amyloidosis, Macular dystrophy, Macular edema, Madelung's disease,

Madras motor neuron disease, Maffucci syndrome, Majeed syndrome, Majewxki ozturk syndrome, Major airway collapse, Meleda disease, Malakoplakia, Malakoplasia, Malaria, Malignant fibrous histiocytoma, Malignant germ cell tumor, Malignant hyperpyrexia, Malignant hyperthermia, Malignant mesenchymal tumor, Malignant paroxysmal ventricular tachycardia, Mallory Weiss syndrome, Malouf syndrome, Maltase-glucoamylase deficiency, Maniac-depressive disorders, Manouvrier syndrome, Mansonellosis, Mantle cell lymphoma, Maple syrup urine disease, Marashi gorlin syndrome, Marble brain disease, Marburg disease, Marchiafava-Micheli disease, Marcus-Gunn syndrome, Marden walker like syndrome, Marfan syndrome, Margarita island ectodermal dysplasia, Marin-Amat syndrome, Marinesco-Sjogren syndrome, Marion mayers syndrome, Markel-Vikkula -Mulliken syndrome, Marles greenberg persaud syndrome, Maroteaux cohen solal bonaventure syndrome, Maroteaux le merrer bensahel syndrome, Maroteaux stanescu cousin syndrome, Maroteaux-Lamy syndrome, Maroteaux-Malamut syndrome, Marsden nyhan sakati syndrome, Marshall syndrome, Marshall-Smith syndrome, Martinez monasterio pinheiro syndrome, Martinez-Frias syndrome, Martsolf syndrome, Massa casaer ceulemans syndrome, Mast cell leukaemia, Mast cell sarcoma, Mastocytosis, Mastroiacovo de rosa satta syndrome, Mathieu de broca bony syndrome, Matsoukas liarikos giannika syndrome, Matthew-Wood syndrome, Mature B-cell tumour, Mature T-cell and NK-cell tumour, May-Hegglin thrombocytopenia, Mayer-Rokitansky-Küster-Hauser syndrome, Mazabraud syndrome, McArdle disease, McCabe's disease, McCune-Albright syndrome, McDonough syndrome, McDowall syndrome, McGrath syndrome, McKusick-Kaufman syndrome, McLeod syndrome, McPherson-Hall syndrome, Mcalister crane syndrome, Mccallum macadam johnston syndrome, Mcgillivray syndrome, Mclain - Dekaban syndrome, Mcpherson clemens syndrome, Meacham winn culler syndrome, Meadows' syndrome, Meckel like syndrome, Meckel syndrome, Meckel-Gruber syndrome, Meconium aspiration syndrome, Medeira dennis donnai syndrome, Mediastinal (thymic) large b-cell lymphoma, Mediastinal diffuse large-cell lymphoma with sclerosis, Mediastinal fibrosis, Medrano roldan syndrome, Medullar disease, Medullary cystic kidney disease, Medulloblastoma, Megacalycosis, Megaduodenum and/or megacystis, Megaloblastic anaemia, Megarbane-Loiselet syndrome, Mehes syndrome, Mehta-Lewis-Patton syndrome, Meier blumberg imahorn syndrome, Meier-Gorlin syndrome, Meige disease, Meinecke pepper syndrome, Meinecke syndrome, Melanoma, Meleda disease, Melhem fahl syndrome, Melioidosis, Melkersson rosenthal syndrome, Melnick-Needles syndrome, Melorheostosis, Membranoproliferative glomerulonephritis, Membranous glomerulopathy, Menetrier's disease, Mengel konigsmark syndrome, Meniere's disease, Meningioma, Meningitis, Menkes syndrome, Mental retardation, Meretoja syndrome, Merkel cell carcinoma

(MCC), Merlob grunebaum reisner syndrome, Mesangial sclerosis, Mesodermic dysplasia, Mesothelioma, Mesulam syndrome, Metabolic intoxication disease, Metabolic liver disease, Metaphyseal dysplasia, Michels syndrome, Mickleson syndrome, Micro syndrome, Microcephaly, Microcoria, Microcystic infiltrating lymphatic malformation, Microcytic anaemia, Microphthalmia, Microscopic colitis Microtia, Microvillous inclusion disease, Mid-aortic dysplastic syndrome, Midas syndrome, Middle aortic syndrome, Midline heart, Mietens syndrome, Mievis verellen dumoulin syndrome, Mikati najjar sahli syndrome, Mikulicz disease, Mild campomelic dysplasia, Miller syndrome, Miller-Dieker syndrome, Miller-Fisher syndrome (MFS), Mills syndrome, Milroy disease, Minimal change nephrotic syndrome (MCNS), Minkowski-Chauffard disease, Mirhosseini-Holmes-Walton syndrome, Mitral valve prolapse disease, Miura syndrome, Mixed connective tissue disease, Mixed phenotype acute leukaemia, Mixed sclerosing bone dystrophy, Miyoshi myopathy, Mls syndrome, Moderate and severe traumatic brain injury, Moebius syndrome, Moerman vandenberghe fryns syndrome, Moersch-Woltman syndrome, Moeschler clarren syndrome, Mohr syndrome, Mohr-Tranebjaerg syndrome, Mollica pavone antener syndrome, Moloney syndrome, Momo syndrome, Monilethrix, Mononen-Karnes-Senac syndrome, Monostotic fibrous dysplasia, Montefiore syndrome, Moore-Federman syndrome, Morava-Mehes syndrome, Morgagni-Stewart-Morel syndrome, Morillo cucci passarge syndrome, Morning glory syndrome, Morquio disease, Morris syndrome, Morse rawnsley sargent syndrome, Morvan syndrome, Moschcowitz disease, Mounier-Kuhn syndrome, Mousa-Al Din-Al Nassar syndrome, Movement disease, Mowat-Wilson syndrome, Moya-moya disease, Moynahan syndrome, Mpo deficiency, Msbd syndrome, Mseleni joint disease (MJD), Mucha Habermann Disease, Muckle-Wells syndrome, Mucoepithelial dysplasia, Mucolipidosis, Mucopolysaccharidosis, Mucormycosis, Mucosal pemphigoid, Mucosulfatidosis, Muenke syndrome, Muir-Torre syndrome, Mullerian aplasia, Multicentric Castleman disease (MCD), Multicentric giant lymph node hyperplasia, Multicentric osteolysis, Multifocal acquired demyelinating sensory and motor neuropathy, Multifocal pattern dystrophy simulating fundus flavimaculatus, Multiglandular hyperplasia, Multiminicore disease (MmD), Multinodular goiter cystic kidney polydactyly, Multiple carboxylase deficiency, Multiple contracture syndrome, Multiple cutaneous and uterine leiomyomas, Multiple endocrine neoplasia, Multiple epiphyseal dysplasia, Multiple fibrofolliculoma, Multiple hamartoma syndrome, Multiple keratoacanthoma, Multiple pterygium syndrome, Multiple sclerosis, Multiple sulfatase deficiency, Multiple system atrophy, Multiple ventricular septal defects, Mulvihill-Smith syndrome, MURCS association, Murray-Puretic-Drescher syndrome, Muscular channelopathy, Muscular dystrophy, Muscular fibrosis multifocal obstructed vessels, Mutchinick syndrome, Myalgia eosinophilia associated with tryptophan,

Myasthenia gravis, Myasthenic syndromes, Mycetoma, Mycoplasma encephalitis, Mycosis fungoides, Myelinoclastic diffuse sclerosis, Myelinosis centralis diffusa, Myelocerebellar disorder, Myelodysplastic or myeloproliferative disease, Myelofibrosis with myeloid metaplasia, Myeloid sarcoma, Myeloma, Myhre syndrome, Myiasis, Myoclonic dystonia, Myoclonic epilepsy, Myodysplasia, Myofibrillar myopathy, Myoglobinuria, Myopathy and diabetes mellitus, Myopathy, Myopia, Myositis ossificans progressiva, Myotilinopathy, Myotonia congenita, Myotonic disease, Myotubular myopathy, Myxofibrosarcoma, Myxoid liposarcoma, Myxoid malignant fibrous histiocytoma, Myxoma with fibrous dysplasia, Möbius syndrome, N syndrome, NACG, NAGS deficiency, NAME syndrome, NAO syndrome, NARP syndrome, NASH syndrome, NBS, NCL, NCMD, NF 1, NFJ syndrome, NHL, NHPP, NISCH syndrome, NOMID syndrome, NPLCA, NSIP, NTD, Naegeli syndrome, Naegeli-Franceschetti-Jadassohn syndrome, Nager syndrome, Naguib syndrome, Nail anomaly, Nail dysplasia, Naito-Oyanagi disease, Nakagawa's angioblastoma, Nakajo nishimura syndrome, Nakajo syndrome, Nakamura osame syndrome, Nance-Horan syndrome, Narcolepsy without cataplexy, Narcolepsy-Cataplexy, Nasodigitoacoustic syndrome, Nasopharyngeal cancer, Nasu-Hakola disease, Nathalie syndrome, Navajo brainstem syndrome, Naxos disease, Necrotising hypophysitis, Necrotizing myelitis, Nemaline myopathy, Neonatal Onset Multisystem Inflammatory Disease, Neonatal death immune deficiency, Neonatal hemochromatosis, Neonatal neutropenia, Neonatal respiratory distress syndrome, Nephroblastoma, Nephrogenic fibrosing dermopathy, Nephrogenic systemic fibrosis, Nephrolithiasis, Nephronophthisis - hepatic fibrosis, Nephropathy, Nephrosis, Nephrotic syndrome with diffuse mesangial sclerosis, Nephrotic syndrome, Nervous system tumour, Netherton disease, Neu-Laxova syndrome, Neuhauser daly magnelli syndrome, Neuhauser eichner opitz syndrome, Neuhauser's anomaly, Neural crest tumour, Neuroacanthocytosis, Neuroaxonal dystrophy, Neuroblastoma, Neurocutaneous melanosis, Neurodegeneration due to 3-hydroxyisobutyryl-CoA hydrolase deficiency, Neurodegeneration with brain iron accumulation (NBIA), Neurodegenerative disease, Neuroectodermal syndrome, Neuroepithelioma, Neurofibromatosis, Neurolipomatosis, Neuromuscular junction disease, Neuromyelitis optica, Neuromyotonia, Neuropathy, Neutral Lipid Storage Disease, Neutropaenia, Nevo syndrome, Nevoid hypermelanosis, Nezelof syndrome, Nicolaides baraitser syndrome, Niemann-Pick disease, Nievergelt syndrome, Niikawa-Kuroki syndrome, Nijmegen breakage syndrome, Nivelon-Nivelon-Mabille syndrome, Noack syndrome, Noble bass sherman syndrome, Nocardiosis, Nodular lymphocyte predominant Hodgkin lymphoma, Nodulosis-arthropathy-osteolysis syndrome, Noma, Non-24-Hour Sleep-Wake syndrome, Non-DYT1 idiopathic torsion dystonia, Non-Hodgkin malignant lymphoma, Non-alcoholic steatohepatitis, Non-

amyloid monoclonal immunoglobulin deposition disease, Non-giant cell granulomatous temporal arteritis with eosinophilia, Non-infectious uveitis affecting the posterior segment of the eye, Nonaka myopathy, Nondysgerminomatous germ cell tumor, Noonan like contracture myopathy hyperpyrexia, Noonan like syndrome, Noonan syndrome, Normomorphic sialidosis, Norrie disease, Norum disease, Nova syndrome, Novak syndrome, Nuclear cell envelopathy, O donnell pappas syndrome, O'Doherty syndrome, O'Sullivan-McLeod syndrome, OA-1, OCA, OCRL1, OFC syndrome, OFCD syndrome, OHSS, OLEDAID, ONMR syndrome, OPPG, ORW 2, OSLAM syndrome, OSMED, OTUDP syndrome, Obliterative portal venopathy, Occlusive infantile arteriopathy, Occupational allergic alveolitis, Ochoa syndrome, Ochronosis, Oculo skeletal renal syndrome, Oculo-osteo-cutaneous syndrome, Oculoectodermal syndrome, Oculogastrointestinal muscular dystrophy, Oculomotor palsy, Oculomotor paralysis, Oculopharyngodistal myopathy, Odontologic disease, Odontomatosis, Oerter-Friedman-Anderson syndrome, Oesophageal atresia, Oguchi disease, Ohaha syndrome, Ohdo madokoro sonoda syndrome, Ohtahara syndrome, Okamoto syndrome, Okihiro syndrome, Oligocone syndrome, Oligomeganephronia, Oliver mcfarlane syndrome, Oliver syndrome, Ollier disease, Olmsted syndrome, Omenn syndrome, Omodysplasia, Onat syndrome, Onchocerciasis, Ondine syndrome, Ondine-Hirschsprung disease, Onychodystrophy, Oochs syndrome, Ophtalmic ichthyosis, Ophtalmoplegia, Opitz BBB/G syndrome, Opitz reynolds fitzgerald syndrome, Opitz-Caltabiano syndrome, Opitz-Frias syndrome, Oppenheim's dystonia, Opsismodysplasia, Opsoclonus-myoclonus syndrome, Optic atrophy, Optic nerve hypoplasia, Optic neuropathy, Optic pathway glioma, Orbital leiomyoma, Ormond's disease, Ornithine aminotransferase deficiency, Orofaciodigital syndrome, Oromandibular dystonia, Oroticaciduria, Oroya fever, Osebold-Remondini syndrome, Osgood-Schlatter disease, Osler-Vaquez disease, Osteoarthropathy, Osteoblastoma, Osteochondritis, Osteochondromas, Osteochondrosis, Osteocraniostenosis, Osteodysplasia, Osteoectasia, Osteogenic sarcoma, Osteolysis, Osteomesopyknosis, Osteonecrosis, Osteopaenia, Osteopathia striata - cranial sclerosis, Osteopetrosis, Osteopoikilosis, Osteoporosis, Osteosarcoma, Osteosclerosis, Ostravik lindemann solberg syndrome, Otosclerosis, Ouvrier billson syndrome, Ovarian Sertoli-Leydig cell tumor, Ovarian cancer, Ovarian germ cell malignant tumor, Ovarioleukodystrophy, Oxalosis, PAF, PAGOD syndrome, PAN, PANDAS, PAP, PAPA syndrome, PARC syndrome, PCA, PCARP, PCH with optic atrophy, PCT, PDALS, PEHO syndrome, PEL, PELVIS syndrome, PFAPA syndrome, PFIC, PHACE syndrome, PIBIDS syndrome, PJS, PLOSL, PMD, PNDM, POADS, POEMS syndrome, POF, POMC deficiency, PPA, PPHS, PPM-X, PPoma, PSEK, PSP, PTC-RCC, PTLAH, PTLD, Pachygyria, Pachyonychia, Pacman dysplasia, Paediatric Autoimmune Disorders Associated with Streptococcus infections,

Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus infections, Paediatric granulomatous arthritis, Paget disease, Pagon stephan syndrome, Pai syndrome, Pallister W syndrome, Pallister-Hall syndrome, Pallister-Killian syndrome, Palmer pagon syndrome, Palpebral disease, Panayiotopoulos syndrome, Pancreatic carcinoma, Pancreatitis, Panner disease, Panniculitis, Panostotic fibrous dysplasia, Papillo-renal syndrome, Papillomatosis, recurrent respiratory, Papillon-Leage-Psaume syndrome, Papillon-Lefevre syndrome, Papular and sclerodermoid lichen myxedematosus, Papular atrichia, Papular mucinosis of infancy, Paramyotonia, Paraneoplastic pemphigus, Paraneoplastic retinopathy, Paraplegia, Parathyroid carcinoma, Parenchymatous liver disease, Paris-Trousseau thrombocytopenia, Parkes-Weber syndrome, Parkinson disease, Parkinsonism-dementia-ALS complex, Paroxysmal cold haemoglobinuria, Paroxysmal exertion-induced dyskinesia, Paroxysmal ventricular fibrillation, Parry-Romberg syndrome, Parsonage-Turner syndrome, Partial deep dermal and full thickness burns, Partington amyloidosis, Partington disease, Partington-Anderson syndrome, Partington-Mulley syndrome, Parvovirus antenatal infection, Pascuel castroviejo syndrome, Pashayan syndrome, Passwell-Goodman-Siprkowski syndrome, Patau syndrome, Patterned dystrophy of the retinal pigment epithelium, Patterson pseudoleprechaunism syndrome, Patterson stevenson syndrome, Patterson-Lowry rhizomelic dysplasia, Pauciarticular chronic arthritis, Pavone fiumara rizzo syndrome, Pearson syndrome, Peeling skin syndrome, Pelget-Huer anomaly, Pelizaeus-Merzbacher brain sclerosis, Pellagra, Pemphigus, Pena-Shokeir syndrome, Pendred syndrome, Penta X syndrome, Pentosuria, Peptide metabolism disease, Peptidic growth factors deficiency, Perheentupa syndrome, Periarteritis nodosa, Pericardial defect diaphragmatic hernia, Pericarditis, Perineurioma, Peripartum cardiomyopathy, Peripheral T-cell lymphoma, Peripheral neuropathy and optic atrophy, Peritoneal leiomyomatosis, Peritumoral oedema derived from brain tumours, Periventricular nodular heterotopia, Perlman syndrome, Pernicious anaemia, Perniola krajewska carnevale syndrome, Peroxisomal beta-oxidation disease, Perrault syndrome, Persistent Mullerian duct syndrome, Peters anomaly, Peters-plus syndrome, Petges-Clejat syndrome, Petit-Fryns syndrome, Petty laxova wiedemann syndrome, Peutz-Jeghers syndrome, Peyronie syndrome, Pfeiffer mayer syndrome, Pfeiffer palm teller syndrome, Pfeiffer rockelein syndrome, Pfeiffer syndrome, Pfeiffer-Kapferer syndrome, Pfeiffer-Singer-Zschiesche syndrome, Pfeiffer-Weber-Christian syndrome, Phacomatosis, Phaeochromocytoma, Phagocyte function anomaly, Phaver syndrome, Phelan-McDermid syndrome, Phenotypic diarrhoea, Phenylketonuria, Phocomelia, Phytosterolemia, Picardi-Lassueur-Little syndrome, Pick disease of brain, Piebaldism, Pierre Robin sequence associated with branchial archs anomalies, Pierre Robin sequence associated with collagen diseases, Pigeon-

breeder's lung disease, Pillay syndrome, Pilomatrixoma, Pilotto syndrome, Pinheiro freire maia miranda syndrome, Pinsky-Di George-Harley syndrome, Pitt-Hopkins syndrome, Pitt-Williams brachydactyly, Pitt-rogers-danks syndrome, Pituitary adenoma, Pituitary agenesis, Pituitary hormone deficiency, Pituitary lactotrophic adenoma, Pityriasis rubra pilaris, Piussan-Lenaerts-Mathieu syndrome, Plasma cell tumour, Platelet function disease, Platyspondylic dysplasia, Plectin deficiency, Pleomorphic liposarcoma, Pleuro-pulmonary blastoma, Pleuro-pulmonary endometriosis, Plott syndrome, Plum syndrome, Plummer-Vinson syndrome, Pneumoblastoma, Pneumocystosis, Pneumonia caused by Pseudomonas Aeruginosa, Poikilo-dermatomyositis, Pollitt syndrome, Polyarteritis nodosa, Polyarthritis, Polycystic kidney disease, Polycystic liver disease, Polycystic ovarian disease, Polycythaemia, Polydactyly, Polyepiphyseal dysplasia, Polymicrogyria, Polymorphic catecholergic ventricular tachycardia, Polymyositis, Polyostotic fibrous dysplasia, Polyposis, Polysyndactyly - cardiac malformation, Pompe disease, Popliteal web syndrome, Porokeratosis, Porphyria, Portal hypertension, Portal vein thrombosis, Post polio syndrome, Post transplantation graft dysfunction, Post-poliomyelitic syndrome, Post-transplant lymphoproliferative disease, Post-traumatic syringomyelia, Postanginal sepsis secondary to orophyngeal infection, Posterior cortical atrophy, Postpartum cardiomyopathy, Postviral Fatigue Syndrome, Potocki-Shaffer syndrome, Potter sequence, Powell chandra saal syndrome, Powell venencie gordon syndrome, Prader-Willi syndrome, Prata liberal goncalves syndrome, Preauricular pits renal disease, Precursor B-cell acute lymphoblastic leukaemia, Precursor T-cell acute lymphoblastic leukaemia, Preeyasombat-Varavithya syndrome, Pregnancy-related cholestasis, Premature aging, Pressure-induced localized lipoatrophy, Prieto-Badia-Mulas syndrome, Prieur-Griscelli syndrome, Primary biliary cirrhosis, Primary ciliary dyskinesia, Primary cutaneous CD30-positive T-cell lymphoproliferative disorders, Primary effusion lymphoma, Primary effusion lymphoma associated with the human immunodeficiency virus (HIV) infection, Primary intestinal lymphangiectasia, Primary lateral sclerosis, Primary lipodystrophy, Primary lymphoedema, Primary pulmonary lymphoma, Primary sclerosing cholangitis, Primerose syndrome, Progeria, Progressive bulbar paralysis of childhood, Progressive cone dystrophy, Progressive diaphyseal dysplasia, Progressive massive osteolysis, Progressive nephropathy with hypertension, Progressive neuronal degeneration of childhood with liver disease, Prolactinoma, Propping Zerres syndrome, Prostate cancer, Proteus syndrome, Proud-Levine-Carpenter syndrome, Prune belly syndrome, Psoriatic arthritis, PTEN Hamartoma syndrome, Pterygia, Pudendal neuralgia, Pudendal neuropathy, Pulmonar arterioveinous aneurysm, Pulmonary Langerhans' cell histiocytosis, Pulmonary alveolar microlithiasis, Pulmonary alveolar proteinosis, Pulmonary aortic stenosis,

Pulmonary arterial hypertension, Pulmonary arterio-veinous fistula, Pulmonary artery hypoplasia, Pulmonary atresia, Pulmonary blastoma, Pulmonary branch stenosis, Pulmonary endometriosis, Pulmonary haemosiderosis, Pulmonary insufficiency, Pulmonary lymphangiectasia, Pulmonary lymphangiomatosis, Pulmonary nodular lymphoid hyperplasia, Pulmonary supravalvular stenosis, Pulmonary surfactant protein anomalies, Pulmonary valve agenesis (PVA), Pulmonary venoocclusive disease, Pulp stones, Pulpal dysplasia, Puretic syndrome, Purtilo syndrome, Pycnodysostosis, Pyknoachondrogenesis, Pyknolepsy, Pyle disease, Pyoderma gangrenosum, Pyomyositis, Pyropoikilocytosis, Q fever, Qazi-Markouizos syndrome, Quattrin mcpherson syndrome, RAEB-1, RAPADILINO syndrome, RB-ILD, RECQ2, RECQL3, RHS, Rabson-Mendenhall syndrome, Radiation syndromes, Radio renal syndrome, Raine syndrome, Rajab-Spranger syndrome, Rambam-Hasharon syndrome, Rambaud galian syndrome, Ramon syndrome, Ramos arroyo clark syndrome, Ramsay hunt syndrome, Randall disease, Rapp-Hodgkin ectodermal dysplasia syndrome, Rapp-Hodgkin syndrome, Rasmussen johnsen thomsen syndrome, Rasmussen syndrome, Rathburn disease, Ray peterson scott syndrome, Raynaud phenomenon, Reardon-Baraitser syndrome, Reardon-Hall-Slaney syndrome, Recurrent hepatitis C virus induced liver disease in liver transplant recipients, Red cell aplasia, Refetoff syndrome, Reflex sympathetic dystrophy syndrome, Refsum disease, Reginato-Schiapachasse syndrome, Reifenstein syndrome, Reinhardt pfeiffer syndrome, Reiter's syndrome, Renal adysplasia, Renal cell carcinoma, Renal dysplasia, Renal glucosuria, Renal hypertension, Renal hypoplasia, Renal nutcracker syndrome, Renal tubular acidosis, Renal tubular disorder, Renal-coloboma syndrome, Rendu-Osler-Weber disease, Renier-Gabreels-Jasper syndrome, Renpenning syndrome, Resistance to activated protein C, Resistance to thyroid stimulating hormone, Respiratory bronchiolitis, Restless legs syndrome, Restrictive cardiomyopathy, Reticular perineurioma, Retinal arteriolar tortuosity, Retinal degeneration, Retinal dystrophy, Retinal hemorrhage, Retinoblastoma, Retinohepatoendocrinologic syndrome, Retinopathy of prematurity, Retinoschisis with early hemeralopia, Retinoschisis, Retraction syndrome, Retroperitoneal fibrosis, Rett like syndrome, Rett syndrome, Revesz-Debuse syndrome, Reye's syndrome, Reynolds syndrome, Rh deficiency syndrome, Rhabdomyosarcoma, Rheumatic fever, Rhizomelic dysplasia, Rhnull syndrome, Richards-Rundle syndrome, Richardson's syndrome, Richieri Costa-Guion Almeida-Cohen syndrome, Richieri costa da silva syndrome, Richieri costa gorlin syndrome, Richieri-Costa-Colletto syndrome, RichieriCosta-Pereira syndrome, Richner-Hanhart syndrome, Ricker syndrome, Rickettesiae disease, Riedel Thyroiditis, Rieger syndrome, Right atrium familial dilatation, Right ventricle hypoplasia, Rigid spine syndrome, Riley-Day syndrome, Riley-Smith syndrome, Rippberger aase syndrome,

Rippling muscle disease, Ritscher schinzel syndrome, Rivera-Perez-Salas syndrome, Roberts syndrome, Robinow syndrome, Robinow-Sorauf syndrome, Robinow-Unger syndrome, Robinow-like syndrome, Roch-Leri mesosomatous lipomatosis, Rocky Mountain spotted fever, Rodini richieri costa syndrome, Roger disease, Roifman-Melamed syndrome, Rokitansky syndrome, Romano-Ward long QT syndrome, Rombo syndrome, Rommen mueller sybert syndrome, Rosai-Dorfman disease, Rosenberg lohr syndrome, Rosenberg Chutorian syndrome, Rothmund-Thomson syndrome, Rotor syndrome, Roy maroteaux kremp syndrome, Rozin-Hertz-Goodman syndrome, Rubinstein-Taybi syndrome, Rudd-Klimek syndrome, Rudiger syndrome, Russell Silver syndrome, Russell weaver bull syndrome, Rutherfurd syndrome, Ruvalcaba syndrome, Ruvalcaba-Myhre-Smith syndrome, SADDAN, SANDO, SAPHO syndrome, SC phocomelia, SCA, SCAN 2, SCAR1, SCARF syndrome, SCASI, SCD, SCID, SCLC, SE(M)D, SGBS, SGS, SHORT syndrome, SIADH, SIBIDS syndrome, SJS, SLK, SMD, SMEI, SMMCI, SOD, SOLAMEN syndrome, SPG, SPONASTRIME dysplasia, SPS, SRP, SUNCT syndrome, Saal-Greenstein syndrome, Saccharopinuria, Sack-Barabas syndrome, Saethre-Chotzen syndrome, Saito kuba tsuruta syndrome, Sakati syndrome, Sakati-Nyhan syndrome, Sakati-Nyhan-Tisdale syndrome, Salcedo syndrome, Salla disease, Salmonellosis, Salti salem syndrome, Sammartino decreccio syndrome, San Luis Valley syndrome, Sandhoff disease, Sandifer syndrome, Sandrow syndrome, Sanfilippo disease, Sanjad-Sakati syndrome, Santavuori disease, Santos-Mateus-Leal syndrome, Sarcocystosis, Sarcoidosis, Sarcosinemia, Sarcosporidiosis, Satoyoshi syndrome, Say barber hobbs syndrome, Say barber miller syndrome, Say field coldwell syndrome, Say meyer syndrome, Scarring in glaucoma filtration surgical procedures, Schaap taylor baraitser syndrome, Scheie syndrome, Scheuermann disease, Schilbach-Rott syndrome, Schilder disease, Schimke syndrome, Schimmelpenning syndrome, Schindler disease, Schinzel syndrome, Schisis association, Schistosomiasis, Schmidt syndrome, Schmitt gillenwater kelly syndrome, Schneckenbecken dysplasia, Schnitzler syndrome, Schofer-Beetz-Bohl syndrome, Scholte begeer van essen syndrome, Schopf-Schulz-Passarge syndrome, Schwannomatosis, Schwartz-Jampel syndrome, Scimitar syndrome, Scleroatrophic syndrome, Scleroderma, Scleromyxedema, Sclerosing mediastinitis, Sclerosteosis, Scott syndrome, Scott-Bryant-Graham syndrome, Scott-Taor syndrome, Seaver cassidy syndrome, Sebastian syndrome, Seckel like syndrome, Seckel syndrome, Sedlackova syndrome, Seemanova lesny syndrome, Segawa syndrome, Seghers syndrome, Seitelberger disease, Selig-Benacerraf-Greene syndrome, Sellars-Beighton syndrome, Sengers syndrome, Sengers-Hamel-Otten syndrome, Senior syndrome, Senior-Boichis syndrome, Senior-Loken syndrome, Sensenbrenner syndrome, Senter syndrome, Sepsis, Septic phlebitis of the internal jugular vein,

Sequeiros sack syndrome, Servelle-Martorell syndrome, Setleis syndrome, Severe closed traumatic brain injury, Severe combined immunodeficiency T- B-, Severe combined immunodeficiency with hypereosinophilia, Severe combined immunodeficiency with leukopenia, Severe pneumococcemia, Sezary's lymphoma, Shapiro syndrome, Sharma kapoor ramji syndrome, Sharp syndrome, Sheehan syndrome, Shigellosis, Shokeir syndrome, Shone syndrome, Short QT syndrome, Short bowel syndrome due to necrotizing enterocolitis, Short bowel syndrome due to thrombosis, Short bowel syndrome, Shprintzen omphalocele syndrome, Shprintzen-Goldberg syndrome, Shulman syndrome, Shwachman-diamond syndrome, Shydrager syndrome, Sialidosis, Sickle cell anaemia, Sideroblastic anaemia, Sidransky-Feinstein-Goodman syndrome, Siegler brewer carey syndrome, Silengo lerone pelizzo syndrome, Sillence syndrome, Simosa penchaszadeh bustos syndrome, Simpson dysmorphia syndrome (SDYS), Simpson-Golabi-Behmel syndrome, Sinding-Larsen-Johansson disease, Singh chhaparwal dhanda syndrome, Singh-Williams-McAlister syndrome, Single ventricular septal defect, Singleton-Merten dysplasia, Singleton-Merten syndrome, Sino-auricular heart block, Sinus node disease and myopia, Sipple syndrome, Sirenomelia, Sitosterolemia, Situs inversus viscerum-cardiopathy, Sjögren syndrome, Sjögren-Larsson syndrome, Skeletal dysplasia, Skeletal muscle disease, Skin collagen disease, Skin vascular disease, Sleep disorder, Sleeping seekness, Sly disease, Small bowel adenocarcinoma, Small bowel leiomyosarcoma, Small non-cleaved cell lymphoma, Smith martin dodd syndrome, Smith-Fineman-Myers syndrome, Smith-Lemli-Opitz syndrome, Smith-Magenis syndrome, Sneddon syndrome, Sneddon-Wilkinson disease, Snyder-Robinson syndrome, Soft tissue perineurioma, Soft tissue sarcomas, Sohval soffer syndrome, Solitary plasmacytoma, Solomon syndrome, Somatotroph adenoma, Sommer hines syndrome, Sommer rathbun battles syndrome, Sommer-Young-Wee-Frye syndrome, Sondheimer syndrome, Sonoda syndrome, Sorsby syndrome, Sorsby's fundus dystrophy, Sotos syndrome, Spastic paraplegia, Spellacy gibbs watts syndrome, Spherophakia-brachymorphia, Sphingolipidosis, Spina bifida, Spinal atrophy, Spirillosis, Splenic marginal zone lymphoma, Spondylarthropathy, Spondylo camptodactyly syndrome, Spondylocostal dysostosis, Spondyloenchondrodysplasia, Spondyloepiphyseal dysplasia, Spongy degeneration of central nervous system, Spongy myocardium, Spontaneous pneumothorax familial type, Sporotrichosis, Squamous cell carcinoma of head and neck, St Louis encephalitis, Stalker chitayat syndrome, Stampe sorensen syndrome, Stapedo-vestibular ankylosis, Staphylococcal necrotizing pneumonia, Staphylococcal scarlet fever, Staphylococcal toxic shock syndrome, Stargardt disease, Stark-Kaeser syndrome, Startle disease, Steatocystoma, Steele-Richardson-Olszewski disease, Stein-Leventhal syndrome, Steinert myotonic dystrophy, Steinfeld syndrome, Stern-

Lubinsky-Durrie syndrome, Stevens-Johnson syndrome, Stickler syndrome, Stiff person syndrome, Still disease, Stimmler syndrome, Stoelinga de koomen davis syndrome, Stoll alembik finck syndrome, Stoll geraudel chauvin syndrome, Stoll kieny dott syndrome, Stoll-Levy-Francfort syndrome, Stomach cancer, Stormorken-Sjaastad-Langslet syndrome, Stratton garcia young syndrome, Stratton parker syndrome, Streptobacillosis, Streptococcal toxic-shock syndrome, Stress cardiomyopathie, Strumpell-Lorrain disease, Sturge-Weber syndrome, Stuve-Wiedemann dysplasia, Subcutaneous panniculitis-like T-cell lymphoma, Subpulmonary stenosis, Sucking/swallowing disorder, Sudden infant death syndrome, Sugarman syndrome, Sujansky-Leonard syndrome, Sulfocysteinuria, Summerskill-Walshe-Tygstrup syndrome, Summitt syndrome, Supravalvar aortic stenosis, Susac syndrome, Sutton disease II, Sweet syndrome, Swyer syndrome, Symphalangism, Syncopal paroxysmal tachycardia, Syncopal tachyarythmia, Syndromatic diarrhea, Synovialosarcoma, Synovitis, Synspondylism, Syntelencephaly, Syringocystadenoma papilliferum, Syringomyelia, Systemic capillary leak syndrome, Systemic lupus erythematosus, Systemic mastocytosis, Systemic scleroderma (systemic sclerosis), Systemic vasculitis, T cell immunodeficiency, T-cell leukaemia, T-cell chronic lymphocytic leukaemia, TAC, TAR syndrome, TCP, TDO syndrome, TEMF, TGA, TINU syndrome, TNF receptor 1 associated periodic syndrome, TOS, TRAPS syndrome, TTP, TTR amyloid cardiopathy, TTR amyloid neuropathy, Tabatznik syndrome, Takatsuki syndrome, Takayasu arteritis, Takotsubo cardiomyopathy, Tang hsi ryu syndrome, Tangier disease, Tardive dyskinesia, Tarsal Tunnel syndrome, Tarui disease, Tauopathy, Taussig-Bing syndrome, Tay syndrome, Tay-Sachs disease, Taybi syndrome, Taybi-Linder syndrome, Teebi al saleh hassoon syndrome, Teebi kaurah syndrome, Teebi naguib alawadi syndrome, Teebi shaltout syndrome, Telangiectasia, Telecanthus, Telfer sugar jaeger syndrome, Temtamy-Shalash syndrome, Ter Haar syndrome, Teratoma, Tetraamelia, Tetralogy of Fallot, Thakker donnai syndrome, Thalassaemia syndrome, Thanatophoric dysplasia, Theodore's syndrome, Thiele syndrome, Thiemann disease, Thies-Reis syndrome, Thomas jewett raines syndrome, Thomas syndrome, Thompson baraitser syndrome, Thomsen and Becker disease, Thong douglas ferrante syndrome, Thoracic aortic aneurysm and/or dissection, Thoracic outlet syndrome, Three M disease, Thromboangiitis obliterans, Thrombocytopaenia, Thrombocytopenic purpura autoimmune, Thrombocytopenic purpura idiopathic, Thrombocytosis, Thromboembolic pulmonary hypertension, Thrombotic disease of haematologic origin, Thymic aplasia, Thymic carcinoma, Thyroid tumor, Tick-borne encephalitis, Tietze syndrome, Timothy syndrome, Tollner horst manzke syndrome, Tolosa-Hunt syndrome, Tomaculous neuropathy, Tome brune fardeau syndrome, Toni-Debré-Fanconi disease, Tonoki-Ohura-Niikawa

syndrome, TORCH syndrome, Toriello syndrome, Toriello-Carey syndrome, Toriello-Higgins-Miller syndrome, Toriello-Lacassie-Droste syndrome, Torres ayber syndrome, Tourette syndrome, Townes-Brocks syndrome, Toxocariasis, Toxoplasma embryopathy, Toxoplasmosis, Tracheopathia osteoplastica, Tranebjaerg-Svejgaard syndrome, Transmissible spongiform encephalopathies, Transposition of the great arteries with pulmonary stenosis, Transthyretin amyloid polyneuropathy, Treacher-Collins syndrome, Aspiration pneumotitis requiring intubation and mechanical ventilation, Cardiogenic shock, Treft-Sanborn-Carey syndrome, Trench fever, Trevor disease, Triatrial heart, Trichinosis, Tricho onychic dysplasia, Tricho-dento-osseous syndrome, Tricho-hepato-enteric syndrome, Trichorhinophalangeal, Trichorrhexis nodosa syndrome, Trichothiodystrophy, Tricuspid atresia, Triopia, Triple A syndrome, Triple H (HHH) syndrome, Triplo-X syndrome, Trisomy, Tritanopia, Trochlear dysplasia, Tropical calcific chronic pancreatitis, Tropical endomyocardial fibrosis, Trueb burg bottani syndrome, Tsao-Ellingson syndrome, Tsukahara-Kajii syndrome, Tsukuhara syndrome, Tsutsugamushi disease, Tsutsugamushi fever, Tuberculosis, Tuberous sclerosis, Tubular duplication of the oesophagus, Tubular dysplasia, Tubular renal disease - cardiomyopathy, Tubulointerstitial nephritis and uveitis syndrome, Tucker syndrome, Tuffli-Laxova syndrome, Tularaemia, Tungiasis, Tungland-Bellman syndrome, Tunnel subaortic stenosis, Turcot syndrome, Turner syndrome, Turner-Kieser syndrome, Twin.twin transfusion syndrome, Tylosis, ULD, UPDM, UPDP, USH, Uhl anomaly, Ulbright hodes syndrome, Ulcerative colitis, Ulerythema ophryogenesis, Ulick syndrome, Ullrich disease, Umbilical cord ulceration, Univentricular cardiopathy, Unverricht-Lundborg disease, Upington disease, Upshaw-Schulman syndrome, Urbach-Wiethe disease, Urban-Rogers-Meyer syndrome, Urban-Schosser-Spohn syndrome, Uremic pruritus, Urrets-Zavalia syndrome, Usher syndrome, Usual interstitial pneumonia (UIP), Uveitis, VIPoma, VMCM, VODI syndrome, VSD, VWS, Vagneur triolle ripert syndrome, Van Allen-Myhre syndrome, Van Benthem-Driessen-Hanveld syndrome, Van Bogaert disease, Van Der Woude syndrome, Van biervliet hendrickx van ertbruggen syndrome, Van de berghe-Dequeker syndrome, Van den Bosch syndrome, Van den ende brunner syndrome, Van der Knapp syndrome, Van goethem syndrome, Van maldergem wetzburger verloes syndrome, Van regemorter pierquin vamos syndrome, Varadi-Papp syndrome, Vascular leukoencephalopathy, Vasculitis, Vasquez-Hurst-Sotos syndrome, Vasterbotten dystrophy, Vein of Galen aneurysm, Venencie powell winkelmann syndrome, Ventricular septal defect, Ventricular septal defect with aortic insufficiency, Verloes - Gillerot - Fryns syndrome, Verloes bourguignon syndrome, Verloes david syndrome, Verloes van maldergem marneffe syndrome, Verloes-Deprez syndrome, Verloove vanhorick brubakk syndrome, Verneuil disease, Viljoen winship syndrome, Viljoen-Kallis-Voges syndrome, Viljoen-Smart syndrome, Viral

hemorrhagic fever, Viral hepatitis, Viral vasculitis, Visceral neuropathy, Vitiligo, Vitreoretinal degeneration, Vogt-Koyanagi-Harada disease, Vohwinkel syndrome, Volcke-Soekarman syndrome, Von Gierke disease, Von Hippel-Lindau disease, Von Recklinghausen disease, Von Voss-Cherstvoy syndrome, Von Willebrand disease, Von hippel anomaly, Vsr syndrome, Vuopala disease, W syndrome, WAGR syndrome, WARBM1, WHIM syndrome, WL syndrome, WT limb-blood syndrome, Waaler-Aarskog syndrome, Waardenburg syndrome, Waardenburg-Shah syndrome, Wagner disease, Waisman syndrome, Waldenström macroglobulinemia, Waldmann disease, Walker-Dyson syndrome, Walker-Warburg syndrome, Wallis cremin beighton syndrome, Wallis zieff goldblatt syndrome, Warburg Micro syndrome, Warburg thomsen syndrome, Warburton-Anyane-Yeboa syndrome, Warman-Mulliken-Hayward syndrome, Water-West syndrome, Waterhouse-Friedrickson syndrome, Watson syndrome, Weaver like syndrome, Weaver syndrome, Weaver-Williams syndrome, Weber-Christian disease (WCD), Weber-Christian panniculitis, Webster deming syndrome, Wegener granulomatosis, Weil syndrome, Weill-Marchesani syndrome, Weismann Netter Stuhl syndrome, Weissenbacher-Zweymuller syndrome, Wellesley-Carman-French syndrome, Wells syndrome, Wells-Jankovic syndrome, Werdnig-Hoffmann disease, Wermer syndrome, Werner syndrome, Wernicke's encephalopathy, Wernicke-Korsakoff syndrome, West syndrome, West-Nile encephalitis, Westerhof-Beemer-Cormane syndrome, Western equine encephalomyelitis, Westphall disease, Whelan syndrome, Whipple disease, Whistling face syndrome, Whooping cough, Whyte-Murphy syndrome, Wieacker-Wolff syndrome, Wiedemann grosse dibbern syndrome, Wiedemann oldigs oppermann syndrome, Wiedemann-Beckwith syndrome, Wiedemann-Rautenstrauch syndrome, Wildervanck syndrome, Wilkes stevenson syndrome, Wilkie-Taylor-Scambler syndrome, Willebrand disease, Willi-Prader syndrome, Williams syndrome, Williams-Beuren syndrome, Wilms tumor, Wilson disease, Wilson-Turner syndrome, Winchester disease, Winkelman bethge pfeiffer syndrome, Winkelmann's cytophagic panniculitis, Winship viljoen leary syndrome, Winter harding hyde syndrome, Winter-Shortland-Temple syndrome, Wiskott-Aldrich syndrome, Wissler-Fanconi syndrome, Witkop syndrome, Wittwer syndrome, Wolcott-Rallison syndrome, Wolf-Hirschhorn syndrome, Wolff zimmermann syndrome, Wolff-Parkinson-White syndrome, Wolfram syndrome, Wolman disease, Woodhouse sakati syndrome, Woods black norbury syndrome, Woods leversha rogers syndrome, Woods-Crouchman-Huson syndrome, Worster drought syndrome, Worth syndrome, Wrinkly skin syndrome, Wyburn-Mason syndrome, XHIGM, XLAG syndrome, XMEA, XP, Xanthic urolithiasis, Xanthinuria, Xanthogranulomatous hypophysitis, Xanthomatosis cerebrotendinous, Xerocytosis, Xeroderma pigmentosum, Yellow fever, Yellow nail syndrome, Yersiniosis, Yorifuji-Okuno syndrome, Yoshimura-takeshita syndrom, Young maders syndrome, Young

syndrome, Young-Hugues syndrome, Young-Simpson syndrome, Yunis-Varon syndrome, ZASP-related myofibrillar myopathy, Zadik-Barak-Levin syndrome, Zellweger syndrome, Zellweger-like syndrome, Zimmer phocomelia, Zimmerman laband syndrome, Zinsser-Cole-Engman syndrome, Zlotogora-Ogur syndrome, Zlotogura-Martinez syndrome, Zollinger-Ellison syndrome, Zori stalker williams syndrome, Zunich-Kaye syndrome, Zygomycosis, 2,8 dihydroxy-adenine urolithiasis, 2-aminoadipic aciduria, 2-hydroxyglutaricaciduria, 2-methylbutyric aciduria, 3 hydroxyisobutyric aciduria, 3-hydroxy-3-methylglutaric aciduria, 3-methylcrotonylglycinuria, 3-methylglutaconic aciduria, 3C syndrome, 3M syndrome, 4-hydroxybutyricaciduria, Visceral leishmaniasis, Vernal keratoconjunctivitis, UV-A and visible light-induced photosensitivity disorders (chronic actinic dermatitis, cutaneous porphyrias, actinic prurigo and solar urticaria), Uremic pruritus, Tricyclic antidepressants poisoning, Traumatic spinal cord injury, Renal cell carcinoma, Superficial bladder cancer, *Staphylococcus aureus* bacteraemia, Spinal cord injury, Spina bifida, Soft tissue sarcoma, Small cell lung cancer, Sickle cell disease, Severe myoclonic epilepsy in infancy, Severe combined immunodeficiency (SCID), Severe closed traumatic brain injury, Retinopathy of prematurity, Retinitis pigmentosa, Respiratory distress syndrome in premature neonates of less than 32 weeks of gestational age, Recurrent hepatitis C virus induced liver disease in liver transplant recipients, Radiation proctitis, *Pseudomonas aeruginosa* lung infection in cystic fibrosis, Progressive myoclonic epilepsies, Primary malignant bone tumors, Primary apnoea of premature newborns, Post-transplant lymphoproliferative disorders, Post-neonatal intracerebral haemorrhage, Post transplantation graft dysfunction, Polycythemia vera, Peritumoral oedema derived from brain tumors, Peripheral T-cell lymphoma (nodal, other extranodal and leukaemic/ disseminated), Ductus arteriosus in premature neonates of less than 34 weeks of gestational age, Partial deep dermal and full thickness burns, Paroxysmal nocturnal haemoglobinuria, Pancreatic cancer, Painful HIV-associated neuropathy, Ovarian cancer, Osteosarcoma, Orthostatic hypotension in patients with pure autonomic failure, Orthostatic hypotension in patients with multiple system atrophy, Ornithine-transcarbamylase deficiency, Oral mucositis in head and neck cancer patients undergoing radiation therapy, Oesophageal cancer, Non-traumatic osteonecrosis, Non-ketotic hyperglycinaemia, Non-infectious uveitis affecting the posterior segment of the eye, Non-24-hour sleep-wake disorders in blind people with no light perception, Neuroblastoma, Neovascular glaucoma, Nephritic syndrome, Myelodysplastic syndromes, Myasthenia gravis, Moderate and severe traumatic brain injury, Metachromatic leukodystrophy, Medullary thyroid carcinoma, Mastocytosis, Mantle cell lymphoma, Malignant melanoma, Malignant gastrointestinal stromal tumors, Malabsorption due to exocrine pancreatic enzyme insufficiency, Low flow priapism, Lipoprotein lipase deficiency,

Ligneous conjunctivitis, Leber's hereditary optic neuropathy, Leber's congenital amaurosis, Late onset sepsis in premature infants of less than or equal to 32 weeks gestational age, Juvenile myelomonocytic leukaemia, Japanese encephalitis, Intestinal graft-versus-host disease, Indolent non-Hodgkin's lymphoma, Inborn errors in primary bile acid synthesis, Hyperphenylalaninemia, Hypereosinophilic syndrome, Glioma, High-grade dysplasia in Barrett´s oesophagus, *Herpes simplex* virus stromal keratitis, Hereditary factor XIII deficiency, Hepatocellular carcinoma, Hepatitis B re-infection following liver transplantation, Hepatic veno-occlusive disease, Gram negative bacterial lung infection in cystic fibrosis, Gastric cancer, Gamma sarcoglycanopathy, Follicular lymphoma, Familial adenomatous polyposis, Emphysema secondary to congenital alpha-1 antitrypsin deficiency, Duchenne muscular dystrophy, Diffuse large B cell lymphoma, Diffuse alveolar haemorrhage, Diarrhoea associated with intestinal microsporidial infection, Cutaneous T-cell lymphoma, Cutaneous forms of lupus erythematosus, Cushing's syndrome secondary to ectopic ACTH secretion, Corneal graft rejection, Congenital venous malformations, Congenital lymphatic malformations, Congenital alpha-1 antitrypsin deficiency, Congenital adrenal hyperplasia, Chronic pain, Cocaine poisoning, Chronic myeloid leukaemia, Chronic lymphocytic leukaemia, Chronic iron overload requiring chelation therapy, Chronic idiopathic myelofibrosis, Chronic eosinophilic leukaemia and the hypereosinophilic syndrome, Cholangiocarcinoma, Charcot-Marie-Tooth disease type 1A, Cardiogenic shock, Bronchopulmonary dysplasia in premature neonates of less than 30 weeks of gestational age, B-cell chronic lymphocytic leukemia, Autoimmune uveitis, Atypical Haemolytic Uraemic Syndrome (aHUS) associated with an inherited abnormality of the complement system, Aspiration pneumonitis requiring intubation and mechanical ventilation, Aneurysmal subarachnoid haemorrhage, Anaplastic thyroid cancer, Anal fistula, Acute sensorineural hearing loss (acute acoustic trauma, sudden deafness and surgery induced acoustic trauma), Acute peripheral arterial occlusion, Acute intermittent porphyria, Active phase of Peyronie's disease, Acanthamoeba keratitis, A-mannosidosis, 5q spinal muscular atrophy, Cavopulmonary Anastomosis, Atrial Septal Defects (ASD), Partial Anomalous Pulmonary Venous Return, Persistent Common Atrio Ventricular Canal Endocardial Cushion Defect. Ostium Primum, Single Atrium, Patent Ductus Arteriosus (PDA), Total Anomalous Pulmonary Venous Return, Ventricular Septal Defects (VSD), Pulmonary Valve Stenosis, Pulmonary Artery Stenosis and Stenosis of Pulmonary Artery Branches, Pulmonary Atresia with Intact Ventricular Septum, Congenital Mitral Valve Disease, Aortic Valvular Stenosis and Congenital Aortic Valvular Regurgitation, Supravalvular Aortic Stenosis, Transposition of the Great Arteries, Double Outlet Right Ventricle, Corrected Transposition of the Great Arteries, Truncus Arteriosus, Aorto Pulmonary Window,

Tricuspid Atresia, Ebstein Anomaly, Malformations of the Vena Cava, Coarctation of the Aorta, Atresia of Aortic Valve, Anomalies of the Aortic Arch, Anomalous Origin of the Right Subclavian Artery with Coarctation of the Aorta, Idiopathic Dilatation of the Pulmonary Artery, Left Pulmonary Artery Arising from Right Pulmonary Artery, Dextrocardia - Situs Inversus Totalis, Association of Heart Malformations with Asplenia, Malformations of the Vena Cava, Congenital Coronary Artery Arterio-Venous Fistula, Abnormal Origin of the Coronary Arteries, Aneurysm of the Sinus of Valsalva (Aortic Sinus Aneurysm), Endocardial Fibroelastosis, Idiopathic Hypertrophic Subaortic Stenosis (IHSS), Mitral Valve Prolapse - Barlow's Syndrome, Hypoplastic Left Heart.

**Pharmaceutical compositions**

Still another aspect of the present invention relates to the use of the peptide of the invention and the inventive peptide combination as an active ingredient, together with at least one pharmaceutically acceptable carrier, excipient and/or diluents for the manufacture of a pharmaceutical composition for the treatment and/or prophylaxis of cancer, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, a lung disease, a heart and vascular disease or a metabolic disease or any other disease disclosed herein.

Such pharmaceutical compositions comprise the peptide or the peptide combination as an active ingredient, together with at least one pharmaceutically acceptable carrier, excipient, binders, disintegrates, glidents, diluents, lubricants, coloring agents, sweetening agents, flavoring agents, preservatives or the like. The pharmaceutical compositions of the present invention can be prepared in a conventional solid or liquid carrier or diluents and a conventional pharmaceutically-made adjuvant at suitable dosage level in a known way. Preferably, the two peptides are contained in the combination in an amount from 20% by weight of peptide 1 to 80% by weight of peptide 2 to 80% by weight of peptide 1 to 20% by weight of peptide 2. More preferably, the two peptides are contained in the combination in an amount from 30% by weight of peptide 1 to 70% by weight of peptide 2 to 70% by weight of peptide 1 to 30% by weight of peptide 2. Still more preferably the two peptides are contained in the combination in an amount from 40% by weight of peptide 1 to 60% by weight of peptide 2 to 60% by weight of peptide 1 to 40% by weight of peptide 2.

Preferably the peptide or the peptide combination is suitable for intravenous administration or suitable for oral administration or suitable for administration by inhalation.

Administration forms include, for example, pills, tablets, film tablets, coated tablets, capsules, liposomal formulations, micro- and nano-formulations, powders and deposits. Furthermore, the present invention also includes pharmaceutical preparations for parenteral application, including dermal, intradermal, intragastral, intracutan, intravasal, intravenous, intramuscular, intraperitoneal, intranasal, intravaginal, intrabuccal, percutan, rectal, subcutaneous, sublingual, topical, or transdermal application, which preparations in addition to typical vehicles and/or diluents contain the peptide or the peptide combination according to the present invention.

The present invention also includes mammalian milk, artificial mammalian milk as well as mammalian milk substitutes as a formulation for oral administration of the peptide combination to newborns, toddlers, and infants, either as pharmaceutical preparations, and/or as dietary food supplements.

The peptide or the peptide combination of the invention can also be administered in form of its pharmaceutically active salts. Suitable pharmaceutically active salts comprise acid addition salts and alkali or earth alkali salts. For instance, sodium, potassium, lithium, magnesium or calcium salts can be obtained.

The peptide or the peptide combination of the invention forms pharmaceutically acceptable salts with organic and inorganic acids. Examples of suitable acids for such acid addition salt formation are hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, acetic acid, citric acid, oxalic acid, malonic acid, salicylic acid, p-aminosalicylic acid, malic acid, fumaric acid, succinic acid, ascorbic acid, maleic acid, sulfonic acid, phosphonic acid, perchloric acid, nitric acid, formic acid, propionic acid, gluconic acid, lactic acid, tartaric acid, hydroxymaleic acid, pyruvic acid, phenylacetic acid, benzoic acid, p-aminobenzoic acid, p-hydroxybenzoic acid, methanesulfonic acid, ethanesulfonic acid, nitrous acid, hydroxyethanesulfonic acid, ethylenesulfonic acid, p-toluenesulfonic acid, naphthylsulfonic acid, sulfanilic acid, camphersulfonic acid, china acid, mandelic acid, o-methylmandelic acid, hydrogen-benzenesulfonic acid, picric acid, adipic acid, D-o-tolyltartaric acid, tartronic acid, α-toluic acid, (o, m, p)-toluic acid, naphthylamine sulfonic acid, and other mineral or carboxylic acids well known to those skilled in the art. The salts are prepared by contacting the free base form with a sufficient amount of the desired acid to produce a salt in the conventional manner.

The pharmaceutical compositions according to the present invention will typically be administered together with suitable carrier materials selected with respect to the intended form of administration, i.e. for oral administration in the form of tablets, capsules (either solid filled, semi-solid filled or liquid filled), powders for constitution, aerosol preparations consistent with conventional pharmaceutical practices. Other suitable formulations are gels, elixirs, dispersible granules, syrups, suspensions, creams, lotions, solutions, emulsions, suspensions, dispersions, and the like. Suitable dosage forms for sustained release include tablets having layers of varying disintegration rates or controlled release polymeric matrices impregnated with the active components and shaped in tablet form or capsules containing such impregnated or encapsulated porous polymeric matrices. The pharmaceutical compositions may be comprised of 5 to 95% by weight of the peptide or the peptide combination, while also up to 100% of the pharmaceutical composition can consist of the peptide combination.

As pharmaceutically acceptable carrier, excipient and/or diluents can be used lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid filled capsules).

Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethyl-cellulose, polyethylene glycol and waxes. Among the lubricants that may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose, guar gum and the like. Sweetening and flavoring agents and preservatives may also be included where appropriate. Some of the terms noted above, namely disintegrants, diluents, lubricants, binders and the like, are discussed in more detail below.

Additionally, the compositions of the present invention may be formulated in sustained release form to provide the rate controlled release of any one or more of the components or active ingredients to optimize the therapeutic effects. Suitable dosage forms for sustained release include layered tablets containing layers of varying disintegration rates or controlled release polymeric matrices impregnated with the active components and shaped in tablet form or capsules containing such impregnated or encapsulated porous polymeric matrices.

Aerosol preparations suitable for inhalation may include solutions and solids in powder form, which may be in combination with a pharmaceutically acceptable carrier such as inert compressed gas, e.g. nitrogen.

For preparing suppositories, a low melting wax such as a mixture of fatty acid glycerides such as cocoa butter is first melted, and the active ingredient is dispersed homogeneously therein by stirring or similar mixing. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool and thereby solidify.

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for either oral or parenteral administration. Such liquid forms include solutions, suspensions and emulsions.

The peptide or the peptide combination of the present invention may also be deliverable transdermally. The transdermal compositions may take the form of creams, lotions, aerosols and/or emulsions and can be included in a transdermal patch of the matrix or reservoir type as are conventional in the art for this purpose.

The transdermal formulation of the peptide or the peptide combination of the invention is understood to increase the bioavailability of said peptide into the circulating blood. One problem in the administration of peptide(s) is the loss of bioactivity due to the formation of insolubles in aqueous environments or due to degradation. Therefore stabilization of peptide(s) for maintaining their fluidity and maintaining their biological activity upon administration to the patients in need thereof needs to be achieved.

Prior efforts to provide active agents for medication include incorporating the medication in a polymeric matrix whereby the active ingredient is released into the systemic circulation. Known sustained-release delivery means of active agents are disclosed, for example, in US4235988, US4188373, US4100271, US447471, US4474752, US4474753, or US4478822 relating to polymeric pharmaceutical vehicles for delivery of pharmaceutically active chemical materials to mucous membranes. The pharmaceutical carriers are aqueous solutions of certain polyoxyethylene-polyoxypropylene condensates. These polymeric pharmaceutical vehicles are described as providing for increased drug absorbtion by the mucous membrane and prolonged drug action by a factor of two or more. The substituents are block copolymers of polyoxypropylene and polyoxyethylene used for stabilization of drugs such as insulin.

Aqueous solutions of polyoxyethylene-polyoxypropylene block copolymers (poloxamers) are useful as stabilizers for peptide(s). Aside from serving as a stabilizer for the peptide(s), poloxamers provide excellent vehicles for the delivery of the peptide(s), and they are physiologically acceptable. Poloxamers, also known by

the trade name Pluronics (e.g. Pluronic F127, Pluronic P85, Pluronic F68) have surfactant properties that make them useful in industrial applications. Among other things, they can be used to increase the water solubility of hydrophobic, oily substances or otherwise increase the miscibility of two substances with different hydrophobicities. For this reason, these polymers are commonly used in industrial applications, cosmetics, and pharmaceuticals. They have also been used as model systems for drug delivery applications. In situ gelation of pharmaceutical compositions based on poloxamer that are biologically triggered are known in the art (e.g. US5256396), describing compositions containing poloxamer 407 and water at specified concentrations.

The term capsule refers to a special container or enclosure made of methyl cellulose, polyvinyl alcohols, or denatured gelatins or starch for holding or containing compositions comprising the active ingredients. Hard shell capsules are typically made of blends of relatively high gel strength bone and pork skin gelatins. The capsule itself may contain small amounts of dyes, opaquing agents, plasticizers and preservatives.

Tablet means compressed or molded solid dosage form containing the active ingredients with suitable diluents. The tablet can be prepared by compression of mixtures or granulations obtained by wet granulation, dry granulation or by compaction well known to a person skilled in the art.

Oral gels refers to the active ingredients dispersed or solubilized in a hydrophilic semi-solid matrix.

Powders for constitution refer to powder blends containing the active ingredients and suitable diluents which can be suspended in water or juices. One example for such an oral administration form for newborns, toddlers and/or infants is a human breast milk substitute which is produced from milk powder and milk whey powder, optionally and partially substituted with lactose.
Human breast milk is a complex fluid, rich in nutrients and in non-nutritional bioactive components. It contains all of the nutrients needed by the newborn baby. These include the metabolic components (fat, protein, and carbohydrates), water, and the raw materials for tissue growth and development, such as fatty acids, amino acids, minerals, vitamins, and trace elements.
More than 98% of the fat in is in the form of triglycerides. Oleic acid and palmitic acid are the most abundant fatty acids in breastmilk triglycerides, with comparatively high

proportions of the essential fatty acids, and linolenic acid, followed by long-chain polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid. These long-chain fatty acids are constituents of brain and neural tissue and are needed in early life for mental and visual development. The lipid component of breast milk is the transport vehicle for fat-soluble micronutrients such as prostaglandins and vitamins A, D, E, and K.

Proteins account for approximately 75 % of the nitrogen-containing compounds in breast milk. Non-protein nitrogen substances include urea, nucleotides, peptides, free amino acids, and DNA. The proteins of breast milk can be divided into two categories: micellar caseins and aqueous whey proteins, present in the ratio of about 40:60. Casein forms micelles of relatively small volume and produces a soft, flocculent curd in the infant's stomach. The major whey proteins are lactalbumin, lactoferrin, secretory IgA, and serum albumin, with a large number of other proteins and peptides present in smaller amounts.

The principal carbohydrate is lactose, a disaccharide produced in the mammary epithelial cell from glucose by a reaction involving lactalbumin.

In addition to the nutritional components, breast milk contains a wealth of bioactive components that have beneficial non-nutritional functions. These include a wide range of specific and non-specific antimicrobial factors; cytokines and anti-inflammatory substances; and hormones, growth modulators, and digestive enzymes (Table 1), many of which have multiple activities. These components may be of particular importance for young infants because of the immaturity of the host defense and digestive systems early in life.

**TABLE 2: Examples of the non-nutritional components of breast milk**

| **Antimicrobial factors** | **Growth factors** |
|---|---|
| secretory IgA, IgM, IgG<br>lactoferrin<br>lysozyme<br>complement C3<br>leucocytes<br>bifidus factor<br>lipids and fatty acids<br>antiviral mucins, GAGs<br>oligosaccharides | epidermal (EGF)<br>nerve (NGF)<br>insulin-like (IGF)<br>transforming (TGF)<br>taurine<br>polyamines |
| **Cytokines and anti-inflammatory factors** | **Digestive enzymes** |

| | |
|---|---|
| tumor necrosis factor<br>interleukins<br>interferons<br>prostaglandins<br>antichymotrypsin<br>antitrypsin<br>platelet-activating factor | amylase<br>bile acid-stimulating esterase<br>bile acid-stimulating lipases<br>lipoprotein lipase |
| **Hormones** | **Transporters** |
| feedback inhibitor of lactation (FIL)<br>insulin<br>prolactin<br>thyroid hormones<br>corticosteroids<br>ACTH<br>oxytocin<br>calcitonin<br>parathyroid hormone<br>erythropoietin | lactoferrin (Fe)<br>folate binder<br>cobalamin binder<br>IgF binder<br>thyroxine binder<br>corticosteroid binder |

Besides breast milk, infant formula is the only other infant milk which the medical community considers nutritionally acceptable for infants under the age of one year. Cow's milk is not recommended because of its high protein and electrolyte (salt) content which may harm infant's immature kidneys. The nutrient content of infant formula should comprise: Protein, Fat, Linoleic acid, Vitamins: A, C, D, E, K, thiamin (B1), riboflavin (B2), B6, B12, Niacin, Folic acid, Pantothenic acid, Calcium, Metals: magnesium, iron, zinc, manganese, copper; Phosphorus, Iodine, Sodium chloride, Potassium chloride. In addition, formulas not made with cow's milk must include biotin, choline, and inositol. Hypoallergenic formulas reduce the likelihood of certain medical complications in babies with specific health problems. Baby formula can be synthesized from raw amino acids. This kind of formula is sometimes referred to as elemental infant formula or as medical food because of its specialized nature.

Powder blends containing the active ingredients and suitable diluents which can be suspended in water or juices can be produced by spray drying.

Spray drying has been found the most suitable process for removing the last part of the water, since spray drying can convert milk concentrate into a powder while still keeping the valuable properties of the milk. The principle of all spray dryers is to transform the concentrate into many small droplets which are then exposed to a fast current of hot air. Because of the very large surface area of the droplets, the water

evaporates almost instantaneously and the droplets are transformed into powder particles.

Powdered milk is a powder made from dried milk solids. Powdered milk has a far longer shelf life than liquid milk and does not need to be refrigerated due to its low moisture content.

Instant milk powder is produced by partially rehydrating the dried milk powder particles causing them to become sticky and agglomerate. The water is then removed by drying resulting in an increased amount of air incorporated between the powder particles.

Milk powder manufacture is a process carried out on a large scale. It involves the gentle removal of water, while retaining all the desirable natural properties of the milk like colour, flavour, solubility, nutritional value.

Milk powder process includes spray drying, fluid bed processing, extraction, evaporation and freeze drying. Other processes are freeze concentration, filteration, and homogenisation.

The artificial mother milk formulations or mother milk substitutes of the present invention are preferably prepared by adding to a mother milk formulation including commercially available mother milk formulations especially in powder form the peptide or inventive peptide combination. The peptide or peptide combination is preferably added in an amount of 3 – 100 µg peptide or peptide combination per 100 ml (commercially available) mother milk formulation, more preferably in an amount of 5 – 70 µg / 100 ml and most preferably in an amount of 10 – 40 µg / 100 ml mother milk formulation.

Suitable diluents are substances that usually make up the major portion of the composition or dosage form. Suitable diluents include sugars such as lactose, sucrose, mannitol and sorbitol, starches derived from wheat, corn rice and potato, and celluloses such as microcrystalline cellulose. The amount of diluents in the composition can range from about 5 to about 95% by weight of the total composition, preferably from about 25 to about 75%, more preferably from about 30 to about 60% by weight, and most preferably from about 40 to 50% by weight.

The term disintegrants refers to materials added to the composition to help it break apart (disintegrate) and release the medicaments. Suitable disintegrants include starches, "cold water soluble" modified starches such as sodium carboxymethyl starch, natural and synthetic gums such as locust bean, karaya, guar, tragacanth and agar, cellulose derivatives such as methylcellulose and sodium carboxymethylcellulose, microcrystalline celluloses and cross-linked microcrystalline celluloses such as sodium croscarmellose, alginates such as alginic acid and sodium

alginate, clays such as bentonites, and effervescent mixtures. The amount of disintegrant in the composition can range from about 1 to about 40% by weight of the composition, preferably 2 to about 30% by weight of the composition, more preferably from about 3 to 20% by weight of the composition, and most preferably from about 5 to about 10% by weight.

Binders characterize substances that bind or "glue" powders together and make them cohesive by forming granules, thus serving as the "adhesive" in the formulation. Binders add cohesive strength already available in the diluents or bulking agent. Suitable binders include sugars such as sucrose, starches derived from wheat, corn rice and potato; natural gums such as acacia, gelatin and tragacanth; derivatives of seaweed such as alginic acid, sodium alginate and ammonium calcium alginate; cellulosic materials such as methylcellulose and sodium carboxymethylcellulose and hydroxypropyl-methylcellulose; polyvinylpyrrolidone; and inorganics such as magnesium aluminum silicate. The amount of binder in the composition can range from about 1 to 30% by weight of the composition, preferably from about 2 to about 20% by weight of the composition, more preferably from about 3 to about 10% by weight, even more preferably from about 3 to about 6% by weight.

Lubricant refers to a substance added to the dosage form to enable the tablet, granules, etc. after it has been compressed, to release from the mold or die by reducing friction or wear. Suitable lubricants include metallic stearates such as magnesium stearate, calcium stearate or potassium stearate; stearic acid; high melting point waxes; and water soluble lubricants such as sodium chloride, sodium benzoate, sodium acetate, sodium oleate, polyethylene glycols and d'l-leucine. Lubricants are usually added at the very last step before compression, since they must be present on the surfaces of the granules and in between them and the parts of the tablet press. The amount of lubricant in the composition can range from about 0.05 to about 15% by weight of the composition, preferably 0.2 to about 5% by weight of the composition, more preferably from about 0.3 to about 3%, and most preferably from about 0.3 to about 1.5% by weight of the composition.

Glidents are materials that prevent caking and improve the flow characteristics of granulations, so that flow is smooth and uniform. Suitable glidents include silicon dioxide and talc. The amount of glident in the composition can range from about 0.01 to 10% by weight of the composition, preferably 0.1% to about 7% by weight of the total composition, more preferably from about 0.2 to 5% by weight, and most preferably from about 0.5 to about 2% by weight.

Coloring agents are excipients that provide coloration to the composition or the dosage form. Such excipients can include food grade dyes and food grade dyes adsorbed onto a suitable adsorbent such as clay or aluminum oxide. The amount of the coloring agent can vary from about 0.01 to 10% by weight of the composition, preferably from about 0.05 to 6% by weight, more preferably from about 0.1 to about 4% by weight of the composition, and most preferably from about 0.1 to about 1%.

Peptide(s) of the invention can be used to form multiparticulates, discrete particles, well known dosage forms, whose totality represents the intended therapeutically useful dose of a drug. When taken orally, multiparticulates generally disperse freely in the gastrointestinal tract, and maximize absorption. A specific example is described in US 6068859, disclosing multiparticulates that provide controlled release of azithromycin. Another advantage of the multiparticulates is the improved stability of the drug. The poloxamer component of the multiparticulate is very inert, thus minimizing degradation of the drug.
However, formulation problems result from the melt-congeal process often used to form multiparticulates. The multiparticulates are preferably formed into round beads or spheres. Some carriers, when melted and then solidified, do not form round beads but may solidify into rods, strings, or other non-spherical shapes. The result is very irregularly shaped multiparticulates that are difficult to process into dosage forms. This problem is solved by e.g. WO 2007104173 where the particles consist of a poloxamer, a resin, and/or a tocopherol, creating together with the medicament (e.g. insulin) micelles. Micelle formation is essential for the absorption of many nutrients within the human body. Bile salts formed in the liver and secreted by the gall bladder allow micelles of fatty acids to form. This allows the absorption of complicated lipids and lipid soluble vitamins within the micelle by the small intestine. Micelles are approximately spherical in shape. Preferably, the peptide or the peptide combination of the invention are formulated with a poloxamer and a resin to form micelles suitable for oral administration to patients in need of the medicament.

Liquid form preparations include solutions, suspensions and emulsions. As an example may be mentioned water or water-propylene glycol solutions for parenteral injections or addition of sweeteners and opacifiers for oral solutions, suspensions and emulsions. Liquid form preparations may also include solutions for intranasal administration.

Other preferred pharmaceutical compositions are buffered solutions. The term buffer, buffer system, buffer solution and buffered solution, when used with reference to hydrogen-ion concentration or pH, refers to the ability of a system, particularly an

aqueous solution, to resist a change of pH on adding acid or alkali, or on dilution with a solvent. Preferred buffer systems can be selected from the group consisting of formate (pKa=3.75), lactate (pKa=3.86), benzoic acid (pKa=4.2) oxalate (pKa=4.29), fumarate (pKa=4.38), aniline (pKa=4.63), acetate buffer (pKa=4.76), citrate buffer (pKa2=4.76,pKa3=6.4), glutamate buffer (pKa=4.3), phosphate buffer (pKa=7.20), succinate (pKa1=4.93;pKa2=5.62), pyridine (pKa=5.23), phthalate (pKa=5.41); histidine (pKa=6.04), MES (2-(N-morpholino)ethanesulphonic acid; pKa=6.15); maleic acid (pKa=6.26); cacodylate (dimethylarsinate, pKa=6.27), carbonic acid (pKa=6.35), ADA (N-(2-acetamido)imino-diacetic acid (pKa=6.62); PIPES (4-piperazinebis-(ethanesulfonic acid; BIS-TRIS-propane (1,3-bis[tris(hydroxymethyl)methylamino]-propane), pKa=6.80), ethylendiamine (pKa=6.85), ACES 2-[(2-amino-2-oxoethyl)amino]ethanesulphonic acid; pKa=6.9), imidazole (pKa=6.95), MOPS (3-(N-morphin)-propansulfonic acid; pKa=7.20), diethylmalonic acid (pKa=7.2), TES (2-[tris (hydroxymethyl) methyl] amino ethanesulphonic acid; pKa=7.50) and HEPES (N-2-hydroxylethylpiperazin-N´-2-ethansulfonic acid; pKa=7.55) buffers or other buffers having a pKa between 3.8 to 7.7.

Preferred is the group of carboxylic acid buffers such as acetate and carboxylic diacid buffers such as fumarate, tartrate and phthalate and carboxylic triacid buffers such as citrate. Another group of preferred buffers is represented by inorganic buffers such as sulfate, borate, carbonate, oxalate, calcium hydroxyde and phosphate buffers. Another group of preferred buffers are nitrogen containing buffers such as imidazole, diethylenediamine, and piperazine.

Also preferred are sulfonic acid buffers such as TES, HEPES, ACES, PIPES, [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS), 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS), 4-Morpholinepropanesulfonic acid (MOPS) and N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES).

Another group of preferred buffers are glycine buffers such as glycine, glycyl-glycine, glycyl-glycyl-glycine, N,N-bis(2-hydroxyethyl)glycine and N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine (Tricine).

Preferred are also amino acid buffers such as glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophane, lysine, arginine, histidine, aspartate, glutamate, asparagine, glutamine, cysteine, methionine, proline, 4-hydroxyproline, N,N,N-trimethyllysine, 3-methylhistidine, 5-hydroxylysine, O-

phosphoserine, γ-carboxyglutamate, ε-N-acetyllysine, ω-N-methylarginine, citrulline, ornithine and derivatives thereof.

Table 3: Also preferred are the following buffers:

| **effective pH range** | **pKa 25°C** | **buffer** |
|---|---|---|
| 2.7-4.2 | 3.40 | malate (pK1) |
| 3.0-4.5 | 3.75 | formate |
| 3.0-6.2 | 4.76 | citrate (pK2) |
| 3.2-5.2 | 4.21 | succinate (pK1) |
| 3.6-5.6 | 4.76 | acetate |
| 3.8-5.6 | 4.87 | propionate |
| 4.0-6.0 | 5.13 | malate (pK2) |
| 4.9-5.9 | 5.23 | pyridine |
| 5.0-6.0 | 5.33 | piperazine (pK1) |
| 5.0-7.4 | 6.27 | cacodylate |
| 5.5-6.5 | 5.64 | succinate (pK2) |
| 5.5-6.7 | 6.10 | MES |
| 5.5-7.2 | 6.40 | citrate (pK3) |
| 5.5-7.2 | 6.24 | maleate (pK2) |
| 5.5-7.4 | 1.70, 6.04, 9.09 | histidine |
| 5.8-7.2 | 6.46 | bis-tris |
| 5.8-8.0 | 7.20 | phosphate (pK2) |
| 6.0-12.0 | 9.50 | ethanolamine |
| 6.0-7.2 | 6.59 | ADA |
| 6.0-8.0 | 6.35 | carbonate (pK1) |
| 6.1-7.5 | 6.78 | ACES |
| 6.1-7.5 | 6.76 | PIPES |
| 6.2-7.6 | 6.87 | MOPSO |
| 6.2-7.8 | 6.95 | imidazole |
| 6.3-9.5 | 6.80, 9.00 | BIS-TRIS propane |
| 6.4-7.8 | 7.09 | BES |
| 6.5-7.9 | 7.14 | MOPS |
| 6.8-8.2 | 7.48 | HEPES |
| 6.8-8.2 | 7.40 | TES |
| 6.9-8.3 | 7.60 | MOBS |

| | | |
|---|---|---|
| 7.0-8.2 | 7.52 | DIPSO |
| 7.0-8.2 | 7.61 | TAPSO |
| 7.0-8.3 | 7.76 | triethanolamine (TEA) |
| 7.0-9.0 | 0.91, 2.10, 6.70, 9.32 | pyrophosphate |
| 7.1-8.5 | 7.85 | HEPPSO |
| 7.2-8.5 | 7.78 | POPSO |

Preferred are the buffers having an effective pH range of from 2.7 to 8.5, and more preferred of from 3.8 to 7.7. The effective pH range for each buffer can be defined as pKa - 1 to pKa + 1, where Ka is the ionization constant for the weak acid in the buffer and pKa = - log K.

Most preferred are buffers suitable for pharmaceutical use e.g. buffers suitable for administration to a patient such as acetate, carbonate, citrate, fumarate, glutamate, lactate, phosphate, phthalate, and succinate buffers. Particularly preferred examples of commonly used pharmaceutical buffers are acetate buffer, citrate buffer, glutamate buffer and phosphate buffer. Also most preferred is the group of carboxylic acid buffers. The term "carboxylic acid buffers" as used herein shall refer to carboxylic mono acid buffers and carboxylic diacid buffers as well as carboxylic triacid buffers. Of course also combinations of buffers, especially of the buffers mentioned herein are useful for the present invention.

Some suitable pharmaceutical buffers are a citrate buffer (preferably at a final formulation concentration of from about 20 to 200 mM, more preferably at a final concentration of from about 30 to 120 mM) or an acetate buffer (preferably at a final formulation concentration of about 20 to 200 mM) or a phosphate buffer (preferably at a final formulation concentration of about 20 to 200 mM).

Techniques for the formulation and administration of the peptide or the peptide combination of the present invention may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton PA. A suitable composition comprising at least one peptide mentioned herein may be a solution of the peptide or the peptide combination in a suitable liquid pharmaceutical carrier or any other formulation such as tablets, pills, film tablets, coated tablets, dragees, capsules, powders and deposits, gels, syrups, slurries, suspensions, emulsions, and the like.

A particularly preferred pharmaceutical composition is a lyophilised (freeze-dried) preparation (lyophilisate) suitable for administration by inhalation or for intravenous

administration. To prepare the preferred lyophilised preparation the peptide or the peptide combination of the invention are solubilised in a 4 to 5% (w/v) mannitol solution and the solution is then lyophilised. The mannitol solution can also be prepared in a suitable buffer solution as described above.

Further examples of suitable cryo- / lyoprotectants (otherwise referred to as bulking agents or stabilizers) include thiol-free albumin, immunoglobulins, polyalkyleneoxides (e.g. PEG, polypropylene glycols), trehalose, glucose, sucrose, sorbitol, dextran, maltose, raffinose, stachyose and other saccharides (cf. for instance WO 97/29782), while mannitol is used preferably. These can be used in conventional amounts in conventional lyophilization techniques. Methods of lyophilisation are well known in the art of preparing pharmaceutical formulations.

For administration by inhalation the particle diameter of the lyophilised preparation is preferably between 2 to 5 µm, more preferably between 3 to 4 µm. The lyophilised preparation is particularly suitable for administration using an inhalator, for example the OPTINEB® or VENTA-NEB® inhalator (NEBU-TEC, Elsenfeld, Germany). The lyophilised product can be rehydrated in sterile distilled water or any other suitable liquid for inhalation adminstration.

Alternatively for intravenous administration the lyophilised product can be rehydrated in sterile distilled water or any other suitable liquid for intravenous administration.

After rehydration for administration in sterile distilled water or another suitable liquid the lyophilised preparation should have the approximate physiological osmolality of the target tissue for the rehydrated peptide preparation i.e. blood for intravenous administration or lung tissue for inhalation administration. Thus it is preferred that the rehydrated formulation is substantially isotonic.

The preferred dosage concentration for either intravenous, oral, or inhalation administration is between 100 to 2000 µmole/ml, and more preferably is between 200 to 800 µmole/ml. These are also the preferred ranges of the peptide combination in the mother milk substitute or artificial mother milk formulation or the pharmaceutical compositions disclosed herein.

**Dietary supplement**

Still another aspect of the present invention relates to the use of disclosed peptide and peptide combination as a dietary supplement. That dietary supplement is preferably for oral administration and especially but not limited to administration to

newborns, toddlers, and/or infants. A dietary supplement is intended to supplement the diet. The "dietary ingredients" in these products may in addition include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements may be manufactured in forms such as tablets, capsules, softgels, gelcaps, liquids, or powders.

**Method of treatment**

Another aspect of the present invention relates to a method of prophylaxis and/or treatment of cancer, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, a lung disease, a heart and vascular disease or a metabolic disease or any other disease disclosed herein comprising administering to a patient in need thereof a pharmaceutical composition comprising the peptide or the peptide combination according to the present invention in a therapeutically effective amount effective to treat the afore-mentioned disease.

Accordingly, the terms "prophylaxis" or "treatment" includes the administration of the peptide or the peptide combination of the present invention to prevent, inhibit, or arrest the symptoms of an infectious disease, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease. In some instances, treatment with the peptide or the peptide combination of the present invention will be done in combination with other protective compounds to prevent, inhibit, or arrest the symptoms of an infectious disease, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease.

The term “active agent” or “therapeutic agent” as used herein refers to an agent that can prevent, inhibit, or arrest the symptoms and/or progression of an infectious, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, a heart and vascular disease or any other disease disclosed herein.

The term "therapeutic effect" as used herein, refers to the effective provision of protection effects to prevent, inhibit, or arrest the symptoms and/or progression of an infectious, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease.

The term "a therapeutically effective amount" as used herein means a sufficient amount of the peptide or the peptide combination of the invention to produce a therapeutic effect, as defined above, in a subject or patient in need of treatment.

The terms "subject" or "patient" are used herein mean any mammal, including but not limited to human beings, including a human patient or subject to which the compositions of the invention can be administered. The term mammals include human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals.

The peptide or the peptide combination of the present invention can be used for the prophylaxis and/or treatment of cancer, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, a lung disease, a heart and vascular disease or a metabolic disease or any other disease mentioned herein in combination administration with another therapeutic compound. As used herein the term "combination administration" of a compound, therapeutic agent or known drug with the peptide or the peptide combination of the present invention means administration of the drug and the peptide or the peptide combination at such time that both the known drug and the peptide or the peptide combination will have a therapeutic effect. In some cases this therapeutic effect will be synergistic. Such concomitant administration can involve concurrent (i.e. at the same time), prior, or subsequent administration of the drug with respect to the administration of the peptide or the peptide combination of the present invention. A person of ordinary skill in the art would have no difficulty determining the appropriate timing, sequence and dosages of administration for particular drugs and peptide(s) of the present invention.

**Definition of peptide activity**

A peptide or peptide combination is deemed to have therapeutic activity if it demonstrated any one of the following activities listed in a) to g).

a) The peptide could inhibit the activity of an over active biological pathway.

b) The peptide could inhibit the production of an over produced biological molecule.

c) The peptide could inhibit the activity of an over produced biological molecule.

d) The peptide could increase the activity of an under active biological pathway.

e) The peptide could increase the production of an under produced biological molecule.

f) The peptide could mimic the activity of an under produced biological molecule.

g) The peptide could prevent, inhibit, or arrest the symptoms and/or progression of cancer, an infectious disease, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease or any other disease disclosed herein.

As used herein "inhibition" is defined as a reduction of the activity or production of a biological pathway or molecule activity of between 10 to 100%. More preferably the reduction of the activity or production of a biological pathway or molecule activity is between 25 to 100%. Even more preferably the reduction of the activity or production of a biological pathway or molecule activity is between 50 to 100%.

As used herein "increase" is defined as an increase of the activity or production of a biological pathway or molecule of between 10 to 100%. More preferably the increase of the activity or production of a biological pathway or molecule activity is between 25 to 100%. Even more preferably the increase of the activity or production of a biological pathway or molecule activity is between 50 to 100%.

As used herein "mimic" is defined as an increase in the activity of a biological pathway dependent on the under produced biological molecule of between 10 to 100%. More preferably the increase of the activity of the biological pathway is between 25 to 100%. Even more preferably the increase of the activity of the biological pathway is between 50 to 100%.

**Peptides and peptide combination**

The following peptides were tested alone and in combination for their activity as a therapeutic agent for the prophylaxis and/or treatment of cancer, an infectious disease, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, or a heart and vascular disease.

peptide 1 having the amino acid sequence: Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH (CNP (1-22)),

and the peptide 2 having the amino acid sequence: Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ (Physalaemin).

The term "CNP (1-22)" in brackets after the peptide sequence Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH is an abbreviation or synonyme of said peptide No. 1.
The term "Physalaemin" in brackets after the peptide sequence Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ is an abbreviation or synonyme of the second peptide No. 2.

Both peptides are preferably contained in the inventive combination in a molar ratio of 1 mole peptide 1 to 5 mole peptide 2 to 5 mole peptide 1 to 1 mole peptide 2, more preferred in a molar ratio of 1 mole peptide 1 to 4 mole peptide 2 to 4 mole peptide 1 to 1 mole peptide 2, still more preferred in a molar ratio of 1 mole peptide 1 to 3 mole peptide 2 to 3 mole peptide 1 to 1 mole peptide 2, still more preferred in a molar ratio of 1 mole peptide 1 to 2 mole peptide 2 to 2 mole peptide 1 to 1 mole peptide 2, and most preferred in a molar ratio of 1 mole peptide 1 to 1.5 mole peptide 2 to 1.5 mole peptide 1 to 1 mole peptide 2. Preferred ratios of the peptides in % by weight are disclosed above which can be used instead of the ratios mentioned as molar rates.

Furthermore the present invention relates to the use of the above-mentioned peptide combination as pharmaceutically active agents in medicine, i.e. as medicament. Advantage of the inventive peptide combination is that the peptides are less toxic in comparison to the commonly used drugs for the certain indications mentioned herein and that the peptide combination has less side effects, can be used for a long term treatment of certain diseases and can be easily administered. Moreover the peptide combination is selective for certain targets and under physiological conditions no toxic or noxious degradation products are formed.

As used herein, the term "peptide(s)" or "peptide combination" shall also refer to salts, deprotected or deacetylated forms, acetylated form, enantiomers, diastereomers, racemates, prodrugs and hydrates of the above-mentioned peptides. Diastereomers of a peptide are obtained when the stereochemical or chiral center of one or more amino acids is changed. The enantiomer has the opposite stereochemistry at all chiral centers.

The term "prodrug" refers to any precursor compound which is able to generate or to release the above-mentioned peptide under physiological conditions. Such prodrugs, i.e. such precursor molecules are for instance larger peptides which are selectively cleaved in order to form one of the above-mentioned peptides. Further

prodrugs are protected amino acids having especially protecting groups at the carboxylic acid and/or amino group.

Suitable protecting groups for amino groups are the benzyloxycarbonyl, t-butyloxycarbonyl (BOC), formyl, and acetyl or acyl group. Suitable protecting groups for the carboxylic acid group are esters such as benzyl esters or t-butyl esters.

The present invention also includes the above peptides having amino acid substitutions, deletions, additions, the substitutions and additions including the standard D and L amino acids and modified amino acids such as for example amidated and acetylated amino acids, wherein the therapeutic activity of the base peptide sequence as shown above is maintained.

In the listed peptide sequences "Ac" indicates an acetylated residue and "$NH_2$" indicates an amidated residue, "cyclo" indicates a cyclic peptide, and "D" indicates a D optical isomer. Deacetyled amino or NH-group refers to the free amino (–$HH_2$) group.

Table 4:

The following abbreviations are used for the common amino acids referred to herein.

| Abbreviation | Amino acid |
|---|---|
| Ala | Alanine |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic acid (Aspartate) |
| Cys | Cysteine |
| Gln | Glutamine |
| Glu | Glutamic acid (Glutamate) |
| Gly | Glycine |
| His | Histidine |
| Ile | Isoleucine |
| Leu | Leucine |
| Lys | Lysine |
| Met | Methionine |
| Phe | Phenylalanine |
| Pro | Proline |
| Pyl | Pyrrolysine |
| Ser | Serine |
| Sec | Selenocysteine |
| Thr | Threonine |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| Val | Valine |
| Asx | Aspartic acid or Asparagine |
| Glx | Glutamine or Glutamic acid |
| Xaa | Any amino acid |
| Xle | Leucine or Isoleucine |

Some modified amino acids are indicated as follows:

"D-2-Nal" is 2-naphthyl-D-alanine,

"SertBu" is t-butyl serine,

"Azagly" is aza glycine,

"Me" is methyl,

Met(O) is methionine sulfoxide,

"Pyr" and "pGlu" are pyroglutamic acid,

"Tyr(SO3H)" is sulphated tyrosine,

"Tyr(Me)" is methyltyrosine,

"NHEt" is ethylamide.

## EXAMPLES

The peptides as listed above and the inventive peptide combination with approximately equimolar amounts of the two peptides (deviation ±10%) were tested for activity using the assays described in Examples 1 to 17. The tested peptides are all commercially available and are all known petides and well described and characterized in the state of the art literature. The inventive peptide combination was prepared by simply mixing the two commercially available peptides in a molar ratio, for instance, between 0.9 to 1.1 and 1.1 to 0.9 (referred to as "approximately equimolar amounts") or other ratios such as from 0.5 – 1.5 to 1.5 – 0.5. It has to be mentioned that the biological data obtained with the peptide combinations in molar ratios between 0.4 to 1.6 mole (peptide 1 to peptide 2) and 1.6 to 0.4 mol (peptide 1 to peptide 2) were in general very similar to the biological data obtained with the equimolar peptide combinations so that in most cases the data for the equimolar (from 0.9 : 1.1 to 1.1 : 0.9) combinations are cited herein. Further data are presented only in these cases where the biological data of the peptide combinations with non equimolar amunts of the peptides differ more than 20% from the biological data of the equimolar combination.

The term "peptides" as used in the following examples refers to peptide 1, peptide 2 and the peptide combination and the concentration of "10 micrograms per ml" refers to 10 µg peptide 1 per ml or 10 µg peptide 2 per ml or 10 µg peptide combination per ml.

Thus the term "peptides" in the following examples indicates that the test disclosed in the corresponding example was conducted with peptide 1 alone and peptide 2 alone and with the peptide combination generally in equimolar ratios (molar ratio about 1 : 1 for peptide 1 : peptide 2) if no other molar ratio is mentioned in the corresponding example.

**EXAMPLE 1:**

**HIV-1 Experiments**

CEM-SS cells were passaged in T-75 flasks prior to use in the antiviral assay. On the day preceding the assay, the cells were split 1:2 to assure they were in an exponential growth phase at the time of infection. Total cell viability quantification was performed using a hemacytometer and trypan blue exclusion. Cell viability was greater than 95% for the cells to be utilized in the assay. The cells were resuspended at 5 X $10^4$ cells/ml in tissue culture medium and added to the peptides-containing microtiter plates in à volume of 50 microliters.

The virus used was the lymphocytotropic strain HIV-$1_{IIIB}$. Virus was obtained from NIH AIDS Research and Reference Reagent Program and was grown in CEM-SS cells for the production of stock virus pools. For each assay, a pre-titered aliquot of virus was removed from the freezer (-80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus was resuspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 microliters was the amount determined to give between 85% to 95% cell killing after 6 days post-infection. $TCID_{50}$ calculations by endpoint titration in CEM-SS cells indicated that the multiplicity of infection was approximately 0.01. AZT (nucleoside reverse transcriptase inhibitor; NRTI) and indinavir (protease inhibitor; PI) were used as positive control antiviral compounds.

Plate format

Each plate contained cell control wells (cells only), virus control wells (cells plus virus), drug cytotoxicity wells (cells plus peptides only), peptide colorimetric control wells (peptide only) as well as experimental wells (peptides - 10 micrograms per ml - plus cells plus virus). Samples were evaluated for antiviral efficacy with triplicate measurements and with duplicate measurements to determine cellular cytotoxicity, if detectable.

At assay termination, the plates were stained with the soluble tetrazolium-based dye MTS (CellTiter 96 Reagent, Promega) to determine cell viability and quantify peptides' toxicity. MTS is metabolized by the mitochondrial enzymes of metabolically active cells to yield a soluble formazan product, allowing the rapid quantitative analysis of cell viability and peptide cytotoxicity. This reagent is a stable, single solution that does not require preparation before use. At assay termination, 20-25 microliters of MTS reagent was added per well and the microtiter plates were then incubated for 5 hours at 37°C, and 5% $CO_2$ to assess cell viability. Adhesive plate sealers were used in place of lids, the sealed plates were inverted several times to mix the soluble formazan product and the plate was read spectrophotometrically at 490/560 nm with a Molecular Devices Vmax plate reader.

The overall assay performance was valid based upon judgement of the positive control compounds AZT and indinavir exhibiting the expected levels of antiviral activity. Macroscopic observation of the cells in each well of the microtiter plate confirmed the cytotoxicity results obtained following staining of the cells with the MTS metabolic dye.

Results from HIV experiments: The peptide combination of the invention showed no inhibition of HIV-1 activity on tested T-cells. In addition, the peptides of the invention did not show any significant inhibitory effects on cell viability in these human T-cells.

**EXAMPLE 2:**

**HBV Experimental Assay System**

HepG2-2.2.15 is a stable cell line containing the hepatitis B virus (HBV) ayw strain genome (ATCC Cat. No. CRL-11997). Antiviral compounds blocking any late step of viral replication such as transcription, translation, pregenome encapsidation, reverse transcription, particle assembly and release can be identified and characterized using this cell line. In this assay, an active compound will reduce the production of secreted HBV from cells, measured by utilizing real time quantitative PCR (TaqMan) assay to directly and accurately measure HBV DNA copies. The analysis of this data allows to calculate:

* Antiviral activity
* Compound Cytotoxicity

HepG2-2.2.15 cells were plated in 96-well microtiter plates. After 16-24 hours the confluent monolayer of HepG2-2.2.15 cells was washed and the medium was replaced with complete medium containing test peptides - 10 micrograms per ml - in duplicate. Lamivudine (3TC) was used as the positive control, while media alone was added to the cells as a negative control (virus control). Three days later the culture medium was replaced with fresh medium containing the peptides. Six days following the initial administration of the peptides, the cell culture supernatants was collected, treated with pronase and DNAse and then used in a real-time quantitative TaqMan PCR assay. The PCR-amplified HBV DNA was detected in real-time by monitoring increases in fluorescence signals that result from the exonucleolytic degradation of a quenched fluorescence probe molecule that hybridizes to the mplified HBV DNA. For each PCR amplification, a standard curve was simultaneously generated using dilutions of purified HBV DNA. Antiviral activity was calculated from the reduction in HBV DNA levels (% virus control). A novel dye uptake assay was then employed to measure cell viability, which is used to calculate toxicity (% cell control).

Results from HBV experiments: Peptide 2 had no effect on inhibition of HBV replication on tested liver cells. Peptide 1 inhibited by 10.1% HBV replication as compared to the virus control infection. The peptide combination did not provide synergistic effects. In addition, the peptides of the invention did not show any significant inhibitory effects on cell viability in these human liver cells.

**EXAMPLE 3:**

**HCMV experimental assay system**

MRC-5 cells (human embryonal lung fibroblasts) were obtained from the American Type Culture Collection (ATCC CCL-171; Rockville, Maryland) and grown in Eagle's Minimum Essential Medium with Earle's BSS (EMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 2.0 mM L-Glutamine, 100 units/ml Pencillinand 100 micrograms/ml Streptomycin. Cells were split twic a week 1:2.

HCMV strain AD169 was obtained from ATCC (ATCC VR-538). Virus stocks were prepared by infecting 80% confluent MRC-5 cells at a minimal multiplicity of infection in MRC-5 growth medium containing 2% FBS. Monolayers were incubated at 37°C, 5% $CO_2$ until 90%-95% viral cytopathic effect (CPE) was observed (10-13 days). Culture medium was then collected from the cells, centrifuged at low speed to remove cellular debris, aliquoted in 1ml volumes and stored at -80°C as stock virus.

MRC-5 cells were seeded at 75,000 cells/well in 24 well plates using MRC-5 growth medium. The plates were incubated overnight at 37°C, 5% $CO_2$. The following day, media was removed and 100 plaque forming units (pfu) of HCMV was added to the wells. Virus was allowed to adsorb onto the cells for 1 hour at 37°C, 5% $CO_2$. Peptides were diluted - 10 micrograms per ml - in assay medium containing 0.5% Methylcellulose. After the incubation period, 1ml of each peptide solution was added to the wells without aspirating the virus inoculums. The plates were incubated for 7-10 days to allow for plaque formation. Ganciclovir was used as positive control. Cultures were examined microscopically and toxicities were noted. The media was the aspirated from the wells and the cells were fixed and stained using 20% methanol containing Crystal Violet followed by enumeration of plaques by microscopic inspection.

For cytotxicity testing, MRC-5 cells were seeded at 2,500 cells/well in 96 well plates using growth medium. The plates were incubated overnight at 37°C, 5% $CO_2$. The following day, peptides were added and tested in duplicates. After a 6 days incubation period, cell viability was measured using CellTiter 96 Solution (Promega). Plates were incubated for additional 4 hours at 37°C. Adhesive plate sealers were used in place of lids, the sealed plates were inverted several times to mix the soluble formazan product and the plate was read spectrophotometrically at 490/560 nm with a Molecular Devices Vmax plate reader.

The overall assay performance was valid based upon judgement of the positive control compound Ganciclovir exhibiting the expected levels of antiviral activity. Macroscopic observation of the cells in each well of the microtiter plate confirmed the cytotoxicity results obtained following staining of the cells with the MTS metabolic dye.

Results from HCMV assay: Peptide 1 and peptide 2 did not show any inhibition of HCMV plaque formation as compared to the virus control experiment. In addition, the peptides of the invention did not show any significant inhibitory effects on cell viability in these human lung cells.

**EXAMPLE 4:**

**Methicillin Resistant *Staphylococcus Aureus* (MRSA) assay**

The antibacterial assay was conducted using clear, U-bottom 96-well microtiter plates. Cation-adjusted Mueller-Hinton Broth (MHB) was used for testing MRSA. The peptides of the invention (0.1 ml of each - 10 micrograms per ml -) were dispensed into wells in duplicate. Then the wells were inoculated with 5 x $10^5$ CFU/mL MRSA in 0.1 ml volume. For control purposes, each plate included 4 wells containing media without bacterial inoculum and 4 wells containing medium with inoculum but without peptides. The plates were incubated for 12 h at 37 °C, and read visually 18-24 hours post-incubation. Growth control of MRSA was examined first to determine adequacy of media preparations and growth conditions. Acceptable growth is defined as ≥ 2mm wide button of cells at the bottom of each sample well, or obvious turbidity in the culture supernatant. Test wells were examined and scored as positive/negative for activity. A positive score for activity is based on complete inhibition of macroscopic growth of the test MRSA.

Results from MRSA assay: Peptides of the invention and the peptide combination showed no inhibitory effects on the growth of MRSA.

**EXAMPLE 5:**

***Pseudomonas aeruginosa* assay**

The antibacterial assay was conducted using clear, U-bottom 96-well microtiter plates. Cation-adjusted Mueller-Hinton Broth (MHB) was used for testing *Pseudomonas aeruginosa*. The peptides of the invention (0.1 ml of each - 10 micrograms per ml -) were dispensed into wells in duplicate. Then the wells were inoculated with 5 x $10^5$ CFU/mL *Pseudomonas aeruginosa* in 0.1 ml volume. For control purposes, each plate included 4 wells containing media without bacterial inoculum and 4 wells containing medium with inoculum but without peptides. The plates were incubated for 12 h at 37 °C, and read visually 18-24 hours post-incubation. Growth control of *Pseudomonas aeruginosa* was examined first to determine adequacy of media preparations and growth conditions. Acceptable growth is defined as ≥ 2mm wide button of cells at the bottom of each sample well, or obvious turbidity in the culture supernatant. Test wells were examined and scored as

positive/negative for activity. A positive score for activity is based on complete inhibition of macroscopic growth of the test *Pseudomonas aeruginosa*.

Results from *Pseudomonas aeruginosa* assay: Peptides of the invention and the peptide combination showed no inhibitory effects on the growth of Pseudomonas aeruginosa.

**EXAMPLE 6:**

***Streptococcus pneumoniae* assay**

The antibacterial assay was conducted using clear, U-bottom 96-well microtiter plates. Cation-adjusted Mueller-Hinton Broth (MHB) was used for testing *Streptococcus pneumoniae*. The peptides of the invention (0.1 ml of each - 10 micrograms per ml -) were dispensed into wells in duplicate. Then the wells were inoculated with 5 x $10^5$ CFU/mL *Streptococcus pneumoniae* in 0.1 ml volume. For control purposes, each plate included 4 wells containing media without bacterial inoculum and 4 wells containing medium with inoculum but without peptides. The plates were incubated for 12 h at 37 °C, and read visually 18-24 hours post-incubation. Growth control of *Streptococcus pneumoniae* was examined first to determine adequacy of media preparations and growth conditions. Acceptable growth is defined as ≥ 2mm wide button of cells at the bottom of each sample well, or obvious turbidity in the culture supernatant. Test wells were examined and scored as positive/negative for activity. A positive score for activity is based on complete inhibition of macroscopic growth of the test *Streptococcus pneumoniae*.

Results from *Streptococcus pneumoniae* assay: Peptides of the invention and the peptide combination showed no inhibitory effects on the growth of Streptococcus pneumoniae.

**EXAMPLE 7:**

***Mycobacterium tuberculosis* assay**

The antibacterial assay was conducted using clear, U-bottom 96-well microtiter plates. Middlebrook 7H12 assay medium was used for testing drug-resistant *Mycobacterium tuberculosis*. The peptides of the invention (0.1 ml of each - 10 micrograms per ml -) were dispensed into wells in duplicate. Then the wells were inoculated with 5 x $10^5$ CFU/mL *Mycobacterium tuberculosis* in 0.1 ml volume. For control purposes, each plate included 4 wells containing media without bacterial inoculum and 4 wells containing medium with inoculum but without peptides. The plates were incubated for seven days at 37 °C, and read visually thereafter. Growth control of *Mycobacterium tuberculosis* was examined first to determine adequacy of

media preparations and growth conditions. Acceptable growth is defined as ≥ 2mm wide button of cells at the bottom of each sample well, or obvious turbidity in the culture supernatant. Test wells were examined and scored as positive/negative for activity. A positive score for activity is based on complete inhibition of macroscopic growth of the test *Mycobacterium tuberculosis*. The drug-resistant *Mycobacterium tuberculosis* that was used in the assay is resistant against following medicaments: para-aminosalicylic acid (PAS), streptomycin and isoniazid (INH).

Results from *Mycobacterium tuberculosis* assay: Peptides of the invention and the peptide combination showed no inhibitory effects on the growth of Mycobacterium tuberculosis.

**EXAMPLE 8:**

**Cell cycle assay**

Human A549 cells (carcinomic human alveolar basal epithelial cells) were utilized in the experiments employing the Propidium iodide cell cycle assay. The eukaryotic cell cycle is a series of events that take place in a cell leading to its replication.

The regulation of the cell cycle involves steps crucial to the cell, including detecting and repairing genetic damage, and provision of various checks to prevent uncontrolled cell division. The molecular events that control the cell cycle are ordered and directional; that is, each process occurs in a sequential fashion.

The cell cycle consists of four distinct phases: $G_1$ phase, S phase, $G_2$ phase (collectively known as interphase) and M phase. M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called $G_0$ phase. The relatively brief M phase consists of nuclear division and cytoplasmic division. The first phase within interphase, from the end of the previous M phase till the beginning of DNA synthesis is called $G_1$ (G indicating gap or growth). During this phase the biosynthetic activities of the cell resume at a high rate. This phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. The ensuing S phase starts when DNA synthesis commences; when it is complete, all of the chromosomes have been replicated. The cell then enters the $G_2$ phase, which lasts until the cell enters mitosis. Significant protein synthesis occurs during this phase, mainly involving the production of microtubules, which are required during the process of mitosis. Inhibition of protein synthesis during $G_2$ phase prevents the cell from undergoing mitosis.

Disregulation of the cell cycle components may lead to tumor formation.

Propidium iodide is an intercalating agent and a fluorescent molecule that can be used to stain DNA. Cells were incubated for 24 hours with test peptides - 10 micrograms per ml - or left untreated. After that cells were trypsinized, suspended in medium + 10% FCS, centrifuged (1000 rpm, 5 min), and the cell pellet resuspended in PBS (1 ml). The cells were pipetted into 2.5 ml absolute EtOH (final concentration approx. 70%) and incubated on ice for 15 min. Thereafter, cells were pelleted at 1500 rpm for 5 min and resuspended in Propidium iodide solution in PBS. After incubation for 40 min at 37°C, cells were analyzed in the FACS.
Results from cell cycle assay: Peptides of the invention and the peptide combination showed no inhibitory or irregular effects on the cell cycle of the tested human lung cells.

**EXAMPLE 9:**

**T cell proliferation assay**

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from normal human donors. The T cell proliferation was induced by stimulation of the cells with the T cell mitogen phytohemagglutinin (PHA), either in the absence (positive proliferation control), or in the presence of test peptides - 10 micrograms per ml - to examine their effects on the T cell proliferating response. $10^5$/well PBMC were plated in 96-well microtiter plates and assayed in duplicate with the peptides. Cell cultures were incubated at 37°C for 3 days in a 5% $CO_2$ incubator and were thereafter pulsed with 1microCi/well $^3$H-thymidine for additional 12 hours of culture. At the end of incubation time, the plates were harvested and the cells counted by liquid scintillation for the incorporation of $^3$H-thymidine as a measure of T cell proliferation.
Results from T cell proliferation assay: Peptides of the invention and the peptide combination showed no significant inhibitory effects on the proliferation of specifically stimutated human T-cells.

**EXAMPLE 10:**

**B cell proliferation assay**

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from normal human donors. The B cell proliferation was induced by stimulation of the cells with the B cell mitogen Staphylococcus aureus Cowans I (SAC) plus Interleukin-2, either in the absence (positive proliferation control), or in the presence of test peptides - 10 micrograms per ml - to examine their effects on the B cell response. $10^5$/well PBMC were plated in 96-well microtiter plates and assayed in duplicate with the peptides.

Cell cultures were incubated at 37°C for 3 days in a 5% $CO_2$ incubator and were thereafter pulsed with 1microCi/well $^3$H-thymidine for additional 12 hours of culture. At the end of incubation time, the plates were harvested and the cells counted by liquid scintillation for the incorporation of $^3$H-thymidine as a measure of B cell proliferation.

Results from B cell proliferation assay: Peptides of the invention and the peptide combination showed no significant inhibitory effects on the proliferation of specifically stimutated human B-cells.

**EXAMPLE 11:**

**Phagocytosis assay**

RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were obtained from ATCC and grown in RPMI 1640 medium containing 10% FBS. Cells were incubated in 12x75 mm tubes at 37°C with test peptides - 10 micrograms per ml - for 30 min prior to adding Fluorescein-labeled *Escherichia coli* bacteria as the agent to be ingested. After the cells were incubated for additional 60 min at 37°C and allowed to ingest the Fluorescein-labeled *Escherichia coli* bacteria, cells were fixed with 1% paraformaldehyde. The samples were then analyzed by flow cytometry to determine the amount of phagocytosis as a function of brightness (the greater the phagocytic activity, the more fluorescence in the macrophage population). Data are reported as % positive and the mean fluorescence intensity (MFI) of positively stained cells.

Results from phagocytosis assay: Peptide 1 induced by 42.9% phagocytosis as compared to the virus control experiment. Peptide 2 did not induce phagocytosis as compared to the virus control experiment. The peptide combination (0.95 mole peptide 1 and 1.05 mole peptide 2) induced by 46.7% phagocytosis and the peptide combination (1.50 mole peptide 1 and 0.50 mole peptide 2) induced by 50.7% phagocytosis as compared to the virus control experiment.

**EXAMPLE 12:**

**Apoptosis induction assay**

Human A549 cells (carcinomic human alveolar basal epithelial cells) were utilized in the experiments employing the Annexin-5 apoptosis assay. Annexin-5 is a member of a highly conserved protein family that binds acidic phospholipids in a calcium-dependent manner. Annexin-5 possesses a high affinity for phosphatidylserine. Phosphatidylserine is translocated from the inner side of the plasma membrane to the outer layer when cells undergo death by apoptosis or cell necrosis and serves as

a signal by which cell destined for death are recognized by phagocytes. Test peptides - 10 micrograms per ml - were exposed for 24 hours to the A549 cells before they were analyzed for signs of apoptosis.

Results from apoptosis induction assay: Peptides of the invention and the peptide combination showed no significant induction of apoptosis on human lung cells.

**EXAMPLE 13:**

**Apoptosis prevention assay**

Human A549 cells (carcinomic human alveolar basal epithelial cells) were utilized in the experiments employing the Annexin-5 apoptosis assay. Annexin-5 is a member of a highly conserved protein family that binds acidic phospholipids in a calcium-dependent manner. Annexin-5 possesses a high affinity for phosphatidylserine. Phosphatidylserine is translocated from the inner side of the plasma membrane to the outer layer when cells undergo death by apoptosis or cell necrosis and serves as a signal by which cell destined for death are recognized by phagocytes. A549 cells were pretreated for 30 min with test peptides - 10 micrograms per ml - followed by the exposure to C2 ceramide. Ceramide mediates cell apoptosis through the activation of the mitogen activating protein kinase (MAPK) and the stress activated kinase (JNK/SAPK). C2 ceramide is a synthetic, membrane soluble analog of ceramide.

Results from apoptosis prevention assay: Peptides of the invention and the peptide combination showed no significant protection against ceramide-induced apoptosis on human lung cells.

**EXAMPLE 14:**

**Th1/Th2 cytokine profiling assay**

The Balb/c mice (originated in 1923, it is a popular strain and is used in many different research disciplines. Also classified as an inbred from the production of 20 or more successive brother-sister matings, the Balb/c mouse is albino and small in size) were immunized on Days 1, 15, and 29 with Ovalbumin (Ovalbumin is the main protein found in egg white, commonly used to stimulate an immunological reaction in test animals) in PBS (5 micrograms/injection). On day 50, spleens of the mice were harvested (3 weeks after last boost with Ovalbumin). Cells were cultured ($2x10^5$/well in triplicate) and incubated with culture medium or test peptides - 10 micrograms per ml - for 30 min. Thereafter, additional Ovalbumin was added to the cells at 10 micrograms/ml for in vitro restimulation of the cells. 72 hours later, cell supernatants were harvested and assayed using the Becton Dickinson Mouse Th1/Th2 Cytokine

CBA Kit. This kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interferon-γ (IFN-γ ), and Tumor Necrosis Factor-α (TNF-α ) protein levels in a single sample. The kit performance has been optimized for analysis of physiologically relevant concentrations (pg/ml levels) of specific cytokine proteins in tissue culture supernatants and serum samples.

Results from Th1/Th2 Cytokine assay:

| Compound | **TNF-a** spleen cells; **TH1** response (% of control) | **IFNγ** spleen cells; **TH1** response (% of control) | **IL-2** spleen cells (% of control) | **IL-4** spleen cells; **TH2** response (% of control) | **IL-5** spleen cells; **TH2** response (% of control) |
|---|---|---|---|---|---|
| CNP (1-22) | 111.8 | 140.2 | 52.2 | 0.0 | 56.3 |
| Physalaemin | 85.7 | 74.2 | 0.0 | 0.0 | 53.0 |
| combination | 97.4 | 87.8 | 34.2 | 0,0 | 36.9 |

**EXAMPLE 15:**

**TNF alpha production assay**

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from normal human donors. The macrophages were prepared by adherence of PBMC to the plastic wells of the plates. After 8 days in culture in the presence of recombinant human macrophage-colony stimulating factor at 2ng/ml, differentiated macrophages were preincubated with test peptides - 10 micrograms per ml - for 30 min, followed by in-well stimulation by the addition of lipopolysaccharide at a final concentration of 200ng/ml. Not stimulated macrophages served as negative background control.

After overnight incubation, supernatants from the control and LPS-stimulated cultures were harvested and assayed for TNF alpha production employing a TNF alpha specific ELISA.

Results from TNF alpha assay: Peptides of the invention and the peptide combination did not significantly change the LPS-induced TNF-alpha production in human macrophages.

**EXAMPLE 16:**

**Endothelial cell migration assay**

Endothelial cell migration is a prerequisite for the process of neo-vascularization or angiogenesis which is crucial for on-site recruitment of blood vessel formation. Primary Human endothelial cells (HUVEC) were seeded in insert chambers with 3 micrometer pore size of multi-transwell plate for 6 hours at 37°C in Endothelial Cell Basal Medium (EBM) supplemented with 0.1% bovine serum albumin. Thereafter, designated concentration of testing peptides - 10 micrograms per ml - was added in duplicate wells. The endothelia were allowed to migrate for 22 hours at 37°C, then, migrated cells were fixed and stained with Hoechst 33342 dye. Images of 3 fields per insert were taken and the number of migrated cells per field were quantified using the ImageProPlus software. Data were analyzed for the average number of the migrated cells and standard deviation of six data points for each treatment condition. Active test peptides against HUVEC migration was determined based on 50% inhibition of migrated cells as compared with the control. Statistic p values were computed using the Student's t-test.

**Results from endothelial cell migration assay:** Peptide 2 induced by 15% the migration of human endothelial cells, peptide 1 inhibited by 7% the migration of endothelial cells, and the peptide combination (peptide 1 : peptide 2 (0.95 mole : 1.05 mole)) did not show any synergistic effect on the inhibition of the migration of human endothelial cells.

**EXAMPLE 17:**

**Endothelial tube formation assay**

The endothelial tube formation assay is based on the ability of endothelial cells to form three-dimensional capillary-like tubular structures when cultured on a gel of basement membrane extract. The endothelial tube formation assay represents a powerful model for studying inhibition and induction of angiogenesis. Pre-labeled HUVEC with Calcein AM were seeded in a 96-well culture plate coated with extracellular metrix (Chemicon international Cat. ECM625) and treated with test peptides - 10 micrograms per ml - in full growth medium. Positive control was vehicle only. The endothelial cells were allowed to form tubes for 20 hours and were then examined under an inverted fluorescent microscope. Duplicate wells for each

treatment were photographed and quantitatevily analyzed for an average tubule length using image analysis software ImageProPlus. Raw data were expressed as average tubule lenghs in pixels ± standard deviation. Statistic p values were computed using the Student's t-test.

Results from endothelial tube formation assay: Peptide 1 inhibited by 22% the tube formation of human endothelial cells, peptide 2 inhibited by 6% the tube formation endothelial cells, and the peptide combination (peptide 1 : peptide 2 (0.95 mole : 1.05 mole) did not show any synergistic effect on the tube formation of human endothelial cells.

**EXAMPLE 18**

**Mother milk formulation**

Methods to prepare mother milk or artifical mother milk formulations or mother milk substitutes are described in WO03043429, US5962062, WO0030461, EP0527283, EP0832565

One example of an artificial mother milk or mother milk substitute formulation is provided in the following while also the other formulations disclosed in the above mentioned references can be used and are included herewith by refernece.

The milk substitute contains, by weight, approximately 15% skimmed milk solids, approximately 75% demineralized water, approximately 9% soya oil, approximately 0.02% of carrageenates, 0.2% lecithin, and approximately 0.2% of disodium hydrogenphosphate.

In a first step, the solubilizing aqueous medium is produced, comprises, by weight, approximately 75% of water, approximately 0.02% of carrageenate and approximately 0.2% of disodium hydrogenphosphate.

The skimmed milk powder is then added to the solution for 10 min at 60°C and dissolved in the liquid.

Then soya oil and lecithin are added to the milk substitute composition at 60°C. The milk composition is allowed to stand 30 min at 55°C. After pasteurization, the peptide 1 of the invention is added in liquid or powder form in such a quantity that the milk composition obtained comprises an amount of 5-50 micrograms, preferably 10-40 micrograms per 100 ml of milk composition. Optionally peptide 2 could be added in similar or smaller amounts to the obtained composition.

**EXAMPLE 19**

**Gel formulation**

0.5 g of peptide 1
1.6 g of isopropanol
1.0 g of glycerol
1.6 g of polyoxyethylene-polyoxypropylene copolymer 12500 (Pluronic F127)
5.3.g of water

are mixed for 10 minutes and then heated to 85°C under continuous stirring for 15 minutes. The solution is cooled to room temperature under stirring. During the cooling phase the solution begins to gel at a temperature of about 45°C to form a clear gel. The gel contains 5% of the peptide 1 for medical use. Optionallly peptide 2 could be added in an amount form 0.01 to 0.5 g.

**EXAMPLE 20**

**Lotion formulation**

0.5 g of peptide 1
1.9 g of isopropanol
1.0 g of dimethylisosorbide
1.0 g of polyoxyethylene-polyoxypropylene copolymer 12500 (Pluronic F127)
5.6 g of water

are stirred and heated at 50°C, until a clear solution has been formed. Then the composition is cooled to room temperature under stirring. The lotion contains 5% of peptide 1 for medical use. Optionallly peptide 2 could be added in an amount form 0.01 to 0.5 g.

**EXAMPLE 21**

**Gel formulation**

0.3 g of peptide 1
0.3 g of peptide 2
1.6 g of isopropanol
1.0 g of glycerol
1.6 g of polyoxyethylene-polyoxypropylene copolymer 12500 (Pluronic F127)
5.3.g of water

are mixed for 10 minutes and then heated to 85°C under continuous stirring for 15 minutes. The solution is cooled to room temperature under stirring. During the cooling phase the solution begins to gel at a temperature of about 45°C to form a clear gel. The gel contains 6% of the peptide combination for medical use.

## Claims

1. Peptide having the formula Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH for use in medicine.

2. Peptide according to claim 1 in combination with peptide having the formula Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ for use in medicine.

3. Combination of the peptides Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH and Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$ and salts and hydrates thereof.

4. Combination according to claim 3, wherein the peptides are contained in the combination in an amount from 30% by weight to 70% by weight to 70% by weight to 30% by weight.

5. Combination according to claim 3 or 4 for use in medicine.

6. Use of the peptide according to claim 1 or 2 or the combination according to claim 3 or 4 or 5 for the manufacture of a pharmaceutical composition for treatment and/or prophylaxis of cancer, autoimmune diseases, fibrotic diseases, inflammatory diseases, neurodegenerative diseases, infectious diseases, lung diseases, heart and vascular diseases and metabolic diseases.

7. Use of the peptide according to claim 6, wherein cancer, the autoimmune disease, fibrotic disease, inflammatory disease, neurodegenerative disease, infectious disease, lung disease, heart and vascular disease or metabolic disease is selected from AIDS, AIDS related diseases, HIV, asthma, Omenn's syndrome, idiopathic pulmonary fibrosis, and atopic disorders in genetically susceptible individuals.

8. Use of the peptide according to claim 1 or 2 or the combination according to claim 3 or 4 or 5 for the preparation of a formulation for oral administration to newborns, toddlers, and/or infants.

9. Use of the peptide according to claim 1 or 2 or the combination according to claim 3 or 4 or 5 for the preparation of a lyophilized formulation or a buffered liquide formulation.

10. Pharmaceutical composition containing the peptide according to claim 1 or the combination according to claim 3 together with at least one pharmaceutically acceptable carrier, cryoprotectant, lyoprotectant, excipient and/or diluent.

11. Pharmaceutical composition according to claim 10 in the form of a lyophilisate or liquide buffer solution.

12. Pharmaceutical composition according to claim 10 or 11 suitable for intravenous administration, oral administration, or for administration by inhalation.

13. Pharmaceutical composition according to claim 10, 11 or 12 in the form of an artificial mother milk formulation or mother milk substitute suitable for oral delivery to newborns, toddlers and infants.

14. Pharmaceutical composition according to claim 10, 11, 12 or 13 suitable for the treatment and/or prophylaxis of cancer, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, a lung disease, a heart and vascular disease or a metabolic disease.

15. Method for treating cancer, an autoimmune disease, a fibrotic disease, an inflammatory disease, a neurodegenerative disease, an infectious disease, a lung disease, a heart and vascular disease or a metabolic disease in a mammal, including a human, which comprises administering to the mammal a pharmaceutically effective amount of the peptides Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-OH or salts and hydrates thereof effective to treat the cancer, the autoimmune disease, the fibrotic disease, the inflammatory disease, the neurodegenerative disease, the infectious disease, the lung disease, the heart and vascular disease or the metabolic disease.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/008027

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/22 A61K38/04 A61P3/00 A61P9/00 A61P11/00
A61P25/28 A61P31/00 A61P35/00 A61P37/00 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, Sequence Search, CHEM ABS Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | WO 01/17548 A (PHARIS BIOTEC GMBH [DE]; KRAUSE ALEXANDER [DE]; MARONDE ERIK [DE]; FOR) 15 March 2001 (2001-03-15) see claims and page 5 | 1-15 |
| Y | US 2001/027181 A1 (KITAKAZE MASAFUMI [JP]) 4 October 2001 (2001-10-04) see claims and Seq. No.9 | 1-15 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
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| Name and mailing address of the ISA/ | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Merckling-Ruiz, V |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | BEPLER G. ET AL.: "In vitro growth inhibition of human small cell lung cancer by physalaemin." CANCER RES., vol. 47, 1987, pages 2371-2375, XP002512763 abstract<br>----- | 1-15 |

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Information on patent family members

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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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(71) Applicant *(for all designated States except US)*: **SCIOS INC.** [US/US]; 617 North Mary Avenue, Sunnyvale, CA 94085 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **SCHREINER, George, F.** [US/US]; 12774 Leander Drive, Los Altos, CA 94022 (US).

(74) Agent: **KAIS, Samuel, M.**; Scios INC., 617 North Mary Avenue, Sunnyvale, CA 94085 (US).

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WO 03/079979 A2

(54) Title: METHOD FOR TREATING CONGESTIVE HEART FAILURE

(57) Abstract: The present invention relates to methods for administration of natriuretic peptide that are especially useful for treatment of a CHF patient who is relatively compensated, or to a CHF patient in an outpatient setting, and especially as an adjunct to oral therapy. The methods are characterized by administration of a composition that provides a dose of natriuretic peptide that is lower than that dose typically administered to a CHF patient who is in need of acute treatment. The methods of the invention are also useful for the treatment of chronic CHF patients, especially subacute chronic CHF patients.

# METHOD FOR TREATING CONGESTIVE HEART FAILURE

## BACKGROUND OF THE INVENTION

### Field of the Invention

The invention is in the field of natriuretic peptide and methods for administration of the same. The methods are especially useful for treating congestive heart failure patients in an outpatient setting.

### Background of the invention

Advanced congestive heart failure (CHF) accounts for over 1 million hospital admissions yearly in the United States (U.S.) and is associated with a 5-year mortality rate of 40%–50%. (American Heart Association. *2001 Heart and Stroke Statistical Update*. Dallas, TX.: American Heart Association, 2000; Massie, B.M., and Shah, N.B., *Am. Heart J. 133*:703-712 (1997)) In the U.S., CHF is currently the most costly cardiovascular disease, with the total estimated direct and indirect costs approaching $56 billion in 1999. (Rich, M.W., and Nease, R.F., *Arch Intern Med 159*:1690-1700 (1999)) The estimated cost of a single hospital admission primarily for CHF is $11,000 (Massie, B.M., and Shah, N.B., *Am. Heart J. 133*:703-712 (1997); Rich, M.W., and Nease, R.F., *Arch Intern Med 159*:1690-1700 (1999)) , whereas the Medicare Diagnostic-Related Group (DRG) reimbursement is approximately $4,300. This represents an increased economic burden for hospitals, as Medicare is the primary provider for this disease.

Efforts to contain rising costs for hospital acute care have resulted in shorter hospital stays for patients with acutely decompensated CHF. These shorter stays may not allow for adequate diuresis or titration of oral medications for patients to receive the full benefit of intravenously (IV) administered medications.

Additionally, there is an unmet need for effective outpatient treatment of advanced chronic CHF. Therapies that improve quality of life and/or reduce the number of hospital admissions for CHF are needed. While there is increasing interest among clinicians in the use of outpatient IV therapy for the treatment of advanced CHF, there is no consensus among clinicians regarding patient

selection, dosing or treatment duration. The use of milrinone and dobutamine in this setting is controversial and may lead to increased mortality. (O'Connell, J.B., *Clin. Cardiol. 23*:III6-10 (2000))

## BRIEF SUMMARY OF THE INVENTION

The present invention relates to methods for administration of natriuretic peptide. The methods of the invention are especially useful for treatment of a CHF patient who is relatively compensated, or to a CHF patient in an outpatient setting, and especially as an adjunct to oral therapy. The methods of the invention are characterized by administration of a composition that provides a dose of natriuretic peptide that is preferably lower than that dose typically administered to a CHF patient who is in need of acute treatment. The methods of the invention are also useful for the treatment of chronic CHF patients, especially subacute chronic CHF patients.

Accordingly, in a first embodiment, the invention is directed to a method for the administration of natriuretic peptide to a patient who has been diagnosed as having advanced congestive heart failure and who is in a relatively compensated state but in need of management of a risk of heart failure, such method comprising administration of a therapeutically effective dose of natriuretic peptide to said patient, such therapeutically effective dose preferably being in adjunct to oral therapy and at a dose that is lower than the dose used to treat acute CHF.

In a further embodiment, the such natriuretic peptide is atrial natriuretic peptide (ANP) or more preferably, B-type natriuretic peptide (BNP).

In a further embodiment, natriuretic peptide is human natriuretic peptide.

In a further embodiment, the such human natriuretic peptide is recombinant human B-type natriuretic peptide (nesiritide).

In a further embodiment, such administration is in the form of an infusion, preferably an intravenous infusion, and most preferably a serial intravenous infusion.

In a further embodiment, the composition of the invention is provided as the primary IV vasoactive therapy for a patient with CHF, and such patient is infused with therapeutically effective doses of natriuretic peptide for 4-6 hours weekly.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1. A schematic representation of the amino acid sequence of human B-type natriuretic peptide (SEQ ID NO:1).

Figure 2. A graph of pulmonary capillary wedge pressure (PCWP) in patients in the VMAC (Vasodilation in the Management of Acute Congestive Heart Failure) study.

Figure 3. Mean hemodynamic change from baseline in patients in the VMAC (Vasodilation in the Management of Acute Congestive Heart Failure) study.

Figure 4. Adverse reactions in patients in the VMAC study.

Figure 5. Calculation of the appropriate bolus volume and infusion flow rate for conventional administration of Natrecor®.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

The term "ameliorate" denotes a lessening of an effect. To ameliorate a condition or disease refers to a lessening of the symptoms of the condition or disease.

An "individual" is a vertebrate, preferably a mammal, more preferably a human.

"Mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sport, or pet animals, such as, for

example, horses, sheep, cows, pigs, dogs, cats, etc. Preferably, the mammal is human.

A "therapeutically effective amount" or an "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be provided in one dose. Alternatively, the effective amount can be provided in multiple doses for a desired period of time, such multiple doses being cumulatively sufficient to effect the beneficial or desired result but each of such multiple doses being at an individual level that may or may not be effective had it been administered by itself.

"Administration" means any manner of providing a desired agent to a subject or patient. Administration "in combination with" one or more further therapeutic agents means any manner with provides for the beneficial effects of the administration of both agents, including simultaneous (concurrent) administration and consecutive administration in any order.

The term "modulate" means to control in a predictable fashion, either by increasing or by decreasing the targeted parameter, as indicated from the context.

A "treatment" is an approach for obtaining a beneficial or desired result, especially a clinical result, especially the administration of an agent to a subject for purposes which may include prophylaxis, amelioration, prevention or cure of an undesired physiological condition or disease. Such treatment need not necessarily completely ameliorate the condition or disorder. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of tissue injury or disease, stabilized (i.e., not worsening) state of tissue injury or disease, delay or slowing of the progression or tissue injury or disease, amelioration or palliation of an undesired physiological condition or disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. "Treatment" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures.

Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed.

The term "pharmaceutically acceptable salt" as used herein refers to salt forms of a substance that are substantially non-toxic to living organisms. Typical pharmaceutically acceptable salts include those salts prepared by reaction of a desired agent, such as a desired form of natriuretic peptide, with a pharmaceutically acceptable mineral or organic acid or an inorganic base. Such salts are known as acid addition and base addition salts.

Acids commonly employed to form acid addition salts are inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like, and organic acids such as p-toluenesulfonic, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid, and the like. Examples of pharmaceutically acceptable salts formed from such acids are the sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, gamma.-hydroxybutyrate, glycollate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate, mesylate, and the like. Preferred pharmaceutically acceptable acid addition salts are those formed with mineral acids such as hydrochloric acid and hydrobromic acid, and those formed with organic acids such as maleic acid and methanesulfonic acid.

Salts of amine groups may also comprise quaternary ammonium salts in which the amino nitrogen carries a suitable organic group such as an alkyl, alkenyl, alkynyl, or aralkyl moiety.

Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, bicarbonates, and the like. Such bases useful in preparing the salts of this invention thus include sodium hydroxide, potassium hydroxide, ammonium hydroxide, potassium carbonate, sodium carbonate, sodium bicarbonate, potassium bicarbonate, calcium hydroxide, calcium carbonate, and the like. The potassium and sodium salt forms are particularly preferred.

It should be recognized that the particular counterion forming a part of any salt of this invention is not of a critical nature, so long as the salt as a whole is pharmacologically acceptable and as long as the counterion does not contribute undesired qualities to the salt as a whole.

The term "essentially free of contaminants" refers to a substance that is purified to a degree such that the substance contains no, or acceptable levels of, undesired or unnecessary substances that arose form, or had been present during, the in vitro or in vivo synthesis of the desired substance.

Congestive heart failure

Congestive heart failure (CHF; cardiac failure) is a condition in which weakened heart function exists together with a build-up of body fluid. Cardiac failure often occurs when cardiac output is insufficient to meet metabolic demands of the body, or when the heart cannot meet the demands of operating at increased levels of filling/diastolic pressure. Therapy involves not only support of the weakened heart function but also treatment to counteract the build up of the body fluid.

Congestive heart failure may be caused by many forms of heart disease. Common causes of congestive heart failure include: narrowing of the arteries supplying blood to the heart muscle (coronary heart disease); prior heart attack

(myocardial infarction) resulting in scar tissue large enough to interfere with normal function of the heart; high blood pressure; heart valve disease due to past rheumatic fever or an abnormality present at birth; primary disease of the heart muscle itself (cardiomyopathy); defects in the heart present at birth (congenital heart disease) and infection of the heart valves and/or muscle itself (endocarditis and/or myocarditis). Each of these disease processes can lead to congestive heart failure by reducing the strength of the heart muscle contraction, by limiting the ability of the heart's pumping chambers to fill with blood due to mechanical problems or impaired diastolic relaxation, or by filling the heart's chambers with too much blood.

Advanced congestive heart failure (CHF) includes both acute and chronic presentations. Patients presenting with acute decompensated CHF usually have an acute injury to the heart, such as a myocardial infarction, mitral regurgitation or ventricular septal rupture. Typically, the injury compromises myocardial performance (for example, a myocardial infarction) or valvular/chamber integrity (for example, mitral regurgitation or ventricular septal rupture). Such injuries result in an acute rise in the left ventricular (LV) filing pressures. The rise in the LV filing pressures results in pulmonary edema and dyspnea. The treatment of patients with acute decompensated CHF focuses on treating the reason behind the myocardial injury. In addition, the heart's function is supported by treatments to reduce LV filling pressures and to improve cardiac performance.

Patients with chronic decompensated heart failure often have symptoms of volume overload and/or low cardiac output - but do not appear to be in a volume overloaded state. Such patients thus have a chronic LV systolic dysfunction.

The Compositions of the Invention

The invention provides pharmaceutically active compositions that are useful for both the prophylactic and therapeutic treatment of CHF for patients that are relatively compensated.. The pharmaceutically active compositions of the

invention invention are characterized in containing a natriuretic peptide, sufficient to provide a therapeutically effective amount of a natriurertic peptide to such patient when administered in an appropriate dose and for an appropriate period of time.

The natriuretic peptide that is present in a composition of the invention can be any of the family of therapeutically effective natriuretic peptides, or a mixture of the same. Examples of useful natriuretic peptides include, for example, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP or B-type natriuretic peptide) and C-type natriuretic peptide (CNP). Of them, ANP and BNP are preferred, and BNP is the most preferred. Sequences of many useful forms of natriuretic peptide are provided in (U.S. Patent Application Publication No. 20010027181A1, incorporated by reference herein).

Specific examples of ANPs that can be used in the methods of the invention include: human ANP (human atrial natriuretic peptide; hANP, Kangawa et al., Biochem. Biophys. Res. Commun., Vol. 118, p. 131, 1984) (Seq. ID No. 1) or rat ANP (Kangawa et al., Biochem. Biophys. Res. Commun., Vol. 121, p. 585, 1984). Such ANPs comprise 28 amino acids. Such ANPs may be administered as a peptide having a ring structure of ANP (formation of a disulfide bond based on Cys), and a C-terminal portion succeeding the ring structure. An example of such a peptide is a peptide having amino acid residues at the 7-position to the 28-position of ANP is provided in U.S. Patent Application Publication No. 20010027181A1. Another example is frog ANP. Of them, human ANP (hANP), and especially recombinant hANP is particularly preferred.

Specific examples of BNPs that can be used in the methods of the invention include human BNP (hBNP). Human BNP comprises 32 amino acids and involves the formation of a disulfide bond, like the above-described ANP (Sudoh et al., Biochem. Biophys. Res. Commun., Vol. 159, p. 1420, 1989). *See also*, US Patent Nos. 5,114,923, 5,674,710, 5,674,710, 5,948,761, each of which is hereby incorporated by reference. Various BNP's of the origin other than human, such as pig BNP and rat BNP, are also known, and can be used similarly. A further example is chicken BNP.

Specific examples of CNPs that can be used in the methods of the invention include pig CNP. Pig CNP comprises 22 amino acids and involves the formation of a disulfide bond, like the above-described ANP and BNP (Sudoh et al., Biochem. Biophys. Res. Commun., Vol. 168, p. 863, 1990) (human and rat also have the same amino acid sequence), chicken CNP (Arimura et al., Biochem. Biophys. Res. Commun., Vol. 174, p. 142, 1991). Frog CNP (Yoshihara et al., Biochem. Biophys. Res. Commun., Vol. 173, p. 591, 1990) can also be used.

Furthermore, any person skilled in the art can apply modification, such as deletion, substitution, addition or insertion, and/or chemical modification to amino acid residues in the amino acid sequence of a known natriuretic peptide (e.g., the aforementioned human ANP; hANP), as desired, by a known method. One skilled in the art can confirm that the resulting compound is a compound which has the activity of acting on a receptor of the starting ANP or BNP or CNP. Derivatives having this activity, therefore, are included in the substance as an active ingredient which is administered to a patient in accordance with the method of the present invention.

A substance that activates the patient's natriuretic peptide receptor could also be uses in the compositions of the invention in place of, or in addition to, one or more of the natriuretic peptides discussed above. Such substance should be capable of acting on a natriuretic peptide receptor to increase intracellular cGMP production. Such substances may be non-peptide compounds.

In the compositions of the invention, the natriuretic peptide is preferably provided as a free (non-salt) form, or as a pharmaceutically acceptable salt. A salt with an inorganic acid preferably includes salts with hydrochloric acid, sulfuric acid, and phosphoric acid. The salt with an organic acid thus may, preferably be, for example, acid addition salts with formic acid, acetic acid, butyric acid, succinic acid, and citric acid. The salt is preferably in the form of a metal salt with sodium, potassium, lithium or calcium, or a salt with an organic base.

To produce compositions for infusion, carriers or additives can be added to provide a desired stability or property to the composition. Examples of such carriers and additives include: (1) tonicity agents such as sodium chloride, D-

mannitol, and D-sorbitol, (2) pH regulators such as hydrochloric acid and citric acid, (3) buffering agents such as sodium citrate, sodium acetate, and boric acid, and (4) soothing agents such as procaine hydrochloride; as well as stabilizers, and surface active agents. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers composed of phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®.

In consideration of the stability, etc. of the active natriuretic peptide ingredient, it can be selected whether the active ingredient should be formed into a preparation to be used after dissolution or suspension when required, or into a liquid preparation.

The Methods of the Invention

According to the invention, serial and intermittent administration of natriuretic peptides provides therapeutic and prophylactic benefit to CHF patients. In a preferred embodiment, CHF patients who are in a relatively stable or compensated state are administered a bolus of natriuretic peptide coupled with serial and intermittent infusion to achieve the desired treatment. By "relatively stable or relatively compensated," it is meant that the CHF patient, at the time of administration of a therapeutic and/or prophylactic dosage of a natriuretic peptide according to the present invention, is not exhibiting acute heart failure symptoms necessitating immediate treatment. Such patients may include, for example, patients in New York Heart Association functional classifications I through IV

as described in Table 2, as well as patients who may have recently been hospitalized for previous treatment of acute heart failure. Preferably, the peptide dosage can range to where the bolus is about, not less than 2 μg/kg and the serial and intermittent infusion is less than about 0.01 μg/kg/min.

The pharmaceutically active compositions that provide the active natriuretic peptide are preferably administered to the patient who is in need of the same in the form of an injection, Such injections can be, for example, *intravenous,* intramuscular, subcutaneous, intradermal, intrasternal, intraperitoneal or intra-articular. Most preferably, the compositions are provided in the form of an infusion, and especially, an intravenous infusion.

In a preferred embodiment, therapeutically effective doses of natriuretic peptides and especially, nesiritide (commercially sold as Natrecor® ) are used in the compositions of the invention in a form intended for injection or infusion. Preferably, such compositions reduce pulmonary capillary wedge pressure and to improve patients with dyspnea at rest or with minimal activity.

The infusion can be administered for any effective period of time, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 hours, or a desired period of time in between. In a further embodiment, infusions for greater than 10 hours are performed. In a preferred embodiment, a patient in need of such treatment is infused for 4-6 hours. Preferably such infusion is continuous although two infusions of shorter duration following one right after the other may be used.

The infusion rate may be any that is tolerated by the patient. Examples of useful bolus amounts and infusion rates are provided in Table 1.

Higher or lower infusion rates can be used if desired. In a preferred embodiment, the infusion rate is about 0.00125 μg/kg/min to about 0.01 μg/kg/min. In a further preferred embodiment, an infusion rate of about 0.005 μg/kg/min is used.

The infusion rate should be sufficient to provide a therapeutically effective amount of the natriuretic peptide during the infusion period or treatment protocol but without compromising patient safety.

If one embodiment, a separate initial bolus of a preparation that contains natriuretic peptide is administered to the patient immediately prior to a subsequent infusion. Such a bolus preferably provides from about 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 or 1.75 μg/kg natriuretic peptide.

The optimal volume of the infusion and amount of the active natriuretic peptide will vary by body weight. For example, a 30 kg (66 pound) patient might first be given a 2.5 ml bolus of 0.5 μg/ml of the natriuretic peptide composition infused at 0.8 ml/hr so as to provide for 0.0025 μg/kg/min, followed by a minimum bolus of 0.25 μg/kg in 1.3 mL infused at 0.4 mL/hr and 0.00125 μg/kg/min to a maximum bolus of 1.0 μg/kg in 5.0 mL infused at a maximal rate of 1.5 mL/hr to provide 0.005 μg/kg/min.

For another example, a 30 kg (66 pound) patient might first be given a 5.0 ml bolus of 1.0 μg/ml of the natriuretic peptide composition infused at 1.5 ml/hr so as to provide for 0.005 μg/kg/min, followed by a minimum bolus of 0.5 μg/kg in 2.5 mL infused at 0.8 mL/hr and 0.0024 μg/kg/min.

In contrast, a 175 kg (386 pound) patient might first be given a 14.6 ml bolus of 0.5 μg/ml of the natriuretic peptide composition infused at 4.4 ml/hr so as to provide for 0.0025 μg/kg/min, followed by a minimum bolus of 0.25 μg/kg in 7.3 mL infused at 2.2 mL/hr and 0.00124 μg/kg/min to a maximum bolus of 1.0 μg/kg in 29.2 mL infused at a maximal rate of 8.8 mL/hr to provide 0.005 μg/kg/min.

Alternatively, a 175 kg (386 pound) patient might first be given a 29.2 ml bolus of 1.0 μg/ml of the natriuretic peptide composition infused at 8.8 ml/hr so as to provide for 0.005 μg/kg/min, followed by a minimum bolus of 0.5 μg/kg in 14.6 mL infused at 4.4 mL/hr and 0.0025 μg/kg/min. Additional examples are provided in Table 1.

In a preferred embodiment, the methods of the invention provide therapeutically effective doses of natriuretic peptides, and especially, nesiritide, to a patient in need of long term management and/or at high risk of heart failure. Such treatment preferably occurs in a subacute or outpatient setting. Infusion of the composition of the invention is therapeutically effective if it provides a

desirable hemodynamic and neurohormonal effects in addition to maintaining the advantageous safety profile of the administered natriuretic peptide.

The method of the invention is especially useful as an add-on therapy to oral medications. In a highly preferred embodiment of the method of the invention, serial IV infusions of a netriuretic peptide, and especially of nesiritide are provided at therapeutically effective doses to patients who are also being treated with oral therapeutic agents to manage such patient's CHF, such therapeutically effective doses of the infused natriuretic peptide resulting at least in part in a more rapid and sustained compensation of CHF in patients with frequent episodes of acutely decompensated CHF, and/or who are in need of chronic CHF treatment in a subacute or outpatient setting.

It is an advantage of the invention that the method of the invention can be used to treat patients who are in a relatively compensated state. According to the invention, the appropriate dose of natriuretic peptide, and especially, the appropriate dose of nesiritide, is provided by infusion and at a lower dose than that generally used in an acutely decompensated setting and maybe better tolerated by the patient in need of treatment.

Having now generally described the invention, the same will become better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless other wise specified. All referenced publications and patents are incorporated, in their entirety by reference herein.

## EXAMPLES

The following abbreviations are used:

ACE angiotensin converting enzyme

AICD automatic implantable cardiac defibrillator

ANOVA analysis of variance

ARB angiotensin receptor blocker

BP blood pressure

| | |
|---|---|
| BNP | B-type natriuretic peptide |
| CI | cardiac index |
| CHF | congestive heart failure |
| CRF | case report form |
| DRG | diagnosis related group |
| EF | ejection fraction |
| EKG | electrocardiogram |
| hBNP | human B-type natriuretic peptide (Figure 1) |
| HR | heart rate |
| IV | intravenous |
| LVEDI | left ventricular end diastolic index |
| NYHA | New York Heart Association |
| MLwHF | Minnesota Living with Heart Failure Questionnaire |
| PE | physical examination |
| QoL | Quality of Life |
| RR | respiratory rate |
| SBP | systolic blood pressure |
| SVI | stroke volume index |
| SVR | systemic vascular resistance |
| VS | vital signs |
| Wt | weight |

## EXAMPLE 1

The exemplified embodiments utilize Natrecor® as the natriuretic peptide. Natrecor® is a proprietary name for a recombinant form of human B-type natriuretic peptide (hBNP), also known as brain natriuretic peptide. It is identical to the endogenous hormone that is produced primarily by the ventricular myocardium. (Ewy, G.A., J. Am. Coll. Cardiol. 33(2):572-575 (1999)) However, it is to be understood that the examples are not restricted to Natrecor®. The

exemplified embodiments provide an outline for the establishment of the use of any natriuretic peptide in the methods and treatments of the invention.

IV administration of Natrecor® produces balanced vasodilation, antagonism of the renin-aldosterone system, and diuresis. (Hosoda, K., *et al., Hypertension 17*:1152-1155 (1991); Marcus, L.S., *et al. Circulation 94*:3184-3189 (1996)) When administered to CHF patients for up to 24 hours, a continuous infusion of Natrecor® resulted in significant dose related reductions in cardiac filling pressures and systemic vascular resistance (SVR), with increases in cardiac index (CI) and no change in heart rate (HR). (Abraham, W.T., *et al., J. Am. Coll. Cardiol.* 236A (1995)) In a 6-hour placebo controlled comparison in patients with acutely decompensated CHF, Natrecor® was also associated with significant improvement in the symptoms of CHF (including dyspnea and fatigue), a decrease in aldosterone, and an increase in urine output. (Mills, R.M., *et al., J. Am. Coll. Cardiol. 34(1)*:155–62 (1999))

Natrecor® generally has been well tolerated in controlled clinical trials involving more than 1000 patients with CHF.

DESCRIPTION

Natrecor* (nesiritide) is a sterile, purified preparation of a now drug class, human B-type natriuretic peptide (hBNP), and is manufactured from E. coli using recombinant DNA technology. Nesiritide has a molecular weight of 3464 g/mol and an empirical formula of $C_{143}H_{244}N_{50}O_{42}S_4$. Nesiritides has the same 32 amino acid sequence as the endogenous peptide, which is produced by the ventricular myocardium.

Natrecor is formulated as the citrate salt of rhBNP, and is provided in a sterile, single-use vial. Each 1.5 mg vial contains a white-to off-white lyophilized powder for intravenous (IV) administration after reconstitution. The quantitative composition of the lyophilized drug per vial is: nesiritide 1.58 mg, mannitol 20.0 mg, citric acid monohydrate 11 mg, and sodium citrate dihydrate 2.94 mg.

Mechanism at Action

Human BNP binds to the particulate guanylate cyclase receptor of vascular smooth muscle and endothelial cells, leading to increased intracellular concentrations of guanosine 3'5'-cyclic monophosphate (cGMP) and smooth muscle cell relaxation. Cyclic GMP serves as a second messenger to dilate veins and arteries. Nesiritide has been shown to relax isolated human arterial and venous tissue preparations that were precontracted with either endothelin-1 or the alpha-adrenergic agonist, phenylephrine.

In human studies, nesiritide produced dose-dependent reductions in pulmonary capillary wedge pressure (PCWP) and systemic arterial pressure in patients with heart failure.

In animals, nesiritide had no effects an cardiac contractility or on measures of cardiac electrophysiology such as atrial and ventricular effective refractory times or atrioventricular node conduction.

Naturally occurring atrial natriuretic peptide (ANP), a related peptide, increases vascular permeability in animals and humors and may reduce intravascular volume. The effect of nesiritide on vascular permeability has not been studied.

Pharmacokinetics

In patients with congestive heart failure (CHF), Natrecor administered intravenously by infusion or bolus exhibits biphasic disposition from the plasma. The mean terminal elimination half-life ($t_{1/2}$) of Natrecor is approximately 10 minutes and was associated with approximately 2/3 of the area-under-the-curve (AUC). The mean initial elimination phase was estimated to be approximately 2 minutes. In these patients, the mean volume of distribution of the control compartment (Vc) of Natrecor was estimated to be 0.073 Mg, the mean steady-state volume of distribution (Vss) was 0.19 L/kg, and the mean clearance (CL) was approximately 9.2 mL/min/kg. At steady state, plasma BNP levels

increase from baseline endogenous levels by approximatly 3-fold to 6-fold with Natrecor infusion doses ranging from 0.01 to 0.03 μg/kg/min.

Elimination

Human BNP is cleared from the circulation via the following three independent mechanisms, in order of decreasing importance: 1) binding to cell surface clearance receptors with subsequent cellular Internalization and lysosomal proteolysis; 2) proteolytic cleavage of the peptide by endopeptidases, such as neutral endopeptidase, which are present on the vascular lumenal surface; and 3) renal filtration.

Special Populations

Although Natrecor is eliminated, in part, through renal clearance, clinical date suggest that dose adjustment is not required in patients with renal insufficiency. The effects of Natrecor on PCWP, cardiac index (CI), and systolic blood pressure (SBP) were not significantly different in patients with chronic renal insufficiency (baseline serum creatinine ranging from 2 mg/dL to 4.,3 mg/dl), and patients with normal renal function. The population pharmacokinetic (PK) analyses carried out to determine the effects of demographics and clinical variables on PK parameters showed that clearance of Natrecor is proportional to body weight supporting the administration of weight-adjusted dosing of Natrecor (i.e., administration on a μg/kg/min basis). Clearance was not influenced significantly by age, gander, race/ethnicity, baseline endogenous hBNP concentration, severity of CHF (as indicated by baseline PCWP, baseline CI, or New York Heart Association [NYHA] classification) or concomitant administration of an ACE inhibitor.

Effects of Concomitant Medications

The co-administration of Natrecor with enalapril did not have significant effects on the PK of Natrecor. The PK effect of co-administration of Natrecor with other IV vasodilators such as nitroglycerin, nitroprusside, milninone, or IV ACE inhibitors; has not been evaluated. During clinical studies, Natrecor was administered concomitantly with other medications, including: diuretics, digoxin, oral ACE inhibitors, anticoagulant, oral nitrates, statins, class III antiarrhythmic agents, beta-blockers, dobutamine, calcium channel blockers, angiotensin II receptor antagonists, and dopamine. Although no PK interactions were specifically assessed, there did not appear to be evidence suggesting any clinically significant PK interaction.

Pharmacodynamics

The recommended dosing regimen of Natrecor is a 2 μg/kg IV bolus followed by an intravenous infusion dose of 0.01 μg/kg/min. With this dosing regimen, 60% of the 3-hour off act on PCWP reduction is achieved within 15 minutes after the bolus, reaching 95% of the 3-hour effect within 1 hour. Approximately seventy percent of the 3-hour effect on SBP reduction is reached within 15 minutes. The pharmacodynamic (PD) half-life of the onset end offset of the hemodynamic effect of Natrecor is longer than what the PK half-life of 18 minutes would predict. For example, in patients who developed symptomatic hypotension in the VMAC (Vasodilation in the Management of Acute Congestive Heart Failure) trial, bag of the recovery of SBP toward the baseline value after discontinuation or reduction of the dose of Natrecor was observed in about 60 minutes. When higher doses of Natrecor were infused, the duration of hypotension was sometimes several hours.

Clinical Trials

Natrecor has been studied in 10,clinical trials including 941 patients with CHF (NYHA class II-III 61%, NYHA class IV 36%; mean age 60 years, women

28%). There were five randomized, multi-center, placebo- or active-controlled studies (comparative agents included nitroglycerin, dobutamine, milrinone, nitroprusside, or dopamine) in which 772 patients with decompensated CHF received continuous infusions of Natrecor at doses ranging from 0.01 to 0.03 μg/kg/min. (See the ADVERSE REACTION section for relative frequency of adverse events at doses ranging from the recommended dose up to 0.03 μg/kg/min. Of these patients, the majority (n = 541, 70%) received the Natrecor infusion for at least 24 hours; 371 (48%) received Natrecor for 24- 48 hours, and 170 (22%) received Natrecor for greater then 48 hours.

In controlled trials, Natrecor has been used alone or in conjunction with other standard therapies, including diuretics (79%), digoxin (62%), oral ACE inhibitors (55%), anticoagulants (36%), oral nitrates (32%), statins (18%), class III antiarrhythmic agents (16%), beta-blockers (15%), dobutamine (15%), calcium channel blockers (11%), angiotensin II receptor antagonists (6%), and dopamine (4%). Natrecor has been studied in a broad range of patients, including the elderly (42% >65 years of age), women (30%), minorities (26% black), and patients with a history of significant morbidities such as hypertension (67%), previous myocardial infarction (50%), diabetes (44%), atrial fibrillation/flutter (34%), nonsustained ventricular tachycardia (25%) ventricular tachycardia/ fibrillation (12%), preserved systolic function (9%), and acute coronary syndromes less than 7 days before the start of Natrecor (4%).

The VMAC (V*asodilation in the Management of Acute, Congestive Heart Failure*) trial was a randomized, double-blind study of 489 patients (246 patients requilring a right heart catheter, 243 patients without a right heart catheter) who required hospitalization for management at shortness of breath at rest due to acutely decompensated CHF. TN study, compared the effects of Natrecor, placebo, and IV nitroglycerin when added to background therapy (IV and oral diuretics, non-IV cardiac medications, dobutamine, and dopamine). Patients with acute coronary syndrome, preserved systolic function, arrhythmia, and renal insufficiency wore not excluded. The primary endpoints of the study were the change from baseline in PCWP and the change from baseline in patients' dyspnea,

evaluated after three hours. Close attention was also paid to the occurrence and persistence of hypotension, given nesiritide's relatively long (compared to nitroglycerin) PK and PD half-life.

Natrecor was administered as a 2 μg/kg bolus over approximately 60 seconds, followed by a continuous fixed dose infusion of 0.01 μg/kg/min. After the 3-hour placebo-controlled period, patients receiving placebo crossed over to double-bladed active therapy with either Natrecor or nitroglycerin. The nitroglycerin dose was titrated at the physician's discretion. A subset of patients in the VMAC trial with central hemodynamic monitoring who were treated with Natrecor (62 of 124 patients) were allowed dose increases of Natrecor after the first 3 hours of treatment it the PCWP was ≥20 mm Hg and the SBP was ≥100 mm Hg. Dose increases of a 1 μg/kg bolus followed by an increase of the infusion dose by 0.005 μg/kg/min were allowed every 3 hours, up to a maximum dose of 0.03 μg/kg/min. Overall, 23 patients in this subset had the dose of Natrecor increased in the VMAC trial.

In a second double-blind study, 127 patients requiring hospitalization (of symptomatic CHF were randomized to placebo or to one of two doses of Natrecor (0.015 μg/kg/min preceded by an IV bolus of 0.3 μg/kg and 0.03 μg/kg/min preceded by an IV bolus of 0.6 μg/kg). The primary endpoint of the trial was the change in PCWP from baseline to 6 hours, bid the effect on symptoms also was examined.

Effects an Symptoms

In the VMAC study, patients receiving Natrecor reported greater improvement in their dyspnea at 3 hours than patients receiving placebo ($p = 0.034$).

In the dose-response study, patients receiving both doses of Natrecor reported greater improvement in dyspnea at 6 hours than patients receiving placebo.

Effects on Hemodynamics

The PCWP, right atrial pressure (RAP), CI, and other hemodynamic variables were monitored in 246 of the patients in the VMAC trial. There was a reduction in mean PCWP within 15 minutes of starting the Natrecor infusion, with most of the effect seen at 3 hours being achieved within the first 60 minutes of the infusion (see Pharmacodynamics).

In several studies, hemodynamic parameters wars measured after Natrecor withdrawal. Following discontinuation of Natrecor, PCWP returns to within 10% of baseline within 2 hours, but no rebound increase to levels above baseline state was observed. There was also no evidence of tachyphylaxis to the hemodynamic effects of Natrecor in the clinical trials.

Figure 2 summarize the changes in the VMAC trial in PCWP and other measures during the first 3 hours. Figure 3 is the mean hemodynamic change from baseline in patients in the VMAC during the first 3 hours. The VMAC study does not constitute an adequate effectiveness comparison with nitroglycerin. In this trial, the nitroglycerin group provides a rough landmark using a familiar therapy and regimen.

End on Urine Output

In the VMAC trial, in which the use of diuretics was not restricted, the mean change in volume status (output minus input) during the first 24 hours in the nitroglycerin and Natrecor groups was similar. 1279±1455 mL and 1257±1657 mL, respectively.

INDICATIONS AND USAGE

Natrecor is indicated for the intravenous treatment of patients with acutely decompensated congestive heart failure who have dyspnea at rest or with minimal

activity. In this population. the use of Natrecor reduced pulmonary capillary wedge pressuire and improved dyspnea.

CONTRAINDICATIONS

Natrecor is contraindicated in patients who are hypersensitive to any of its components. Natrecor should not be used as primary therapy for patients with cardiovenic shock or in patients with a systolic blood pressure <90 mm Hg.

WARNINGS

Administration of Natrecor should be avoided in patients; suspected of having, of known to have. low cardiac filling pressures.

PRECAUTIONS

General: Parenteral administration of protein pharmaceuticals or *E. coli* derived products should be attended by appropriate precautions in case of an allergic or untoward reaction. No serious allergic or anaphylactic reactions have been reported with Natrecor.

Natrecor is not recommended for patients for whom vasodilating agents arit not appropriate, such as patients with significant valvular stenosis, restrictive or obstructive cardiomyopethy, constrictive pericarditis, pericardial tamponade, or other conditions in which cardiac output is dependent upon venous return, or for patients suspected to have low cardiac filling pressures. (See CONTRAINDICATIONS)

Renal: Natrecor may affect renal function in susceptible individuals. In patients with severe heart failure whose renal function may depend on the activity of the renin-angiotensin-aldosterone system, treatment with Natrecor may be associated with azotemia. When Natrecor was initiated at doses higher than 0.01 μg/kg/min (0.015 and 0.03 μg/kg/min), there was an increased rate of elevated serum creatinine over baseline compared with standard therapies, although the

rate of acute renal failure and need for dialysis was not increased. In the 30-day follow-up period in the VMAC trial, 5 patients in the nitroglycerin group (2%) and 9 patients in the Natrecor group (3%) required first-time dialysis.

Cardiovascular: Natrecor may cause hypotension. In the VMAC trial, in patients given the recommended dose (2 μg/kg bolus followed by a 0.01 μg/kg/min infusion) or the adjustable dose, the incidence of symptomatic hypotansion in the first 24 hours was similar for Natrecor (4%) and IV nitroglycerin (5%). When hypotension occurred, however, the duration of symptomatic hypotension was longer with Natrecor (mean duration was 2.2 hours) than with nitroglycerin (mean duration was 0.7 hours). In earlier trials, when Narrator was initiated at doses higher than the 2 μg/kg bolus followed by a 0.01 μg/kg/min infusion (i.e., 0.015 and 0.03 μg/kg/min preceded by a small bolus), there wets more hypotensive episodes and these episodes were of greater imengity and duration. They were also more often symptomatic and/or more likely to require medical intervention (see ADVERSE REACTIONS and Figure 4). Natrecor should be administered only in settings where blood pressure cart be monitored closely, and the dose of Natrecor should be reduced or the drug discontinued in patients who develop hypotension (see Dosing Instructions). The rate of symptomatic hypotension may be increased in patients with a blood pressure <100 mm Hg at baseline, and Natrecor should be used cautiously in these patients. The potential for hypotension may be increased by combining Natrecor with other drugs that may cause hypertension. For example, in the VMAC trial in pedants treated with either Natrecor or nitroglycerin thereapy, the frequency of symptomatic hypotension in patents who received an oral ACE inhibitor was 6%, compared to a frequency of symptomatic hypotension of 1% in patients who did not receive on oral ACE inhibitor.

Drug Interaction: No trials specifically examining potential drug interactions with Natrecor were conducted, although many concomitant drugs were used in clinical trials. No drug interactions were detected except for an increase in symptomatic hypotension in patients receiving oral ACE inhibitors (see PRECAUTIONS, Cardiovascular).

The co-administration of Natrecor with IV vasodilators such as nitroglycerin, nitroprusside, milrinone, or IV ACE inhibitors has not been evaluated (these drugs were not co-administered with Natrecor in clinical trials).

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term studies in animals have not been performed to evaluate the carcinogenic potential or the effect on fertility of nesiritide. Nesiritide did not increase the frequency of mutations when used in an in vitro bacterial cell assay (Ames test). No other genotoxicity studies were performed.

Pregnancy: Category C: Animal developmental and reproductive toxicity studies have not been conducted with nasiritide. It is also not known whether Natrecor can cause total harm when administered to pregnant women or can affect reproductive capacity. Natrecor should be used during pregnancy only if the potential benefit justifies any possible risk lo the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Therefore, caution should be exercised when Natrecor is administered to a nursing woman.

Pediatric Use: The safety and effectiveness of Natrecor in pediatric patents has not been established:

Geriatric Use: Of the total number of subjects in clinical trials treated with Natrecor (n=941), 38% were 65 years or older and 16% were 75 years or older. No overall differences in effectiveness were observed between these subjects and younger subjects, and other reported clinical experience has not identified differences in responses between the elderly and younger patients. Some older individuals may be more sensitive to the effect of Natrecor than younger individuals.

ADVERSE REACTIONS

Adverse events that occurred with at least a 3% frequency during the first 24 hours of Natrecor infusion are shown in Table 6. Adverse events that we not fisted in the above table that occurred in at least 1% of patients who received any

of the above Natrecor doses included: Tachycardia, atrial fibrillation, AV mods conduction abnormalities, catheter pain, injection site reaction, confusion, paresthesia, somnolence, tremor, increased cough, hemoptysis, apnea, increased creatinine, sweating, pruritus, rash, leg cramps, amblyopia, anemia. All reported events (at least 1%) are included except time already listed, those too general to be informative, and those not reasonably associated with the use of the drug because they were associated with the condition being treated or are very common in the treated population.

In placebo and active-controlled clinical trials, Natrecor has not been associated with an increase in atrial or ventricular tachyarrhythmias. In placebo-controlled trials, the incidence of VT in both Natrecor and placebo patients was 2%. In the PRECEDENT (*Prospective Randomized Evaluation of Cardiac Ectopy with Dobutamine or Natrecor Therapy*) trial, the affects of Natrecor (n = 163) and dobutamine (n=83) onto provocation or aggravation of existing ventricular arrhythmias in patients with decompensated CHF was compared using Holter monitoring. Treatment with Natrecor (0.015 and 0.03 μg/kg/min without an initial bolus) for 24 hours did not aggravate pre-existing VT or the frequency of premature ventricular beats, compared to a baseline 24-hour Holtor tape.

Clinical Laboratory

In the PRECEDENT trial, the incidence of elevations in serum creatinine to >0.5 mg/dL above baseline through day 14 was higher in the Natrecor 0.015 μg/kg/min group (17%) and the Natrecor 0.03 μg/kg/min group (19%) than with standard therapy (11%). In the VMAC trial, through day 30, the incidence of elevations in creatinfris to >0.5 mg/dL above baseline was 20% and 21% in the Natrecor (2 μg/kg bolus followed by 0.01 μg/kg/min) and nitroglycerin groups, respectively.

Effect on Mortality

In the VMAC trial, the mortality rates at six months in the patients receiving Natrecor and nitroglycerin wars 25.1% (95% confidence interval, 20.0% to 30.5%) snul 20.8% (95% confidence interval, 15.5% to 26.5%), respectively. In all controlled trials combined, the mortality rates for Natrecor and active control (including nitroglycerin, dobutamine, nitroprusside, milrinone. amrinone, and dopamine) patients wire 21.5% and 21.7%, respectively.

OVERDOSAGE

No data are available with respect to overdosage in humans. The expected reaction would be excessive hypotension, which should be treated with drug discontinuation or reduction (see PRECAUTIONS) and appropriate measures.

DOSAGE AND ADMINISTRATION

Natrecor (nesiritide), as currently approved by the Food and Drug Administration (FDA), is for intravenous use only. There is limited experience with administering Natrecor for longer then 48 hours. Blood pressure should be monitored closely during Natrecor administration. If hypotension occurs during the administration of Natrecor, the dose should be reduced or discontinued and other measures to support blood pressure should be started (IV fluids, changes in body position). In the VMAC trial, when symptomatic hypotension occurred, Natrecor was discontinued and subsequently could be restarted at a dose that was reduced by 30% (with no bolus administration) once the patient was stabilized. Because hypotension caused by Natrecor may be prolonged (up to hours), a period of observation may be necessary before restarting the drug.

Preparation

1. Reconstitute one 1.5 mg vial of Natrecor by adding 5 mL of diluent removed from a pre-filled 250 mL plastic IV bag containing the diluent of choice. The following preservative-free diluents are recommended for reconstitution: 5% Dextrose Injection (D5W), USP; 0.9% Sodium Chloride Injection, USP; 5% Dextrose and 0.45% Sodium Chloride Injection, USP; or 5% Dextrose and 0.2% Sodium Chloride Injection, USP.
2. Do not shake the vial. Rock the vial gently so that all surfaces, including the stopper, are in contact with the diluent to ensure complete reconstitution. Use only a clear, essentially colorless solution.
3. Withdraw the entire contents of the reconstituted Natrecor vial and add to the 250 mL plastic IV bag. This will yield a solution with a concentration of Natrecor of approximately 6 μg/mL. The IV bag should be inverted several times to ensure complete mixing of the solution.
4. Use the reconstituted solution within 24 hours, as Natrecor contains no antimicrobial preservative. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Reconstituted vials of Natrecor may be left at Controlled Room Temperature (20 - 25°C; 68-77°F) as per United States Pharmecopeia (USP) or may be refrigerated (2-8°C; 36-46°F) for up to 24 hours.

Dosing Instructions

The recommended dose of Natrecor is an IV bolus of 2 μg/kg followed by a continuous infusion of 0.01 μg/kg/min. Natrecor should not be initiated at a dose that is above the recommended dose.

Prime the IV tubing with an infusion of 25 mL prior to connecting to the patient's vascular access port and prior to administering the bolus or starting the infusion.

Bolus followed by infusion: After preparation of the infusion bag. as described previously, withdraw the bolus volume (see table below) from the Natrecor infusion bag, and administer it over approximately 60 seconds through an IV port in the tubing. Immediately following the administration of the bolus, infuse Natrecor at a flow rate of 0.1 mL/kg/hr. This will deliver a Natrecor infusion dose of 0.01 μg/kg/min.

To calculate the appropriate bolus volume and infusion flow rate to deliver a 0.01 μg/kg/min dose, use the following formulas: Bolus Volume (mL) = Patient Weight (kg) ÷ 3; Infusion Flow Rate (mL/hr) = 0.1 x Patient Weight (kg) (or refer to Figure 5).

Dose Adjustments: The dose-limiting side effect of Natrecor is hypotension. Do not initiate Natracor at a dose that is higher then the recommended dose of a 2 μg/kg bolus followed by an infusion of 0.01 μg/kg/min. In the VMAC trial there was limited experience with increasing the dose of Natrecor above the recommended dose (23 patients, all whom had central hemodynamic monitoring). In those patients, fine infusion dose of Natrecor was increased by 0.005 μg/kg/min (preceded by a bolus of 1 μg/kg), no more frequently than every 3 hours up to a maximum dose of 0.03 μg/kg/min. Natrecor should not be titrated at frequent intervals as is dome with other IV agents that have a shorter half-life.

Chemical/Physical Interactions

Natrecor is physically and/or chemically incompatible with injectable formulations of heparin, insulin, ethacrynate sodium, bumetanide, enalaprilat, hydralazine, and furosemide. These drugs should not be co-administered as infusions with Natrecor through the same IV catheter. The preservative sodium metabisulfate is incompatible with Natrecor. Injectable drugs that contain sodium metabisulfate should not be administered in the same infusion line as Natrecor. The catheter must be flushed between administration of Natrecor and incompatible drugs. Natrecor binds to heparin and therefore could bind to the heparin lining of heparin-coated catheter, decreasing the amount of Natrecor delivered the patient for some period of time. Therefore, Natrecor must not be administered through a central heparin-coated catheter. Concomitant administration of a heparin infusion through a separate catheter is acceptable.

Storage

Store Natrecor at controlled room temperature (20-25°C; 68-77°F), excursions permitted to 15-30°C (59-86°F; see USP Controlled Room Temperature),or refrigerated (2-8°C; 36-46°F). Keep in carton until time of use.

HOW SUPPLIED

Natrecor is provided as a sterfie lyophilized powder in 1.5 mg, single-use vials. Each carton contains one vial and is available in the following package:

1 vial/carton (NDC 65847-205-25)

US patent No. 5,114,923 and 5,674,710.

Distributed by Scios Inc.

820 West Maude Ave

Sunnyvale, CA 94085

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Natrecor® is approved for marketing in the U.S. by the Food and Drug Administration and is indicated for the intravenous treatment of patients with acutely decompensated congestive heart failure who have dyspnea at rest or with minimal activity.

Procedures

Week 1

Baseline Procedures, All Patients

- Confirm the patient's continued eligibility since the screening visit
- Obtain and record the patient's current weight and vital signs (BP and HR)
- Evaluate hydration status (refer to section 7.1.5)
- For patients randomized into Group A, determine if weekly inotropic therapy will be utilized
- Perform pregnancy test (if applicable)
- Draw blood for baseline plasma BNP levels, chemistry, and hematology samples (see section 6.1)
- Draw blood for baseline endothelin-1 and aldosterone (only at selected sites)
- Have patient complete the Minnesota Living with Heart Failure (MlwHF) questionnaire
- Request randomization from the study pharmacist or designee

Weeks 2–12

All patients, including patients receiving usual long term cardiac medications therapy, are to be evaluated at least once each week during the study period for evaluation even if, in the investigator's judgment, an infusion is not needed at one or more of those visits.

Weekly Procedures

Prior to Natrecor® , if applicable:

For all patients, regardless of treatment group:

- Record patient's vital signs (BP and HR)
- Record patient's weight
- Obtain blood for plasma BNP level
- Weekly BUN and creatinine are recommended at each visit to aid in assessing hydration status
- Document all known BUN and creatinine values
- Evaluate patients hydration status (refer to section 7.1.5)
- Document cardiac medications taken since last visit
- Query the patients for any adverse events and changes or additions to concomitant medications since the last visit
- Record all medications administered during infusions of Natrecor®.

Non-Weekly Procedures

For all patients regardless of treatment group:

- Obtain Blood samples for endothelin-1 and aldosterone levels at Weeks 4, 8, and 12 prior to any infusions (at selected sites)
- BUN and creatinine must be obtained for all patients during Weeks 4, 8, and 12

- Have patient complete the Minnesota Living with Heart Failure Questionnaire at Weeks 4, 8, and 12 (may be done before or during the infusion)
- Have patient complete the Patient Global Assessment at Weeks 4, 8, and 12 (before the start of each infusion, if applicable)
- Have patient perform a 6-minute walk test at Weeks 6 and 12. The test may be obtained prior to the infusion on the same day or on a day prior to the infusion day, but in the same week
- A Global Assessment is done by the investigator at Weeks 4, 8, and 12 after the infusion.

Associated Laboratory Assessment

Laboratory

Blood is drawn for white blood count, hemoglobin and hematocrit at baseline and Week 13. Specimens are analyzed.

Blood is drawn for sodium, potassium, chloride, bicarbonate, blood urea nitrogen (BUN) and creatinine for all patients at baseline and Week 13. Specimens are analyzed.

BUN and creatinine must be obtained for all patients during Week 4, 8, and 12 prior to any infusions (if applicable).

Blood is drawn for plasma B-type natriuretic peptide (BNP), at baseline and prior to Natrecor® infusions at Weeks 4, 8, and 12.

Endothelin-1 and aldosterone levels (at selected sites) at baseline and prior to Natrecor® infusions at Weeks 4, 8, and 12.

Plasma BNP – within 30 minutes of drawing samples, spin the sample in a refrigerated centrifuge for 15 minutes; store plasma in a –70°C freezer until ready for shipment to Scios Inc. for processing.

Endothelin-1 – as soon as possible after drawing, spin in a refrigerated centrifuge for 15 minutes; freeze plasma specimen in a –70°C freezer until ready for shipment to a central laboratory for processing.

Aldosterone – allow specimen to clot at room temperature for 15–30 minutes, then spin in a refrigerated centrifuge for 15 minutes; store frozen serum in a –70°C freezer until ready for shipment to a central laboratory for processing.

Assessments

New York Heart Association Classification

Assess patients NYHA class using criteria in Table 2 are assessed at screening and Week 13.

Quality of Life Instruments

The Minnesota Living with Heart Failure (MLwHF) Questionnaire is a validated, self-administered questionnaire comprised of 21 questions which cover signs and symptoms relevant to CHF, physical activity, social interaction, sexual activity, work, and emotions during the previous month. (LeJemtel, T.H., *et al., Am. J. Cardiol., 31(2)*:A83–A (1998)) The MLwHF is administered to the patient at baseline, and at Weeks 4, 8, and 12. The patient may complete the questionnaire prior to or during the infusion.

6-Minute Walk Test

To measure functional exercise capacity the patient performs a 6-minute walk test at screening and at Weeks 6 and 12. The test at Week 6 and Week 12 can be obtained prior to the infusion or on a day prior to the infusion day, but in the same week. The walking test is conducted in an enclosed corridor on a course that is approximately 33 m long. The patient is instructed to walk from end to end of the corridor, covering as much distance as they can in the allotted 6-minute time period. (Berry, C., and McMurray, J., *Pharmacoeconomics 16(3)*:247–271 (1999)) The total time walked and the total distance walked is recorded on the

CRF. Further information on the 6-minute walk test is included in the Study Guide.

2-D Echocardiogram

A two dimensional echocardiogram may be performed on each patient within 28 days after the Week 12 study visit to obtain SVI, LVEDI, and EF measurements.

Steps for Natrecor® Administration

Step 1: Administer an IV bolus of Natrecor® (obtained from the Natrecor® bag) over approximately 60 seconds directly into the IV catheter through an injection port that is as close as possible to the IV catheter insertion site.

Step 2: Immediately after the Natrecor® bolus is administered, begin the Natrecor® infusion by administering the appropriate Group B or Group C weight-adjusted flow rate. Infusions must be administered via a programmable infusion pump.

The initial Week 1 Natrecor® infusion is started at the initial bolus dose and initial infusion dose rate specified in each Natrecor® treatment arm (Table 1). At the discretion of the investigator, after the initial Natrecor® dose has been tolerated for at least 3 hours, an increased bolus and infusion may be given for the remainder of the treatment (6 hours maximum) for that study visit. At the following visits the Natrecor® dose may be adjusted at the investigator's discretion within a range that is half of the initial Week 1 dose to double the initial Week 1 dose. Those skilled in the art shall adjust dosage such that it is best tolerated by the patient.

Special Administration Instructions

Natrecor® binds to heparin and therefore could bind to the lining of a heparin-coated catheter, decreasing the amount of Natrecor® study drug delivered to the patient for some period of time. Therefore, Natrecor® must NOT be administered through a heparin-coated catheter. Concomitant administration of a heparin infusion through a separate catheter is permitted.

Natrecor® may be administered through a peripheral or central venous access line but must be infused via a programmable infusion pump. No fluid other than Natrecor® or heparin flush solution should be administered through this line.

Duration of Natrecor®

All patients randomized to receive Natrecor® are expected to be treated with Natrecor® as the primary IV vasoactive therapy for CHF, and should be treated with Natrecor® for 4–6 hours weekly for 12 weeks in an infusion clinic or other qualified unit.

Discontinuation of Natrecor® at the End of Each Infusion

Because of the 18 minute half-life of Natrecor® , it is not necessary to titrate down the dose of Natrecor® before discontinuation. Natrecor® may be discontinued by simply stopping the infusion. Obtain vital signs at 30 minutes and 1 hour after Natrecor® is discontinued.

Guidelines for Dose Selection at Each Visit

In the Natrecor® treatment arms, the goal is to titrate Natrecor® and continue to treat patients at the maximum dose within the assigned treatment dose range, if tolerated. However, because of fluctuations in hydration status from

week to week, adjustments in the dose of study drug may be necessary. Before each infusion is started, the investigator should assess the patient's hydration status and CHF symptoms in order to select the appropriate bolus and infusion for that visit. Natrecor® has vasodilating properties and may lead to increased diuresis and natriuresis in some patients. Repeated infusions of Natrecor® or aggressive diuresis may lead to volume depletion that may necessitate a lower dose of study drug. Alternatively, due to patient's non-compliance with dietary salt restriction or use of diuretics, patients may present with increased volume overload. During weeks when patients are noted to have more severe volume depletion or hypotension, the investigator may consider it appropriate to skip the infusion for that week. Conversely, if patients present with fluid overload, the dose of Natrecor® may be increased up to the maximum dose within the assigned treatment group.

The evaluation of hydration status or CHF symptoms may include a consideration of the following:

- Orthostatic blood pressures
- Systolic blood pressure < 90 mm Hg before the start of the infusion
- Changes in laboratory values (e.g., BUN, creatinine, BUN/creatinine ratio) over 1–2 weeks
- Changes in weight over previous 1–2 weeks
- Physical signs and symptoms of volume depletion or volume overload, which may include skin turgor, mucosal hydration, thirst, rales, jugular venous distention, peripheral edema, ascites, hepatomegaly, changes in dyspnea or orthopnea, urine specific gravity, and extra heart sounds
- Recent changes in concomitant medications
- Recent intercurrent illnesses such as gastroenteritis, gastrointestinal bleed, fever or other signs of infections

Increasing Natrecor®

If, during a weekly evaluation, the investigator believes that the patient's clinical status is such that the patient may receive a clinical benefit from an increased dose, the dose may be increased up to the maximum allowable infusion dose for that treatment group. The following are guidelines for increasing the Natrecor® infusion if additional clinical effect is desired:

- DO NOT increase the Natrecor® dose until the patient has tolerated the lower dose at a previous visit or has received at least 3 hours of the infusion at a lower rate at the current visit.
- If, after at least 3 hours of infusion, it is believed that the patient will benefit from a higher dose of Natrecor® and the patient has a SBP =90 mm Hg, the Natrecor® dose may be increased up to the maximum dose level within the patient's treatment group.
- To increase the Natrecor® dose, administer the bolus dose that corresponds to the next higher dose level for that treatment group (bolus volume obtained from the Natrecor® IV bag) over approximately 60 seconds, followed by an increase to the next higher infusion rate. For example: If the previous infusion rate was 0.0025 μg/kg/min (preceded by a bolus of 0.5 μg/kg), the patient should receive a Natrecor® bolus of 1 μg/kg followed by an infusion rate of 0.005 μg/kg/min. (See Table 1 for the weight adjusted volume of the IV bolus and the higher infusion flow rate).
- Natrecor® may be increased only one time during a weekly infusion.
- If the dose of Natrecor® is increased for any reason, monitor BP closely. Record vital signs (BP, HR) at the following times:
  - Immediately before the dose increase,
  - At 30 minutes and 1 hour after the dose increase.

Note: The measurements obtained immediately before the dose increase must meet the qualifying criteria for the dose increase (i.e., SBP =90 mm Hg).

Decreasing Natrecor®

If, prior to a weekly infusion, the patient appears "dry" or the SBP is less than 90 mm Hg, the investigator may opt to decrease the bolus dose and infusion rate of Natrecor® for the current weekly infusion to a dose as low as half of the initial dose administered at Week 1.

Due to Excessive or Rapid Decreases in Blood Pressure. If the patient experiences an excessive or rapid decrease in blood pressure at any time during study drug administration, perform the following procedures:

- Discontinue Natrecor® or decrease infusion rate by 50%
- Monitor BP and HR closely until the systolic blood pressure (SBP) stabilizes above 90 mm Hg
- If the infusion has been discontinued, and the SBP has stabilized above 90 mm Hg, Natrecor® infusion may be restarted at a flow rate that is 50% lower than the infusion rate administered before the infusion was stopped, Natrecor® should not be readministered during that day's treatment period)
- Intravenous fluids such as normal saline may be administered to treat an episode of hypotension, if appropriate
- After the 50% lower flow rate has been administered for at least 3 hours, if the patient's SBP has stabilized above 90 mm Hg, the Natrecor® infusion rate may be increased. An increase should not be higher than the dose at which hypotension occurred, at that infusion visit.

The previous procedures may be repeated, as clinically appropriate.

If the Natrecor® infusion rate is decreased, or is terminated for any reason, record vital signs (BP, HR) at the following times:

- Immediately before the dose decrease, interruption, or termination of Natrecor®,
- At 30 minutes and 1 hour, after dose decrease, interruption or discontinuation of the infusion,
- If appropriate, record any adverse events

Guidelines for Infusion Frequency

Patients may receive infusions as frequently as twice a week or as infrequently as once every other week.

Patients may be more clinically unstable following discharge as they are titrating back onto oral medications and may benefit from more frequent infusions initially. Therefore, the investigator is encouraged to treat patients twice during the first week in order to more rapidly titrate to the best dose for that patient within the specified dose range.

Natrecor® infusions should be administered at least once per week, unless clinically contraindicated. Patients may receive an additional infusion of Natrecor® per week if clinically indicated at any time during the study.

A weekly infusion may be omitted if, in the opinion of the investigator, the patient may not tolerate the infusion for reasons such as systolic blood pressure below 90 mm Hg, dehydration, volume depletion, cardiogenic shock or hospitalization for CHF symptoms. However, in order to maintain compliance with this protocol, infusions must be administered no less than every other week.

Having now fully described the invention, the same will be understood by those with skill in the art that the scope may be performed with a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

# TABLE 1

## Examples of Useful Doses According to the Invention

**Group B Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 0.5 µg/kg | Rate of Initial Infusion 0.0025 µg/kg/min | Volume of Minimum Bolus 0.25 µg/kg | Rate of Minimum Infusion 0.00125 µg/kg/min | Volume of Maximum Bolus 1.0 µg/kg | Rate of Maximum Infusion 0.005 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 30 | 66 | 2.5 | 0.8 | 1.3 | 0.4 | 5.0 | 1.5 |
| 31 | 68 | 2.6 | 0.8 | 1.3 | 0.4 | 5.2 | 1.6 |
| 32 | 71 | 2.7 | 0.8 | 1.3 | 0.4 | 5.3 | 1.6 |
| 33 | 73 | 2.8 | 0.8 | 1.4 | 0.4 | 5.5 | 1.7 |
| 34 | 75 | 2.8 | 0.9 | 1.4 | 0.4 | 5.7 | 1.7 |
| 35 | 77 | 2.9 | 0.9 | 1.5 | 0.4 | 5.8 | 1.8 |
| 36 | 79 | 3.0 | 0.9 | 1.5 | 0.5 | 6.0 | 1.8 |
| 37 | 82 | 3.1 | 0.9 | 1.5 | 0.5 | 6.2 | 1.9 |
| 38 | 84 | 3.2 | 1.0 | 1.6 | 0.5 | 6.3 | 1.9 |
| 39 | 86 | 3.3 | 1.0 | 1.6 | 0.5 | 6.5 | 2.0 |
| 40 | 88 | 3.3 | 1.0 | 1.7 | 0.5 | 6.7 | 2.0 |
| 41 | 90 | 3.4 | 1.0 | 1.7 | 0.5 | 6.8 | 2.1 |
| 42 | 93 | 3.5 | 1.1 | 1.8 | 0.5 | 7.0 | 2.1 |
| 43 | 95 | 3.6 | 1.1 | 1.8 | 0.5 | 7.2 | 2.2 |
| 44 | 97 | 3.7 | 1.1 | 1.8 | 0.6 | 7.3 | 2.2 |
| 45 | 99 | 3.8 | 1.1 | 1.9 | 0.6 | 7.5 | 2.3 |
| 46 | 101 | 3.8 | 1.2 | 1.9 | 0.6 | 7.7 | 2.3 |
| 47 | 104 | 3.9 | 1.2 | 2.0 | 0.6 | 7.8 | 2.4 |
| 48 | 106 | 4.0 | 1.2 | 2.0 | 0.6 | 8.0 | 2.4 |
| 49 | 108 | 4.1 | 1.2 | 2.0 | 0.6 | 8.2 | 2.5 |
| 50 | 110 | 4.2 | 1.3 | 2.1 | 0.6 | 8.3 | 2.5 |
| 51 | 112 | 4.3 | 1.3 | 2.1 | 0.6 | 8.5 | 2.6 |
| 52 | 115 | 4.3 | 1.3 | 2.2 | 0.7 | 8.7 | 2.6 |
| 53 | 117 | 4.4 | 1.3 | 2.2 | 0.7 | 8.8 | 2.7 |
| 54 | 119 | 4.5 | 1.4 | 2.3 | 0.7 | 9.0 | 2.7 |
| 55 | 121 | 4.6 | 1.4 | 2.3 | 0.7 | 9.2 | 2.8 |
| 56 | 123 | 4.7 | 1.4 | 2.3 | 0.7 | 9.3 | 2.8 |
| 57 | 126 | 4.8 | 1.4 | 2.4 | 0.7 | 9.5 | 2.9 |
| 58 | 128 | 4.8 | 1.5 | 2.4 | 0.7 | 9.7 | 2.9 |
| 59 | 130 | 4.9 | 1.5 | 2.5 | 0.7 | 9.8 | 3.0 |
| 60 | 132 | 5.0 | 1.5 | 2.5 | 0.8 | 10.0 | 3.0 |
| 61 | 135 | 5.1 | 1.5 | 2.5 | 0.8 | 10.2 | 3.1 |
| 62 | 137 | 5.2 | 1.6 | 2.6 | 0.8 | 10.3 | 3.1 |
| 63 | 139 | 5.3 | 1.6 | 2.6 | 0.8 | 10.5 | 3.2 |
| 64 | 141 | 5.3 | 1.6 | 2.7 | 0.8 | 10.7 | 3.2 |
| 65 | 143 | 5.4 | 1.6 | 2.7 | 0.8 | 10.8 | 3.3 |

**Group B Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 0.5 µg/kg | Rate of Initial Infusion 0.0025 µg/kg/min | Volume of Minimum Bolus 0.25 µg/kg | Rate of Minimum Infusion 0.00125 µg/kg/min | Volume of Maximum Bolus 1.0 µg/kg | Rate of Maximum Infusion 0.005 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 66 | 146 | 5.5 | 1.7 | 2.8 | 0.8 | 11.0 | 3.3 |
| 67 | 148 | 5.6 | 1.7 | 2.8 | 0.8 | 11.2 | 3.4 |
| 68 | 150 | 5.7 | 1.7 | 2.8 | 0.9 | 11.3 | 3.4 |
| 69 | 152 | 5.8 | 1.7 | 2.9 | 0.9 | 11.5 | 3.5 |
| 70 | 154 | 5.8 | 1.8 | 2.9 | 0.9 | 11.7 | 3.5 |
| 71 | 157 | 5.9 | 1.8 | 3.0 | 0.9 | 11.8 | 3.6 |
| 72 | 159 | 6.0 | 1.8 | 3.0 | 0.9 | 12.0 | 3.6 |
| 73 | 161 | 6.1 | 1.8 | 3.0 | 0.9 | 12.2 | 3.7 |
| 74 | 163 | 6.2 | 1.9 | 3.1 | 0.9 | 12.3 | 3.7 |
| 75 | 165 | 6.3 | 1.9 | 3.1 | 0.9 | 12.5 | 3.8 |
| 76 | 168 | 6.3 | 1.9 | 3.2 | 1.0 | 12.7 | 3.8 |
| 77 | 170 | 6.4 | 1.9 | 3.2 | 1.0 | 12.8 | 3.9 |
| 78 | 172 | 6.5 | 2.0 | 3.3 | 1.0 | 13.0 | 3.9 |
| 79 | 174 | 6.6 | 2.0 | 3.3 | 1.0 | 13.2 | 4.0 |
| 80 | 176 | 6.7 | 2.0 | 3.3 | 1.0 | 13.3 | 4.0 |
| 81 | 179 | 6.8 | 2.0 | 3.4 | 1.0 | 13.5 | 4.1 |
| 82 | 181 | 6.8 | 2.1 | 3.4 | 1.0 | 13.7 | 4.1 |
| 83 | 183 | 6.9 | 2.1 | 3.5 | 1.0 | 13.8 | 4.2 |
| 84 | 185 | 7.0 | 2.1 | 3.5 | 1.1 | 14.0 | 4.2 |
| 85 | 187 | 7.1 | 2.1 | 3.5 | 1.1 | 14.2 | 4.3 |
| 86 | 190 | 7.2 | 2.2 | 3.6 | 1.1 | 14.3 | 4.3 |
| 87 | 192 | 7.3 | 2.2 | 3.6 | 1.1 | 14.5 | 4.4 |
| 88 | 194 | 7.3 | 2.2 | 3.7 | 1.1 | 14.7 | 4.4 |
| 89 | 196 | 7.4 | 2.2 | 3.7 | 1.1 | 14.8 | 4.5 |
| 90 | 198 | 7.5 | 2.3 | 3.8 | 1.1 | 15.0 | 4.5 |
| 91 | 201 | 7.6 | 2.3 | 3.8 | 1.1 | 15.2 | 4.6 |
| 92 | 203 | 7.7 | 2.3 | 3.8 | 1.2 | 15.3 | 4.6 |
| 93 | 205 | 7.8 | 2.3 | 3.9 | 1.2 | 15.5 | 4.7 |
| 94 | 207 | 7.8 | 2.4 | 3.9 | 1.2 | 15.7 | 4.7 |
| 95 | 209 | 7.9 | 2.4 | 4.0 | 1.2 | 15.8 | 4.8 |
| 96 | 212 | 8.0 | 2.4 | 4.0 | 1.2 | 16.0 | 4.8 |
| 97 | 214 | 8.1 | 2.4 | 4.0 | 1.2 | 16.2 | 4.9 |
| 98 | 216 | 8.2 | 2.5 | 4.1 | 1.2 | 16.3 | 4.9 |
| 99 | 218 | 8.3 | 2.5 | 4.1 | 1.2 | 16.5 | 5.0 |
| 100 | 221 | 8.3 | 2.5 | 4.2 | 1.3 | 16.7 | 5.0 |
| 101 | 223 | 8.4 | 2.5 | 4.2 | 1.3 | 16.8 | 5.1 |
| 102 | 225 | 8.5 | 2.6 | 4.3 | 1.3 | 17.0 | 5.1 |
| 103 | 227 | 8.6 | 2.6 | 4.3 | 1.3 | 17.2 | 5.2 |
| 104 | 229 | 8.7 | 2.6 | 4.3 | 1.3 | 17.3 | 5.2 |
| 105 | 232 | 8.8 | 2.6 | 4.4 | 1.3 | 17.5 | 5.3 |

**Group B Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 0.5 µg/kg | Rate of Initial Infusion 0.0025 µg/kg/min | Volume of Minimum Bolus 0.25 µg/kg | Rate of Minimum Infusion 0.00125 µg/kg/min | Volume of Maximum Bolus 1.0 µg/kg | Rate of Maximum Infusion 0.005 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 106 | 234 | 8.8 | 2.7 | 4.4 | 1.3 | 17.7 | 5.3 |
| 107 | 236 | 8.9 | 2.7 | 4.5 | 1.3 | 17.8 | 5.4 |
| 108 | 238 | 9.0 | 2.7 | 4.5 | 1.4 | 18.0 | 5.4 |
| 109 | 240 | 9.1 | 2.7 | 4.5 | 1.4 | 18.2 | 5.5 |
| 110 | 243 | 9.2 | 2.8 | 4.6 | 1.4 | 18.3 | 5.5 |
| 111 | 245 | 9.3 | 2.8 | 4.6 | 1.4 | 18.5 | 5.6 |
| 112 | 247 | 9.3 | 2.8 | 4.7 | 1.4 | 18.7 | 5.6 |
| 113 | 249 | 9.4 | 2.8 | 4.7 | 1.4 | 18.8 | 5.7 |
| 114 | 251 | 9.5 | 2.9 | 4.8 | 1.4 | 19.0 | 5.7 |
| 115 | 254 | 9.6 | 2.9 | 4.8 | 1.4 | 19.2 | 5.8 |
| 116 | 256 | 9.7 | 2.9 | 4.8 | 1.5 | 19.3 | 5.8 |
| 117 | 258 | 9.8 | 2.9 | 4.9 | 1.5 | 19.5 | 5.9 |
| 118 | 260 | 9.8 | 3.0 | 4.9 | 1.5 | 19.7 | 5.9 |
| 119 | 262 | 9.9 | 3.0 | 5.0 | 1.5 | 19.8 | 6.0 |
| 120 | 265 | 10.0 | 3.0 | 5.0 | 1.5 | 20.0 | 6.0 |
| 121 | 267 | 10.1 | 3.0 | 5.0 | 1.5 | 20.2 | 6.1 |
| 122 | 269 | 10.2 | 3.1 | 5.1 | 1.5 | 20.3 | 6.1 |
| 123 | 271 | 10.3 | 3.1 | 5.1 | 1.5 | 20.5 | 6.2 |
| 124 | 273 | 10.3 | 3.1 | 5.2 | 1.6 | 20.7 | 6.2 |
| 125 | 276 | 10.4 | 3.1 | 5.2 | 1.6 | 20.8 | 6.3 |
| 126 | 278 | 10.5 | 3.2 | 5.3 | 1.6 | 21.0 | 6.3 |
| 127 | 280 | 10.6 | 3.2 | 5.3 | 1.6 | 21.2 | 6.4 |
| 128 | 282 | 10.7 | 3.2 | 5.3 | 1.6 | 21.3 | 6.4 |
| 129 | 284 | 10.8 | 3.2 | 5.4 | 1.6 | 21.5 | 6.5 |
| 130 | 287 | 10.8 | 3.3 | 5.4 | 1.6 | 21.7 | 6.5 |
| 131 | 289 | 10.9 | 3.3 | 5.5 | 1.6 | 21.8 | 6.6 |
| 132 | 291 | 11.0 | 3.3 | 5.5 | 1.7 | 22.0 | 6.6 |
| 133 | 293 | 11.1 | 3.3 | 5.5 | 1.7 | 22.2 | 6.7 |
| 134 | 295 | 11.2 | 3.4 | 5.6 | 1.7 | 22.3 | 6.7 |
| 135 | 298 | 11.3 | 3.4 | 5.6 | 1.7 | 22.5 | 6.8 |
| 136 | 300 | 11.3 | 3.4 | 5.7 | 1.7 | 22.7 | 6.8 |
| 137 | 302 | 11.4 | 3.4 | 5.7 | 1.7 | 22.8 | 6.9 |
| 138 | 304 | 11.5 | 3.5 | 5.8 | 1.7 | 23.0 | 6.9 |
| 139 | 306 | 11.6 | 3.5 | 5.8 | 1.7 | 23.2 | 7.0 |
| 140 | 309 | 11.7 | 3.5 | 5.8 | 1.8 | 23.3 | 7.0 |
| 141 | 311 | 11.8 | 3.5 | 5.9 | 1.8 | 23.5 | 7.1 |
| 142 | 313 | 11.8 | 3.6 | 5.9 | 1.8 | 23.7 | 7.1 |
| 143 | 315 | 11.9 | 3.6 | 6.0 | 1.8 | 23.8 | 7.2 |
| 144 | 318 | 12.0 | 3.6 | 6.0 | 1.8 | 24.0 | 7.2 |
| 145 | 320 | 12.1 | 3.6 | 6.0 | 1.8 | 24.2 | 7.3 |

**Group B Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 0.5 µg/kg | Rate of Initial Infusion 0.0025 µg/kg/min | Volume of Minimum Bolus 0.25 µg/kg | Rate of Minimum Infusion 0.00125 µg/kg/min | Volume of Maximum Bolus 1.0 µg/kg | Rate of Maximum Infusion 0.005 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 146 | 322 | 12.2 | 3.7 | 6.1 | 1.8 | 24.3 | 7.3 |
| 147 | 324 | 12.3 | 3.7 | 6.1 | 1.8 | 24.5 | 7.4 |
| 148 | 326 | 12.3 | 3.7 | 6.2 | 1.9 | 24.7 | 7.4 |
| 149 | 329 | 12.4 | 3.7 | 6.2 | 1.9 | 24.8 | 7.5 |
| 150 | 331 | 12.5 | 3.8 | 6.3 | 1.9 | 25.0 | 7.5 |
| 151 | 333 | 12.6 | 3.8 | 6.3 | 1.9 | 25.2 | 7.6 |
| 152 | 335 | 12.7 | 3.8 | 6.3 | 1.9 | 25.3 | 7.6 |
| 153 | 337 | 12.8 | 3.8 | 6.4 | 1.9 | 25.5 | 7.7 |
| 154 | 340 | 12.8 | 3.9 | 6.4 | 1.9 | 25.7 | 7.7 |
| 155 | 342 | 12.9 | 3.9 | 6.5 | 1.9 | 25.8 | 7.8 |
| 156 | 344 | 13.0 | 3.9 | 6.5 | 2.0 | 26.0 | 7.8 |
| 157 | 346 | 13.1 | 3.9 | 6.5 | 2.0 | 26.2 | 7.9 |
| 158 | 348 | 13.2 | 4.0 | 6.6 | 2.0 | 26.3 | 7.9 |
| 159 | 351 | 13.3 | 4.0 | 6.6 | 2.0 | 26.5 | 8.0 |
| 160 | 353 | 13.3 | 4.0 | 6.7 | 2.0 | 26.7 | 8.0 |
| 161 | 355 | 13.4 | 4.0 | 6.7 | 2.0 | 26.8 | 8.1 |
| 162 | 357 | 13.5 | 4.1 | 6.8 | 2.0 | 27.0 | 8.1 |
| 163 | 359 | 13.6 | 4.1 | 6.8 | 2.0 | 27.2 | 8.2 |
| 164 | 362 | 13.7 | 4.1 | 6.8 | 2.1 | 27.3 | 8.2 |
| 165 | 364 | 13.8 | 4.1 | 6.9 | 2.1 | 27.5 | 8.3 |
| 166 | 366 | 13.8 | 4.2 | 6.9 | 2.1 | 27.7 | 8.3 |
| 167 | 368 | 13.9 | 4.2 | 7.0 | 2.1 | 27.8 | 8.4 |
| 168 | 370 | 14.0 | 4.2 | 7.0 | 2.1 | 28.0 | 8.4 |
| 169 | 373 | 14.1 | 4.2 | 7.0 | 2.1 | 28.2 | 8.5 |
| 170 | 375 | 14.2 | 4.3 | 7.1 | 2.1 | 28.3 | 8.5 |
| 171 | 377 | 14.3 | 4.3 | 7.1 | 2.1 | 28.5 | 8.6 |
| 172 | 379 | 14.3 | 4.3 | 7.2 | 2.2 | 28.7 | 8.6 |
| 173 | 381 | 14.4 | 4.3 | 7.2 | 2.2 | 28.8 | 8.7 |
| 174 | 384 | 14.5 | 4.4 | 7.3 | 2.2 | 29.0 | 8.7 |
| 175 | 386 | 14.6 | 4.4 | 7.3 | 2.2 | 29.2 | 8.8 |

**Group C Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 1.0 µg/kg | Rate of Initial Infusion 0.005 µg/kg/min | Volume of Minimum Bolus 0.5 µg/kg | Rate of Minimum Infusion 0.0025 µg/kg/min | Volume of Maximum Bolus 2.0 µg/kg | Rate of Maximum Infusion 0.01 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 30 | 66 | 5.0 | 1.5 | 2.5 | 0.8 | 10.0 | 3.0 |
| 31 | 68 | 5.2 | 1.6 | 2.6 | 0.8 | 10.3 | 3.1 |
| 32 | 71 | 5.3 | 1.6 | 2.7 | 0.8 | 10.7 | 3.2 |
| 33 | 73 | 5.5 | 1.7 | 2.8 | 0.8 | 11.0 | 3.3 |
| 34 | 75 | 5.7 | 1.7 | 2.8 | 0.9 | 11.3 | 3.4 |
| 35 | 77 | 5.8 | 1.8 | 2.9 | 0.9 | 11.7 | 3.5 |
| 36 | 79 | 6.0 | 1.8 | 3.0 | 0.9 | 12.0 | 3.6 |
| 37 | 82 | 6.2 | 1.9 | 3.1 | 0.9 | 12.3 | 3.7 |
| 38 | 84 | 6.3 | 1.9 | 3.2 | 1.0 | 12.7 | 3.8 |
| 39 | 86 | 6.5 | 2.0 | 3.3 | 1.0 | 13.0 | 3.9 |
| 40 | 88 | 6.7 | 2.0 | 3.3 | 1.0 | 13.3 | 4.0 |
| 41 | 90 | 6.8 | 2.1 | 3.4 | 1.0 | 13.7 | 4.1 |
| 42 | 93 | 7.0 | 2.1 | 3.5 | 1.1 | 14.0 | 4.2 |
| 43 | 95 | 7.2 | 2.2 | 3.6 | 1.1 | 14.3 | 4.3 |
| 44 | 97 | 7.3 | 2.2 | 3.7 | 1.1 | 14.7 | 4.4 |
| 45 | 99 | 7.5 | 2.3 | 3.8 | 1.1 | 15.0 | 4.5 |
| 46 | 101 | 7.7 | 2.3 | 3.8 | 1.2 | 15.3 | 4.6 |
| 47 | 104 | 7.8 | 2.4 | 3.9 | 1.2 | 15.7 | 4.7 |
| 48 | 106 | 8.0 | 2.4 | 4.0 | 1.2 | 16.0 | 4.8 |
| 49 | 108 | 8.2 | 2.5 | 4.1 | 1.2 | 16.3 | 4.9 |
| 50 | 110 | 8.3 | 2.5 | 4.2 | 1.3 | 16.7 | 5.0 |
| 51 | 112 | 8.5 | 2.6 | 4.3 | 1.3 | 17.0 | 5.1 |
| 52 | 115 | 8.7 | 2.6 | 4.3 | 1.3 | 17.3 | 5.2 |
| 53 | 117 | 8.8 | 2.7 | 4.4 | 1.3 | 17.7 | 5.3 |
| 54 | 119 | 9.0 | 2.7 | 4.5 | 1.4 | 18.0 | 5.4 |
| 55 | 121 | 9.2 | 2.8 | 4.6 | 1.4 | 18.3 | 5.5 |
| 56 | 123 | 9.3 | 2.8 | 4.7 | 1.4 | 18.7 | 5.6 |
| 57 | 126 | 9.5 | 2.9 | 4.8 | 1.4 | 19.0 | 5.7 |
| 58 | 128 | 9.7 | 2.9 | 4.8 | 1.5 | 19.3 | 5.8 |
| 59 | 130 | 9.8 | 3.0 | 4.9 | 1.5 | 19.7 | 5.9 |
| 60 | 132 | 10.0 | 3.0 | 5.0 | 1.5 | 20.0 | 6.0 |
| 61 | 135 | 10.2 | 3.1 | 5.1 | 1.5 | 20.3 | 6.1 |
| 62 | 137 | 10.3 | 3.1 | 5.2 | 1.6 | 20.7 | 6.2 |
| 63 | 139 | 10.5 | 3.2 | 5.3 | 1.6 | 21.0 | 6.3 |
| 64 | 141 | 10.7 | 3.2 | 5.3 | 1.6 | 21.3 | 6.4 |
| 65 | 143 | 10.8 | 3.3 | 5.4 | 1.6 | 21.7 | 6.5 |
| 66 | 146 | 11.0 | 3.3 | 5.5 | 1.7 | 22.0 | 6.6 |
| 67 | 148 | 11.2 | 3.4 | 5.6 | 1.7 | 22.3 | 6.7 |
| 68 | 150 | 11.3 | 3.4 | 5.7 | 1.7 | 22.7 | 6.8 |
| 69 | 152 | 11.5 | 3.5 | 5.8 | 1.7 | 23.0 | 6.9 |
| 70 | 154 | 11.7 | 3.5 | 5.8 | 1.8 | 23.3 | 7.0 |

**Group C Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 1.0 µg/kg | Rate of Initial Infusion 0.005 µg/kg/min | Volume of Minimum Bolus 0.5 µg/kg | Rate of Minimum Infusion 0.0025 µg/kg/min | Volume of Maximum Bolus 2.0 µg/kg | Rate of Maximum Infusion 0.01 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 71 | 157 | 11.8 | 3.6 | 5.9 | 1.8 | 23.7 | 7.1 |
| 72 | 159 | 12.0 | 3.6 | 6.0 | 1.8 | 24.0 | 7.2 |
| 73 | 161 | 12.2 | 3.7 | 6.1 | 1.8 | 24.3 | 7.3 |
| 74 | 163 | 12.3 | 3.7 | 6.2 | 1.9 | 24.7 | 7.4 |
| 75 | 165 | 12.5 | 3.8 | 6.3 | 1.9 | 25.0 | 7.5 |
| 76 | 168 | 12.7 | 3.8 | 6.3 | 1.9 | 25.3 | 7.6 |
| 77 | 170 | 12.8 | 3.9 | 6.4 | 1.9 | 25.7 | 7.7 |
| 78 | 172 | 13.0 | 3.9 | 6.5 | 2.0 | 26.0 | 7.8 |
| 79 | 174 | 13.2 | 4.0 | 6.6 | 2.0 | 26.3 | 7.9 |
| 80 | 176 | 13.3 | 4.0 | 6.7 | 2.0 | 26.7 | 8.0 |
| 81 | 179 | 13.5 | 4.1 | 6.8 | 2.0 | 27.0 | 8.1 |
| 82 | 181 | 13.7 | 4.1 | 6.8 | 2.1 | 27.3 | 8.2 |
| 83 | 183 | 13.8 | 4.2 | 6.9 | 2.1 | 27.7 | 8.3 |
| 84 | 185 | 14.0 | 4.2 | 7.0 | 2.1 | 28.0 | 8.4 |
| 85 | 187 | 14.2 | 4.3 | 7.1 | 2.1 | 28.3 | 8.5 |
| 86 | 190 | 14.3 | 4.3 | 7.2 | 2.2 | 28.7 | 8.6 |
| 87 | 192 | 14.5 | 4.4 | 7.3 | 2.2 | 29.0 | 8.7 |
| 88 | 194 | 14.7 | 4.4 | 7.3 | 2.2 | 29.3 | 8.8 |
| 89 | 196 | 14.8 | 4.5 | 7.4 | 2.2 | 29.7 | 8.9 |
| 90 | 198 | 15.0 | 4.5 | 7.5 | 2.3 | 30.0 | 9.0 |
| 91 | 201 | 15.2 | 4.6 | 7.6 | 2.3 | 30.3 | 9.1 |
| 92 | 203 | 15.3 | 4.6 | 7.7 | 2.3 | 30.7 | 9.2 |
| 93 | 205 | 15.5 | 4.7 | 7.8 | 2.3 | 31.0 | 9.3 |
| 94 | 207 | 15.7 | 4.7 | 7.8 | 2.4 | 31.3 | 9.4 |
| 95 | 209 | 15.8 | 4.8 | 7.9 | 2.4 | 31.7 | 9.5 |
| 96 | 212 | 16.0 | 4.8 | 8.0 | 2.4 | 32.0 | 9.6 |
| 97 | 214 | 16.2 | 4.9 | 8.1 | 2.4 | 32.3 | 9.7 |
| 98 | 216 | 16.3 | 4.9 | 8.2 | 2.5 | 32.7 | 9.8 |
| 99 | 218 | 16.5 | 5.0 | 8.3 | 2.5 | 33.0 | 9.9 |
| 100 | 221 | 16.7 | 5.0 | 8.3 | 2.5 | 33.3 | 10.0 |
| 101 | 223 | 16.8 | 5.1 | 8.4 | 2.5 | 33.7 | 10.1 |
| 102 | 225 | 17.0 | 5.1 | 8.5 | 2.6 | 34.0 | 10.2 |
| 103 | 227 | 17.2 | 5.2 | 8.6 | 2.6 | 34.3 | 10.3 |
| 104 | 229 | 17.3 | 5.2 | 8.7 | 2.6 | 34.7 | 10.4 |
| 105 | 232 | 17.5 | 5.3 | 8.8 | 2.6 | 35.0 | 10.5 |
| 106 | 234 | 17.7 | 5.3 | 8.8 | 2.7 | 35.3 | 10.6 |
| 107 | 236 | 17.8 | 5.4 | 8.9 | 2.7 | 35.7 | 10.7 |
| 108 | 238 | 18.0 | 5.4 | 9.0 | 2.7 | 36.0 | 10.8 |
| 109 | 240 | 18.2 | 5.5 | 9.1 | 2.7 | 36.3 | 10.9 |
| 110 | 243 | 18.3 | 5.5 | 9.2 | 2.8 | 36.7 | 11.0 |

**Group C Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 1.0 µg/kg | Rate of Initial Infusion 0.005 µg/kg/min | Volume of Minimum Bolus 0.5 µg/kg | Rate of Minimum Infusion 0.0025 µg/kg/min | Volume of Maximum Bolus 2.0 µg/kg | Rate of Maximum Infusion 0.01 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 111 | 245 | 18.5 | 5.6 | 9.3 | 2.8 | 37.0 | 11.1 |
| 112 | 247 | 18.7 | 5.6 | 9.3 | 2.8 | 37.3 | 11.2 |
| 113 | 249 | 18.8 | 5.7 | 9.4 | 2.8 | 37.7 | 11.3 |
| 114 | 251 | 19.0 | 5.7 | 9.5 | 2.9 | 38.0 | 11.4 |
| 115 | 254 | 19.2 | 5.8 | 9.6 | 2.9 | 38.3 | 11.5 |
| 116 | 256 | 19.3 | 5.8 | 9.7 | 2.9 | 38.7 | 11.6 |
| 117 | 258 | 19.5 | 5.9 | 9.8 | 2.9 | 39.0 | 11.7 |
| 118 | 260 | 19.7 | 5.9 | 9.8 | 3.0 | 39.3 | 11.8 |
| 119 | 262 | 19.8 | 6.0 | 9.9 | 3.0 | 39.7 | 11.9 |
| 120 | 265 | 20.0 | 6.0 | 10.0 | 3.0 | 40.0 | 12.0 |
| 121 | 267 | 20.2 | 6.1 | 10.1 | 3.0 | 40.3 | 12.1 |
| 122 | 269 | 20.3 | 6.1 | 10.2 | 3.1 | 40.7 | 12.2 |
| 123 | 271 | 20.5 | 6.2 | 10.3 | 3.1 | 41.0 | 12.3 |
| 124 | 273 | 20.7 | 6.2 | 10.3 | 3.1 | 41.3 | 12.4 |
| 125 | 276 | 20.8 | 6.3 | 10.4 | 3.1 | 41.7 | 12.5 |
| 126 | 278 | 21.0 | 6.3 | 10.5 | 3.2 | 42.0 | 12.6 |
| 127 | 280 | 21.2 | 6.4 | 10.6 | 3.2 | 42.3 | 12.7 |
| 128 | 282 | 21.3 | 6.4 | 10.7 | 3.2 | 42.7 | 12.8 |
| 129 | 284 | 21.5 | 6.5 | 10.8 | 3.2 | 43.0 | 12.9 |
| 130 | 287 | 21.7 | 6.5 | 10.8 | 3.3 | 43.3 | 13.0 |
| 131 | 289 | 21.8 | 6.6 | 10.9 | 3.3 | 43.7 | 13.1 |
| 132 | 291 | 22.0 | 6.6 | 11.0 | 3.3 | 44.0 | 13.2 |
| 133 | 293 | 22.2 | 6.7 | 11.1 | 3.3 | 44.3 | 13.3 |
| 134 | 295 | 22.3 | 6.7 | 11.2 | 3.4 | 44.7 | 13.4 |
| 135 | 298 | 22.5 | 6.8 | 11.3 | 3.4 | 45.0 | 13.5 |
| 136 | 300 | 22.7 | 6.8 | 11.3 | 3.4 | 45.3 | 13.6 |
| 137 | 302 | 22.8 | 6.9 | 11.4 | 3.4 | 45.7 | 13.7 |
| 138 | 304 | 23.0 | 6.9 | 11.5 | 3.5 | 46.0 | 13.8 |
| 139 | 306 | 23.2 | 7.0 | 11.6 | 3.5 | 46.3 | 13.9 |
| 140 | 309 | 23.3 | 7.0 | 11.7 | 3.5 | 46.7 | 14.0 |
| 141 | 311 | 23.5 | 7.1 | 11.8 | 3.5 | 47.0 | 14.1 |
| 142 | 313 | 23.7 | 7.1 | 11.8 | 3.6 | 47.3 | 14.2 |
| 143 | 315 | 23.8 | 7.2 | 11.9 | 3.6 | 47.7 | 14.3 |
| 144 | 318 | 24.0 | 7.2 | 12.0 | 3.6 | 48.0 | 14.4 |
| 145 | 320 | 24.2 | 7.3 | 12.1 | 3.6 | 48.3 | 14.5 |
| 146 | 322 | 24.3 | 7.3 | 12.2 | 3.7 | 48.7 | 14.6 |
| 147 | 324 | 24.5 | 7.4 | 12.3 | 3.7 | 49.0 | 14.7 |
| 148 | 326 | 24.7 | 7.4 | 12.3 | 3.7 | 49.3 | 14.8 |
| 149 | 329 | 24.8 | 7.5 | 12.4 | 3.7 | 49.7 | 14.9 |
| 150 | 331 | 25.0 | 7.5 | 12.5 | 3.8 | 50.0 | 15.0 |

**Group C Natrecor® Doses**

| Subject Weight | | Initial | | Minimum | | Maximum | |
|---|---|---|---|---|---|---|---|
| | | Volume of Initial Bolus 1.0 µg/kg | Rate of Initial Infusion 0.005 µg/kg/min | Volume of Minimum Bolus 0.5 µg/kg | Rate of Minimum Infusion 0.0025 µg/kg/min | Volume of Maximum Bolus 2.0 µg/kg | Rate of Maximum Infusion 0.01 µg/kg/min |
| kg | lb | mL | mL/hr | mL | mL/hr | mL | mL/hr |
| 151 | 333 | 25.2 | 7.6 | 12.6 | 3.8 | 50.3 | 15.1 |
| 152 | 335 | 25.3 | 7.6 | 12.7 | 3.8 | 50.7 | 15.2 |
| 153 | 337 | 25.5 | 7.7 | 12.8 | 3.8 | 51.0 | 15.3 |
| 154 | 340 | 25.7 | 7.7 | 12.8 | 3.9 | 51.3 | 15.4 |
| 155 | 342 | 25.8 | 7.8 | 12.9 | 3.9 | 51.7 | 15.5 |
| 156 | 344 | 26.0 | 7.8 | 13.0 | 3.9 | 52.0 | 15.6 |
| 157 | 346 | 26.2 | 7.9 | 13.1 | 3.9 | 52.3 | 15.7 |
| 158 | 348 | 26.3 | 7.9 | 13.2 | 4.0 | 52.7 | 15.8 |
| 159 | 351 | 26.5 | 8.0 | 13.3 | 4.0 | 53.0 | 15.9 |
| 160 | 353 | 26.7 | 8.0 | 13.3 | 4.0 | 53.3 | 16.0 |
| 161 | 355 | 26.8 | 8.1 | 13.4 | 4.0 | 53.7 | 16.1 |
| 162 | 357 | 27.0 | 8.1 | 13.5 | 4.1 | 54.0 | 16.2 |
| 163 | 359 | 27.2 | 8.2 | 13.6 | 4.1 | 54.3 | 16.3 |
| 164 | 362 | 27.3 | 8.2 | 13.7 | 4.1 | 54.7 | 16.4 |
| 165 | 364 | 27.5 | 8.3 | 13.8 | 4.1 | 55.0 | 16.5 |
| 166 | 366 | 27.7 | 8.3 | 13.8 | 4.2 | 55.3 | 16.6 |
| 167 | 368 | 27.8 | 8.4 | 13.9 | 4.2 | 55.7 | 16.7 |
| 168 | 370 | 28.0 | 8.4 | 14.0 | 4.2 | 56.0 | 16.8 |
| 169 | 373 | 28.2 | 8.5 | 14.1 | 4.2 | 56.3 | 16.9 |
| 170 | 375 | 28.3 | 8.5 | 14.2 | 4.3 | 56.7 | 17.0 |
| 171 | 377 | 28.5 | 8.6 | 14.3 | 4.3 | 57.0 | 17.1 |
| 172 | 379 | 28.7 | 8.6 | 14.3 | 4.3 | 57.3 | 17.2 |
| 173 | 381 | 28.8 | 8.7 | 14.4 | 4.3 | 57.7 | 17.3 |
| 174 | 384 | 29.0 | 8.7 | 14.5 | 4.4 | 58.0 | 17.4 |
| 175 | 386 | 29.2 | 8.8 | 14.6 | 4.4 | 58.3 | 17.5 |

**TABLE 2**

**New York Heart Association Functional Classification**

| |
|---|
| 1. Patients with cardiac disease but without resulting limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain. |
| 2. Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain. |
| 3. Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain. |
| 4. Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of cardiac insufficiency or of the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased. |

WHAT IS CLAIMED IS:

1. A method for treating congestive heart failure in a mammal comprising administering a therapeutically effective dose of a natriuretic peptide to said mammal.

2. The method of claim 1, wherein said dose is an adjunct to oral therapy.

3. The method of claim 1, wherein the said mammal is in a compensated state of congestive heart failure.

4. The method of claim 1, wherein the said mammal is in a decompensated state of congestive heart failure.

5. The method of claim 1, wherein said natriuretic peptide is selected from the group consisting of an atrial natriuretic peptide (ANP), a B-type natriuretic peptide (BNP) and a C-type natriuretic peptide (CNP).

6. The method of claim 5, wherein the said natriuretic peptide is B-type natriuretic peptide.

7. The method of claim 6, wherein said B-type natriuretic peptide (BNP) is selected from the group consisting of human BNP, pig BNP, rat BNP, and chicken BNP.

8. The method of claim 6, wherein said natriuretic peptide is a human B-type natriuretic peptide.

9. The method of claim 6, wherein said natriuretic peptide is a recombinant B-type natriuretic peptide.

10. The method of claim 5, wherein said natriuretic peptide is atrial natriuretic peptide (ANP).

11. The method of claim 10, wherein said atrial natriuretic peptide (ANP) is selected from the group consisting of frog ANP and human ANP.

12. The method of claim 10, wherein said atrial natriuretic peptide (ANP) is human ANP.

13. The method of claim 10, wherein said atrial natriuretic peptide (ANP) is recombinant ANP.

14. The method of claim 5, wherein said natriuretic peptide is C-type natriuretic peptide (CNP).

15. The method of claim 14, wherein said C-type natriuretic peptide (CNP) is selected from the group consisting of chicken CNP, rat CNP and human CNP.

16. The method of claim 15, wherein said C-type natriuretic peptide (CNP) is a human C-type natriuretic peptide.

17. The method of claim 15, wherein said natriuretic peptide is a recombinant C-type natriuretic peptide.

18. The method of claim 1, wherein said natriuretic peptide is administered by bolus, infusion or a combination of bolus and infusion.

19. The method of claim 18, wherein said natriuretic peptide is administered by bolus.

20. The method of claim 18, wherein said natriuretic peptide is administered by infusion.

21. The method of claim 18, wherein said natriuretic peptide is administered by a combination of bolus and infusion.

22. The method of claim 18, wherein said natriuretic peptide is administered by bolus and intermittent infusion.

23. The method of claim 20 and 21, wherein said infusion is intermittant.

24. The method of claim 20 and 21, wherein said infusion is serial.

25. The method of claim 20 and 21, wherein said infusion is continuous.

26. The method of claim 19, wherein said natriuretic peptide is administered by bolus at a dose of not less than 2 μg/kg body weight.

27. The method of claim 24, wherein said natriuretic peptide is administered by serial infusion at a dose less than 0.01 μg/kg body weight.

28. The method of claim 23, wherein said natriuretic peptide is administered by intermittent infusion at a dose less than 0.01 μg/kg body weight.

29. The method of claim 1, wherein said natriuretic peptide is administered by intravenous, intramuscular, subcutaneous, intradermal, intrasternal, intraperitoneal or intra-articular injection.

30. The method of claim 1, wherein the said natriuretic peptide is administered prophylactically to said mammal.

31. The method of claim 1, wherein said natriuretic peptide is administered to said mammal for 10 hours.

32. The method of claim 16, wherein said natriuretic peptide is administered to said mammal for 4-6 hours weekly.

33. The method of claim 1, wherein said natriuretic peptide is administered to said mammal at an infusion rate of 0.00125 μg/kg body weight/min to 0.01 μg/kg body weight/min.

34. The method of claim 19, wherein said natriuretic peptide is administered to said at 0.005 μg/kg body weight/min.

35. The method of claim 1, wherein said therapeutically effective dose comprises 0.25 μg/kg body weight to 1.75 μg/kg body weight of said natriuretic peptide.

36. A kit for the treatment of congestive heart failure by administration of a therapeutically effective dose of a natriuretic peptide, wherein the natriuretic peptide is packaged separately.

37. The kit as claimed in claim 34, wherein said natriuretic peptide is selected from the group consisting of an atrial natriuretic peptide (ANP), a B-type natriuretic peptide (BNP) and a C-type natriuretic peptide (CNP).

38. The kit as claimed in claim 35, wherein said natriuretic peptide is a human B-type natriuretic peptide.

39. The kit as claimed in claim 35, wherein said natriuretic peptide is a recombinant human B-type natriuretic peptide.

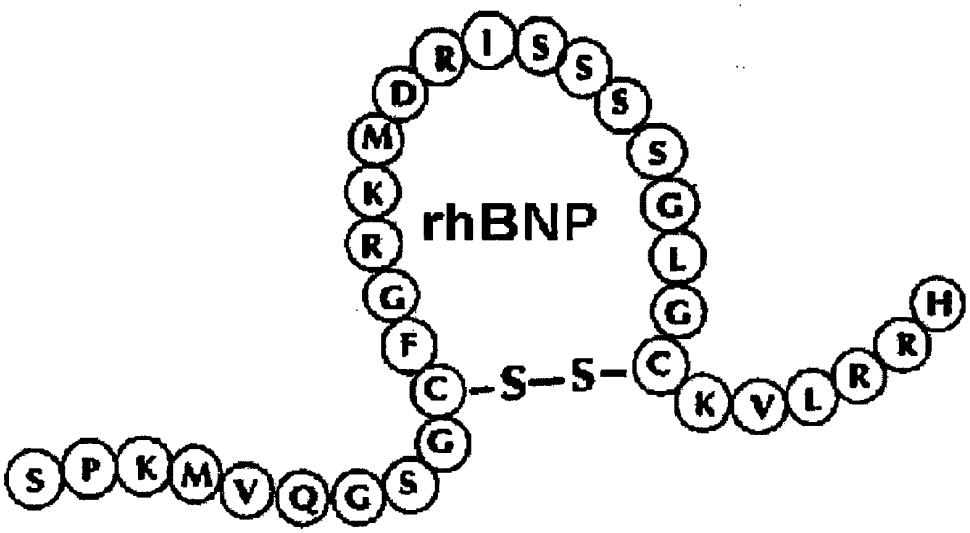
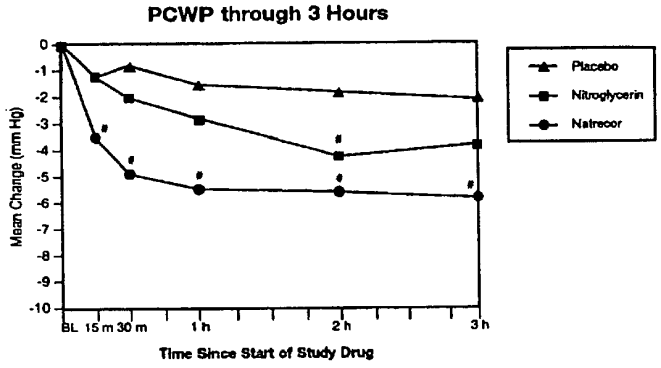



**Figure 1**

Figure 2

## Pulmonary Capillary Wedge Pressure (PCWP)




*p<0.05 compared to placebo

**Figure 3**

**Mean Hemodynamic Change from Baseline**

| **Effects at 3 Hours** | **Placebo** (n=62) | **Nitroglycerin** (n=60) | **Natrecor** (n=124) |
|---|---|---|---|
| Pulmonary capillary wedge pressure (mm Hg) | -2.0 | -3.8 | -5.8‡ |
| Right atrial pressure (mm Hg) | 0.0 | -2.6 | -3.1‡ |
| Cardiac index (L/min/$M^2$) | 0.0 | 0.2 | 0.1 |
| Mean pulmonary artery pressure (mm Hg) | -1.1 | -2.5 | -5.4‡ |
| Systemic vascular resistance (dynes*sec*$cm^{-5}$) | -44 | -105 | -144 |
| Systolic blood pressure† (mm Hg) | -2.5 | -5.7‡ | -5.6‡ |

†Based on all treated subject: placebo n=142, nitroglycerin n=143, Natrecor n=204

‡$p<0.05$ compared to placebo

Figure 4

**Adverse Reactions**

| | VMAC Trial | | Other Long Infusion Trials | | |
|---|---|---|---|---|---|
| | | Natrecor Recommended Dose | | Natrecor µg/kg/min | |
| Adverse Events | Nitroglycerin (n=216) | (n=273) | Control* (n=256) | 0.015 (n=253) | 0.03 (n=246) |
| **Cardiovascular** | | | | | |
| Hyopotension | 25 (12%) | 31 (11%) | 20 (8%) | 56 (22%) | 87 (35%) |
| Symptomatic Hypotension | 10 (5%) | 12 (4%) | 8 (3%) | 28 (11%) | 42 (17%) |
| Asymptomatic Hypotension | 17 (8%) | 23 (8%) | 13 (5%) | 31 (12%) | 49 (20%) |
| Ventricular Tachycardia (VT) | 11 (5%) | 9 (3%) | 25 (10%) | 25 (10%) | 10 (4%) |
| Non-sustained VT | 11 (5%) | 9 (3%) | 23 (9%) | 24 (9%) | 9 (4%) |
| Ventricular Extrasystoles | 2 (1%) | 7 (3%) | 15 (6%) | 10 (4%) | 9 (4%) |
| Angina Pectoris | 5 (2%) | 5 (2%) | 6 (2%) | 14 (6%) | 6 (2%) |
| Bradycardia | 1 (<1%) | 3 (1%) | 1 (<1%) | 8 (3%) | 13 (5%) |
| **Body as a Whole** | | | | | |
| Headache | 44 (20%) | 21 (8%) | 23 (9%) | 23 (9%) | 17 (7%) |
| Abdominal Pain | 11 (5%) | 4 (1%) | 10 (4%) | 6 (2%) | 8 (3%) |
| Back Pain | 7 (3%) | 10 (4%) | 4 (2%) | 5 (2%) | 3 (1%) |
| **Nervous** | | | | | |
| Insomnia | 9 (4%) | 6 (2%) | 7 (3%) | 15 (6%) | 15 (6%) |
| Dizziness | 4 (2%) | 7 (3%) | 7 (3%) | 16 (6%) | 12 (5%) |
| Anxiety | 6 (3%) | 8 (3%) | 2 (1%) | 8 (3%) | 4 (2%) |
| **Digestive** | | | | | |
| Nausea | 13 (6%) | 10 (4%) | 12 (5%) | 24 (9%) | 33 (13%) |
| Vomiting | 4 (2%) | 4 (1%) | 2 (1%) | 6 (2%) | 10 (4%) |

**Figure 5**

**Calculation of Appropriate Bolus Volume and Infusion Rate for Conventional Administration of Natrecor®**

| Natrecor Weight-Adjusted Bolus Volume and Infusion Flow Rate (2 µg/kg Bolus Followed by a 0.01 µg/kg/min Dose) | | |
|---|---|---|
| **Patient Weight (kg)** | **Volume of Bolus (mL)** | **Rate of Infusion (mL/h)** |
| 60 | 20.0 | 6 |
| 70 | 23.3 | 7 |
| 80 | 26.7 | 8 |
| 90 | 30.0 | 9 |
| 100 | 33.3 | 10 |
| 110 | 36.7 | 11 |

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Reissue Application of:
Daniel J. Wendt et al.

| | |
|---|---|
| Application No.: 15/646,822 | Confirmation No.: 7727 |
| Filed: July 11, 2017 | Art Unit: 3991 |
| For: Variants of C-Type Natriuretic Peptide | Examiner: J. F. Railey |

## INFORMATION DISCLOSURE STATEMENT (IDS)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 C.F.R. §§ 1.56, 1.97, and 1.98, the attention of the United States Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of the above-identified application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed after the mailing date of either a Final Action under 37 C.F.R. § 1.113, a Notice of Allowance under 37 C.F.R. § 1.311, or an action that otherwise closed prosecution in this application, but on or before payment of the Issue Fee (37 C.F.R. § 1.97(d)).

Pursuant to 37 C.F.R. § 1.97(e)(1), each item of information contained in this Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

In accordance with 37 C.F.R. § 1.98(a)(2)(ii), copies of U.S. patents and U.S. patent application publications are not submitted. Submitted herewith are copies of foreign patents and non-patent literature in accordance with 37 C.F.R. § 1.98(a)(2).

In accordance with 37 C.F.R. § 1.97(g), the filing of this Information Disclosure Statement shall not be construed as a representation that a search has been made. In accordance with 37 C.F.R. § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in this Information Disclosure Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

It is submitted that the Information Disclosure Statement is in compliance with 37 C.F.R. § 1.98, and the Examiner is respectfully requested to consider the listed references.

Please charge our Credit Card in the amount of $240.00 covering the fee set forth in 37 C.F.R. § 1.17(p). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

Dated: June 10, 2020

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

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|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 06/26/2020

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
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| CAMPELL, BRUCE R | |

| ART UNIT | PAPER NUMBER |
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| 3991 | |

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| 15/646,822 | 07/11/2017 | Wendt et al. | 30610/44556C1-R2 |

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/BRUCE R CAMPELL/
Patent Reexam Specialist, Art Unit 3991

PTO-90C (Rev.04-03)

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|---|---|
| Application Number | 15/646,822-Conf. #7727 |
| Filing Date | July 11, 2017 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | J. F. Railey |
| Attorney Docket Number | 30610/44556C1-R2 |

## U. S. PATENT DOCUMENTS

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## FOREIGN PATENT DOCUMENTS

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|---|---|---|---|---|---|---|
| /B.R.C/ | | WO-2009/046861-A1 | 04-16-2009 | Mondobiotech Lab AG | | |
| /B.R.C/ | | WO-03/079979-A2 | 10-02-2003 | Scios Inc | | |
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|---|---|
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| **Filing Date** | July 11, 2017 |
| **First Named Inventor** | Daniel J. Wendt |
| Art Unit | 3991 |
| Examiner Name | J. F. Railey |
| Attorney Docket Number | 30610/44556C1-R2 |

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| APPLICATION NO | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO | CONFIRMATION NO |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

TITLE OF INVENTION: Variants of C-Type Natriuretic Peptide

| APPLN: TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| Re-Issue | UNDISCOUNTED | $1,000.00 | | | $1,000.00 | 09/02/2020 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| Bruce R. Campell | 3991 | 514-012.400 |

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(A) NAME OF ASSIGNEE: BioMarin Pharmaceutical Inc.

(B) RESIDENCE: (CITY and STATE or COUNTRY): Novato, California

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Authorized Signature /Katherine L. Neville/ Date June 29, 2020

Typed or printed name Katherine L. Neville Registration No. 53,379

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
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| REISSUE ISSUE FEE | 1511 | 1 | 1000 | 1000 |

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| **Extension-of-Time:** | | | | |
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# Electronic Acknowledgement Receipt

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|---|---|
| **EFS ID:** | 39859488 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville/Tiffany Sevcik |
| **Filer Authorized By:** | Katherine Lynn Neville |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 29-JUN-2020 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 17:11:36 |
| **Application Type:** | Utility under 35 USC 111(a) |

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| 2 | Fee Worksheet (SB06) | fee-info.pdf | 30119<br>d3c3ea8ca26dc80caabd20b363706cbad9c 9b9e6 | no | 2 |
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| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | 3991 | 9740 | 30610/44556C1-R2 | 16 | 6 |

**CONFIRMATION NO. 7727**

**CORRECTED FILING RECEIPT**

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606
UNITED STATES OF AMERICA

Date Mailed: 07/06/2020

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**Inventor(s)**

Daniel J. Wendt, Novato, CA;
Shinong Long, Novato, CA;
Sianna Castillo, Novato, CA;
Christopher P. Price, Aachen, GERMANY;
Mika Aoyagi-Scharber, Novato, CA;
Michel Claude Vellard, Novato, CA;
Augustus O. Okhamafe, Concord, CA;

**Applicant(s)**

BioMarin Pharmaceutical Inc., Novato, CA, Assignee (with 37 CFR 1.172 Interest);

**Assignment For Published Patent Application**

BioMarin Pharmaceutical Inc., Novato, CA

**Power of Attorney:** The patent practitioners associated with Customer Number 90849

**Domestic Priority data as claimed by applicant**

This application is a CON of 14/604,262 01/23/2015 PAT RE46707
which is a REI of 13/466,672 05/08/2012 PAT 8598121
which is a CON of 12/784,117 05/20/2010 PAT 8198242
which claims benefit of 61/180,112 05/20/2009
and claims benefit of 61/254,563 10/23/2009

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/646,822**

**Projected Publication Date:** None, application is not eligible for pre-grant publication

**Non-Publication Request:** No

**Early Publication Request:** No

**Title**

Variants of C-Type Natriuretic Peptide

**Preliminary Class**

514

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

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Doc code: IDS
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PTO/SB/08a (03-15)
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

**( Not for submission under 37 CFR 1.99)**

| | |
|---|---|
| Application Number | 15646822 |
| Filing Date | 2017-07-11 |
| First Named Inventor | Daniel J. Wendt |
| Art Unit | 1629 |
| Examiner Name | N/A |
| Attorney Docket Number | 135576-010627/REI/CON |

**U.S.PATENTS** Remove

| Examiner Initial* | Cite No | Patent Number | Kind Code[1] | Issue Date | Name of Patentee or Applicant of cited Document | Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear |
|---|---|---|---|---|---|---|
| | 1 | 5252714 | | 1993-10-12 | Harris et al. | |
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
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| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

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|---|---|---|---|---|---|---|---|---|
| | 1 | 0466174 | EP | A1 | 1992-01-15 | Matsuo Hisayuki | | |
| | 2 | 0497368 | EP | A1 | 1992-08-05 | Suntory Ltd et al. | | |
| | 3 | 1743653 | EP | A1 | 2007-01-17 | Nakao Kazuwa | | |
| | 4 | 1994/20534 | WO | A1 | 1994-09-15 | Mayo Foundation | | |

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| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

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| | 5 | 2000/61631 | WO | | 2000-10-19 | AstraZeneca Ab et al. | | |
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| | 1 | AGOSTON et al., C-type natriuretic peptide regulates endochondral bone growth through p38 MAP kinase-dependent and -independent pathways, BMC Dev. Biol., 7:18 (2007). | |
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| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
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| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
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| | Application Number | 15646822 |
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| | Attorney Docket Number | 135576-010627/REI/CON |

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

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| | 38 | International Search Report and Written Opinion for corresponding International Application No. PCT/US2010/035586, mailing date November 24, 2010. | |

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
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| | Application Number | 15646822 |
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| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

| | | | |
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.R./

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

| | | |
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| /J.R./ | 50 | MELO et al., Chronic regulation of arterial blood pressure by ANP: role of endogenous vasoactive endothelial factors, Am. J. Physiol., 275:H1826-33 (1998). |

If you wish to add additional non-patent literature document citation information please click the Add button Add

**EXAMINER SIGNATURE**

| Examiner Signature | /JOHNNY F RAILEY II/ | Date Considered | 06/18/2018 |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | 15646822 |
| | Filing Date | 2017-07-11 |
| | First Named Inventor | Daniel J. Wendt |
| | Art Unit | 1629 |
| | Examiner Name | N/A |
| | Attorney Docket Number | 135576-010627/REI/CON |

**CERTIFICATION STATEMENT**

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

**OR**

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

**SIGNATURE**

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

| Signature | /Leslie Serunian/ | Date (YYYY-MM-DD) | 2017-10-11 |
|---|---|---|---|
| Name/Print | Leslie Serunian | Registration Number | 35,353 |

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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| APPLICATION NO. | ISSUE DATE | PATENT NO. | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 10/20/2020 | RE48267 | 30610/44556C1-R2 | 7727 |

90849 7590 09/30/2020

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

# ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

**Determination of Patent Term Extension or Adjustment under 35 U.S.C. 154 (b)**

A reissue patent is for "the unexpired part of the term of the original patent." See 35 U.S.C. 251. Accordingly, the above-identified reissue application is not eligible for Patent Term Extension or Adjustment under 35 U.S.C. 154(b).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Daniel J. Wendt, Novato, CA;
BioMarin Pharmaceutical Inc., Novato, CA, Assignee (with 37 CFR 1.172 Interest);
Shinong Long, Novato, CA;
Sianna Castillo, Novato, CA;
Christopher P. Price, Aachen, GERMANY;
Mika Aoyagi-Scharber, Novato, CA;
Michel Claude Vellard, Novato, CA;
Augustus O. Okhamafe, Concord, CA;

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Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No.: RE48,267

Issued: October 20, 2020

For: Variants Of C-Type Natriuretic Peptide

**REQUEST FOR CERTIFICATE OF CORRECTION**
**PURSUANT TO 37 C.F.R. § 1.322**

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted the following typographical errors, which should be corrected:

| In the Application | | | In the Patent | | Error |
|---|---|---|---|---|---|
| **On the Cover Page:** | | | | | |
| **Document** | | | **Field** | | |
| Issue Fee Payment (Pto-85b) Dated June 29, 2020, Page 1 | Under Attorney's Name, Lines 1-2 | | (74) | line 1 | PTO |
| **In the Claims:** | | | | | |
| **Amendment** | **Claim** | **Line** | **Column** | **Line** | |
| March 26, 2020, Page 9 | 27 | 37-38 | 276 | 42 | PTO |
| March 26, 2020, Page 10 | 31 | 6 | 276 | 63 | Applicant |
| March 26, 2020, Page 10 | 35 | 3 | 277 | 19 | Applicant |
| March 26, 2020, Page 11 | 40 | 6 | 277 | 53 | Applicant |
| March 26, 2020, Page 11 | 44 | 3 | 278 | 10 | Applicant |
| March 26, 2020, Page 12 | 49 | 6 | 278 | 45 | Applicant |

| | | | | | |
|---|---|---|---|---|---|
| March 26, 2020, Page 12 | 51 | 2 | 278 | 61 | Applicant |
| March 26, 2020, Page 13 | 54 | 6 | 279 | 20 | Applicant |
| March 26, 2020, Page 13 | 56 | 2 | 279 | 35 | Applicant |
| March 26, 2020, Page 13 | 59 | 6 | 279 | 61 | Applicant |
| March 26, 2020, Page 14 | 63 | 2 | 280 | 13 | Applicant |
| March 26, 2020, Page 14 | 68 | 6 | 280 | 42 | Applicant |
| March 26, 2020, Page 15 | 72 | 2 | 280 | 61 | Applicant |

The errors now sought to be corrected are inadvertent typographical errors the correction of which does not involve new matter or require reexamination.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

Please charge our Credit Card in the amount of $160.00 covering the fee set forth in 37 C.F.R. § 1.20(a). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

Dated: August 12, 2021

Respectfully submitted,

By: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicants

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# UNITED STATES PATENT AND TRADEMARK OFFICE
## CERTIFICATE OF CORRECTION



PATENT NO. : RE48,267 E

APPLICATION NO. : 15/646,822

ISSUE DATE : October 20, 2020

INVENTOR(S) : Daniel J. Wendt et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Cover Page:

At field (74), line 1, "Marshall Gerstein & Borun LLP" should be – Marshall, Gerstein & Borun LLP --.

In the Claims:

At column 276, line 42, "(SEQ ID NO. 36)" should be -- (SEQ ID NO: 36) --.

At column 276, line 63, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 277, line 19, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 277, line 53, "(Gly-CNP37)." should be -- (Gly-CNP37); --.

At column 278, line 10, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 278, line 45, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 278, line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 279, line 20, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
MARSHALL, GERSTEIN & BORUN LLP 1
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# UNITED STATES PATENT AND TRADEMARK OFFICE
## CERTIFICATE OF CORRECTION



PATENT NO. : RE48,267 E

APPLICATION NO. : 15/646,822

ISSUE DATE : October 20, 2020

INVENTOR(S) : Daniel J. Wendt et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At column 279, line 35, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 279, line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 280, line 13, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 280, line 42, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At column 280, line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
MARSHALL, GERSTEIN & BORUN LLP 2
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 15646822 |
| **Filing Date:** | 11-Jul-2017 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Filer:** | Katherine Lynn Neville/Valeria Rodriguez |
| **Attorney Docket Number:** | 30610/44556C1-R2 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |
| CERTIFICATE OF CORRECTION | 1811 | 1 | 160 | 160 |

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| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | | **Total in USD ($)** | **160** |

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 43496959 |
| **Application Number:** | 15646822 |
| **International Application Number:** | |
| **Confirmation Number:** | 7727 |
| **Title of Invention:** | Variants of C-Type Natriuretic Peptide |
| **First Named Inventor/Applicant Name:** | Daniel J. Wendt |
| **Customer Number:** | 90849 |
| **Filer:** | Katherine Lynn Neville |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 30610/44556C1-R2 |
| **Receipt Date:** | 12-AUG-2021 |
| **Filing Date:** | 11-JUL-2017 |
| **Time Stamp:** | 16:01:37 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $160 |
| RAM confirmation Number | E20218BG02124831 |
| Deposit Account | 132855 |
| Authorized User | Katherine Neville |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:<br>37 CFR 1.17 (Patent application and reexamination processing fees)<br>37 CFR 1.20 (Post Issuance fees) | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Request for Certificate of Correction | Request_for_Certificate_of_Correction.PDF | 21955<br>b03a271272b7ae8b4552e9d471a82b08484f113d | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Request for Certificate of Correction | Certificate_of_Correction.PDF | 19633<br>6d4a2ecd2db9fe35699af37e217175dcd5544456a | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Fee Worksheet (SB06) | fee-info.pdf | 37799<br>3e8b399126cbe91a6dc72f8a17f8e3f632f4dece | no | 2 |
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**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
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UNITED STATES PATENT AND TRADEMARK OFFICE

# CERTIFICATE OF CORRECTION



| | |
|---|---|
| PATENT NO. | : RE48,267 E |
| APPLICATION NO. | : 15/646822 |
| DATED | : October 20, 2020 |
| INVENTOR(S) | : Daniel J. Wendt et al. |

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

At item (74), Column 2, Line 1, "Marshall Gerstein & Borun LLP" should be -- Marshall, Gerstein & Borun LLP --.

In the Claims

At Column 276, Line 42, "(SEQ ID NO. 36)" should be -- (SEQ ID NO: 36) --.

At Column 276, Line 63, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 277, Line 19, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 277, Line 53, "(Gly-CNP37)." should be -- (Gly-CNP37); --.

At Column 278, Line 10, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 278, Line 45, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 278, Line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 279, Line 20, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 279, Line 35, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 279, Line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 280, Line 13, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

Signed and Sealed this
Seventh Day of September, 2021

Drew Hirshfeld
*Performing the Functions and Duties of the*
*Under Secretary of Commerce for Intellectual Property and*
*Director of the United States Patent and Trademark Office*

At Column 280, Line 42, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

At Column 280, Line 61, "(Gly-CNP37)" should be -- (Gly-CNP37); --.

# PATENT ASSIGNMENT DRAFT COVER SHEET

Electronic Version v1.1
Stylesheet Version v1.2

Assignment ID: 1249174

| | |
|---|---|
| **SUBMISSION TYPE:** | CORRECTIVEASSIGNMENT |
| **NATURE OF CONVEYANCE:** | Corrective Assignment to Correct the MISSING ASSIGNOR: Michel Claude Vellard ; Execution Date 07/26/2010 previously recorded on Reel 52962 Frame 859 .Assignor (s) hereby confirms the Assignment |

**CONVEYING PARTY DATA**

| Name | Execution Date |
|---|---|
| DANIEL J. WENDT | 10/04/2010 |
| SHINONG LONG | 07/08/2010 |
| SIANNA CASTILLO | 07/09/2010 |
| CHRISTOPHER P. PRICE | 07/12/2010 |
| MIKA AOYAGI-SCHARBER | 07/15/2010 |
| MICHAEL CLAUDE VELLARD | 07/26/2010 |
| AUGUSTUS O. OKHAMAFE | 06/16/2010 |

**RECEIVING PARTY DATA**

| | |
|---|---|
| **Company Name:** | BIOMARIN PHARMACEUTICAL INC. |
| **Street Address:** | 105 DIGITAL DRIVE |
| **City:** | NOVATO |
| **State/Country:** | CALIFORNIA , UNITED STATES |
| **Postal Code:** | 94949 |

**PROPERTIES NUMBERS Total: 1**

| **Property Type** | **Number** |
|---|---|
| **Application Number:** | 15646822 |

**CORRESPONDENCE DATA**

**Fax Number:** 3124740448

**Phone Number:** (312)474-6820

**Email:** docket@marshallip.com

*Correspondence will be sent to the email address first; if that is unsuccessful, it will be sent using a fax number, if provided; if that is unsuccessful, it will be sent via US Mail.*

**Correspondent Name:** Marshall Gerstein & Borun LLP

**Address Line 1:** 233 South Wacker Drive

**Address Line 2:** Suite 6300

**City** Chicago

| | |
|---|---|
| **State** | ILLINOIS |
| **Country/Postal:** | UNITED STATES, 60606 |
| **Docket number:** | 30610/44556C1 -R2 |

**Total Attachments : 1**

source =Corrective assignment .pdf

UNITED STATES PATENT AND TRADEMARK OFFICE
UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

JUNE 18, 2020

PTAS

MARSHALL, GERSTEIN & BORUN LLP
233 SOUTH WACKER DRIVE
SUITE 6300
CHICAGO, IL 60606

**506111001**

UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT RECORDATION BRANCH OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE ASSIGNMENT RECORDATION BRANCH AT 571-272-3350. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, MAIL STOP: ASSIGNMENT RECORDATION BRANCH, P.O. BOX 1450, ALEXANDRIA, VA 22313.

RECORDATION DATE: 06/17/2020

REEL/FRAME: 052962/0859
NUMBER OF PAGES: 8

BRIEF: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

DOCKET NUMBER: 30610/44556C1-R2

ASSIGNOR:
WENDT, DANIEL J. DOC DATE: 10/04/2010

ASSIGNOR:
LONG, SHINONG DOC DATE: 07/08/2010

ASSIGNOR:
CASTILLO, SIANNA DOC DATE: 07/09/2010

ASSIGNOR:
PRICE, CHRISTOPHER P. DOC DATE: 07/12/2010

ASSIGNOR:
AOYAGI-SCHARBER, MIKA DOC DATE: 07/15/2010

ASSIGNOR:
OKHAMAFE, AUGUSTUS O. DOC DATE: 06/16/2010

ASSIGNEE:
BIOMARIN PHARMACEUTICAL INC.
105 DIGITAL DRIVE
NOVATO, CALIFORNIA 94949

APPLICATION NUMBER: 15646822 FILING DATE: 07/11/2017
PATENT NUMBER: ISSUE DATE:
TITLE: VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

ASSIGNMENT RECORDATION BRANCH
PUBLIC RECORDS DIVISION

# PATENT ASSIGNMENT COVER SHEET

Electronic Version v1.1
Stylesheet Version v1.2

| | |
|---|---|
| **SUBMISSION TYPE:** | NEW ASSIGNMENT |
| **NATURE OF CONVEYANCE:** | ASSIGNMENT |

**CONVEYING PARTY DATA**

| Name | Execution Date |
|---|---|
| DANIEL J. WENDT | 10/04/2010 |
| SHINONG LONG | 07/08/2010 |
| SIANNA CASTILLO | 07/09/2010 |
| CHRISTOPHER P. PRICE | 07/12/2010 |
| MIKA AOYAGI-SCHARBER | 07/15/2010 |
| AUGUSTUS O. OKHAMAFE | 06/16/2010 |

**RECEIVING PARTY DATA**

| | |
|---|---|
| **Name:** | BIOMARIN PHARMACEUTICAL INC. |
| **Street Address:** | 105 DIGITAL DRIVE |
| **City:** | NOVATO |
| **State/Country:** | CALIFORNIA |
| **Postal Code:** | 94949 |

**PROPERTY NUMBERS Total: 1**

| Property Type | Number |
|---|---|
| **Application Number:** | 15646822 |

**CORRESPONDENCE DATA**

**Fax Number:** (312)474-0448
**Email:** anagelberg@marshallip.com
***Correspondence will be sent to the e-mail address first; if that is unsuccessful, it will be sent using a fax number, if provided; if that is unsuccessful, it will be sent via US Mail.***
**Correspondent Name:** MARSHALL, GERSTEIN & BORUN LLP
**Address Line 1:** 233 SOUTH WACKER DRIVE
**Address Line 2:** SUITE 6300
**Address Line 4:** CHICAGO, ILLINOIS 60606

| | |
|---|---|
| **ATTORNEY DOCKET NUMBER:** | 30610/44556C1-R2 |
| **NAME OF SUBMITTER:** | AUDREY NAGELBERG |
| **Signature:** | /Audrey Nagelberg/ |

| | |
|---|---|
| **Date:** | 06/17/2020 |

**Total Attachments: 6**
source=Assignment Recorded#page1.tif
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source=Assignment Recorded#page4.tif
source=Assignment Recorded#page5.tif
source=Assignment Recorded#page6.tif

**RECEIPT INFORMATION**

| | |
|---|---|
| **EPAS ID:** | PAT6157724 |
| **Receipt Date:** | 06/17/2020 |

## ASSIGNMENT

Serial No: 12/784,117 Filed: May 20, 2010
PCT No: PCT/US10/35586 Filed: May 20, 2010

Title: VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

This assignment is effective *nunc pro* tunc as of May 20, 2010. For $10.00 (Ten Dollars) and other good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, we, the undersigned, Daniel J. Wendt, a citizen of the United States, residing at 496 La Vista Road, Walnut Creek, CA 94588; Shinong Long, a citizen of the People's Republic of China, residing at 988 Cameron Circle, Milpitas, CA 95035; Sianna Castillo, a citizen of the United States, residing at 400 25th Avenue, #5, San Francisco, CA 94121; Christopher P. Price, a citizen of the United States, residing at 847 Sir Francis Drake, Kentfield, CA 94904; Mika Aoyagi-Scharber, a citizen of Japan, residing at 383 Durant Way, Mill Valley, CA 94941; Michel Claude Vellard, a citizen of France, residing at 191 Oak Drive, San Rafael, CA 94901 and Augustus O. Okhamafe, a citizen of the United States, residing at 5310 Crystyl Ranch Drive, Concord, CA 94521 , hereby assign to BIOMARIN PHARMACEUTICAL INC., 105 Digital Drive, Novato, California 94949 (hereinafter "Assignee"), its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in the above-identified application for letters patent of the United States, executed by the undersigned, and in said application and any and all other applications, both in the United States and in other countries, which the undersigned may file or be named as an inventor, either solely or jointly with others, on said invention or improvements, and in any and all letters patent of the United States and other countries, which may be obtained on any of said applications, and in any reissue or extension thereof.

The undersigned hereby authorize and request the Commissioner for Patents to issue said letters patent to said Assignee.

The undersigned warrant themselves to be the owners of the interest herein assigned and to have the right to make this assignment and further warrant that there are no outstanding prior assignments, licenses, or other rights in the interest herein assigned except in favor of Assignee.

For said consideration the undersigned hereby agree, upon the request and at the expense of said Assignee, its successors and assigns, to execute any and all applications on said invention or improvements, and any necessary oath or affidavit relating thereto, and any application for the reissue or extension of any letters patent that may be granted upon any and all of said applications, and any and all applications and other documents for letters patent in other countries on said invention or improvements, that said Assignee, its successors or assigns, may deem necessary or expedient, and for said consideration the undersigned further agree upon the request of said Assignee, its successors or assigns, in the event of any application or letters patent assigned herein becoming involved in Interference or Opposition, to cooperate to the best of the ability of the undersigned with said Assignee, its successors or assigns, in the matters of preparing and executing the preliminary statement and giving and producing evidence in support thereof, the undersigned hereby agreeing to perform, upon request, any and all affirmative acts to obtain said letters patent, both in the United States and in other countries, and vest all rights therein hereby conveyed in said Assignee, its successors and assigns, whereby said letters patent will be held and enjoyed by said Assignee, its successors and assigns, to the full end of the term for which said letters patent

MARSHALL, GERSTEIN & BORUN LLP, 233 S. Wacker Drive, Suite 6300, Sears Tower, Chicago, Illinois 60606-6357

may be granted as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment and sale had not been made.

10/4/10
Date

Daniel J. Wendt

State of California
County of Marin

On Oct 4, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Daniel J. Wendt, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769882
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Signature

07/08/2010
Date

Shinong Long

State of California
County of Marin

On July 8, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Shinong Long, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769882
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Signature

07/07/10 — Sianna Castillo
Date — Sianna Castillo

State of California
County of Marin

On July 7, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Sianna Castillo, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that ~~he/she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signature~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 25, 2011

Signature

(seal)

07/12/10
Date — Christopher P. Price

State of California
County of Marin

On July 12, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Christopher P. Price, who proved to me on the basis of satisfactory evidence to be the person~~(s)~~ whose name~~(s)~~ is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signatures~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 25, 2011

Signature

07/15/2010
Date

Mika Aoyagi-Scharber

State of California
County of Marin

On July 15, 2010 before me, Mary Ann J. Flores Notary Public, personally appeared Mika Aoyagi-Scharber, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that ~~he~~/she/~~they~~ executed the same in ~~his~~/her/~~their~~ authorized capacity(~~ies~~), and that by ~~his~~/her/~~their~~ signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) ~~acted~~, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Nov 29, 2011

Signature

(Seal)

7.26.10
Date

Michel Claude Vellard

State of California
County of Marin

On July 26, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Michel Claude Vellard, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that he/she/~~they~~ executed the same in his/~~her~~/~~their~~ authorized capacity(~~ies~~), and that by his/~~her~~/~~their~~ signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) ~~acted~~, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Nov 29, 2011

Signature

(Seal)

June 16, 2010
Date

Augustus O. Okhamafe
Augustus O. Okhamafe

State of California
County of Marin

On June 16, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Augustus O. Okhamafe, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signature~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Jan 23, 2011

Mary Ann J. Flores
Signature

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF CHRISTOPHER P. PRICE UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Christopher P. Price, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 27-Aug-2025

DocuSigned by:
Christopher Price
915C8C48B56846D...

Christopher P. Price

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF JOSHUA R. BLIESATH UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Joshua R. Bliesath, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and myself. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the application/patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 28-Aug-2025

DocuSigned by:
Joshua Bliesath
372D022FDA90440...

Joshua R. Bliesath

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF MIKA AOYAGI-SCHARBER UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Mika Aoyagi-Scharber, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 27-Aug-2025

DocuSigned by:
68EFEE1EB308487...
Mika Aoyagi-Scharber

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF AUGUSTUS O. OKHAMAFE UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Augustus O. Okhamafe, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 28-Aug-2025

DocuSigned by:
Augustus Okhamafe
EBFD6B5C37934C7...

Augustus O. Okhamafe

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF SEAN BELL UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Sean Bell, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors myself and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the application/patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 27-Aug-2025

DocuSigned by:
Sean Bell
1036E9A24C214CA...
Sean Bell

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**REQUEST FOR CORRECTION OF INVENTORSHIP UNDER 37 C.F.R. §1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Patentees hereby request correction of the inventorship of the above-identified patent to add as inventors Sean Bell and Joshua R. Bliesath. With that correction, the co-inventors of the patent will be as follows:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

In accordance with 37 C.F.R. § 1.324, please find attached to this request:

1. Statements agreeing to the correction of inventorship from the two (2) co-inventors to be added, namely, (i) Sean Bell and (ii) Joshua R. Bliesath;

2. A statement from each of the originally named co-inventors, Daniel J. Wendt, Shinong Long, Sianna Castillo, Christopher P. Price, Mika Aoyagi-Scharber, Michel Claude Vellard and Augustus O. Okhamafe, agreeing to the correction of inventorship to add the two co-inventors identified in paragraph 1 above; and

3. A statement from the assignee agreeing to the correction of inventorship to add the two co-inventors identified in paragraph 1 above.

A Corrected Application Data Sheet, marked to show the requested correction of inventorship, is submitted herewith.

This request is concurrently submitted with payment of the required fee under 37 C.F.R. § 1.20(b). The Director is hereby authorized to charge any deficiency of the fee due with submission of this petition to Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

Dated: August 29, 2025

Respectfully submitted,

By: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF SHINONG LONG UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Shinong Long, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 26-Aug-2025

Signed by:
Shinong Long
9C869C1ABA6E420...

Shinong Long

**Corrected Application Data Sheet**

**Inventor Information**

| | |
|---|---|
| Inventor Number:: | 1 |
| Given Name:: | Daniel |
| Middle Name:: | J. |
| Family Name:: | Wendt |
| City of Residence:: | Novato |
| State or Province of Residence:: | CA |
| Country of Residence:: | US |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

| | |
|---|---|
| Inventor Number:: | 2 |
| Given Name:: | Shinong |
| Family Name:: | Long |
| City of Residence:: | Novato |
| State or Province of Residence:: | CA |
| Country of Residence:: | US |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |

| | |
|---|---|
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

| | |
|---|---|
| Inventor Number:: | 3 |
| Given Name:: | Sianna |
| Family Name:: | Castillo |
| City of Residence:: | Novato |
| State or Province of Residence:: | CA |
| Country of Residence:: | US |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

| | |
|---|---|
| Inventor Number:: | 4 |
| Given Name:: | Christopher |
| Middle Name:: | P. |
| Family Name:: | Price |
| City of Residence:: | Munich |
| Country of Residence:: | Germany |
| Street of mailing address:: | Rugendosstrasse 1 |

| | |
|---|---|
| City of mailing address:: | Munich |
| Country of mailing address:: | Germany |
| Postal or Zip Code of mailing address:: | 81479 |

| | |
|---|---|
| Inventor Number:: | 5 |
| Given Name:: | Mika |
| Family Name:: | Aoyagi-Scharber |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |
| State or Province of mailing address:: | California |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

| | |
|---|---|
| Inventor Number:: | 6 |
| Given Name:: | Michel |
| Middle Name:: | Claude |
| Family Name:: | Vellard |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |
| State or Province of mailing address:: | California |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

| | |
|---|---|
| Inventor Number:: | 7 |
| Given Name:: | Augustus |
| Middle Name:: | O. |
| Family Name:: | Okhamafe |
| City of Residence:: | Concord |
| State or Province of Residence:: | California |
| Country of Residence:: | US |
| Street of mailing address:: | 5310 ~~Crystal~~ Crystyl Ranch Drive |
| City of mailing address:: | Concord |
| State or Province of mailing address:: | California |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94521 |

| | |
|---|---|
| Inventor Number:: | 8 |
| Given Name:: | Sean |
| Family Name:: | Bell |
| City of Residence:: | San Rafael |
| State or Province of Residence:: | CA |
| Country of Residence:: | US |
| Street of mailing address:: | 19 Ridge Ave. |
| City of mailing address:: | San Rafael |
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |

| | |
|---|---|
| Postal or Zip Code of mailing address:: | 94901 |

| | |
|---|---|
| Inventor Number:: | 9 |
| Given Name:: | Joshua |
| Middle Name:: | R. |
| Family Name:: | Bliesath |
| City of Residence:: | San Diego |
| State or Province of Residence:: | CA |
| Country of Residence:: | US |
| Street of mailing address:: | 1306 Scenic Drive |
| City of mailing address:: | Escondido |
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 92029 |

**Correspondence Information**

| | |
|---|---|
| Correspondence Customer Number:: | ~~35893~~ 90849 |

**Application Information**

| | |
|---|---|
| Application Type:: | Continuation Reissue |
| Subject Matter:: | Utility |
| CD-ROM or CD-R?:: | None |
| Sequence submission?:: | Paper |

| | |
|---|---|
| Computer Readable Form (CRF)?:: | Yes |
| Title:: | Variants of C-Type Natriuretic Peptide |
| Attorney Docket Number:: | 30610/44556C1-R2 ~~135576.010627/REI/CON~~ |
| Request for Early Publication?:: | No |
| Request for Non-Publication?:: | No |
| Total Drawing Sheets:: | 57 |
| Small Entity?:: | No |
| Petition included?:: | No |
| Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2:: | No |
| This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013:: | No |

**Representative Information**

| | |
|---|---|
| Representative Customer Number:: | ~~35893~~ 90849 |

**Domestic Priority Information**

| Application:: | Continuity Type:: | Parent Application:: | Parent Filing Date:: | Status:: |
|---|---|---|---|---|
| This Application | Continuation of | 14/604,262 | 01/23/2015 | Patented |

| | | | | |
|---|---|---|---|---|
| And This Application | Reissue of | 14/604,262 | 01/23/2015 | Patented |
| 14/604,262 | Reissue of | 13/466,672 | 5/08/2012 | Patented |
| 13/466,672 | Continuation of | 12/784,117 | 5/20/2010 | Patented |
| 12/784,117 | An application claiming the benefit under 35 USC 119(e) | 61/254,563 | 10/23/2009 | Expired |
| 12/784,117 | An application claiming the benefit under 35 USC 119(e) | 61/180,112 | 05/20/2009 | Expired |

**Foreign Priority Information**

| Country:: | Application number:: | Filing Date:: | Priority Claimed:: | DAS Access:: |
|---|---|---|---|---|
| | | | | |

**Applicant Information**

| | |
|---|---|
| Applicant Number:: | 1 |
| Applicant Type:: | Assignee |
| Organization Name:: | BIOMARIN PHARMACEUTICAL INC. |
| Street of mailing address:: | 105 Digital Drive |
| City of mailing address:: | Novato |
| State or Province of mailing address:: | CA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 94949 |

## Signature:

**NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

| | | | |
|---|---|---|---|
| **Signature** | /Katherine L. Neville/ | Date (YYYY-MM-DD) | 2025-08-29 |
| Name | Katherine L. Neville | Registration Number | 53,379 |

PTO/AIA/96 (11-23)
Approved for use through 11/30/2027. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# STATEMENT UNDER 37 CFR 3.73(c)

First Named Inventor/Patent Owner: Michel Claude Vellard, Sean Bell, Joshua R. Bliesath, and BIOMARIN PHARMACEUTICAL INC.

Application No./Patent No.: RE48,267 Filed/Issue Date: October 20, 2020

Titled: Variants of C-Type Natriuretic Peptide

BIOMARIN PHARMACEUTICAL INC. , a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. [x] The assignee of the entire right, title, and interest.

2. [ ] An assignee of less than the entire right, title, and interest (check applicable box):
   - [ ] The extent (by percentage) of its ownership interest is _____ %. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.
   - [ ] There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. [ ] The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. [ ] The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. [ ] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached*.

B. [x] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Okhamafe, Augustus O. To: BIOMARIN PHARMACEUTICAL INC.
   The document was recorded in the United States Patent and Trademark Office at
   Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

2. From: Shinong Long To: BIOMARIN PHARMACEUTICAL INC.
   The document was recorded in the United States Patent and Trademark Office at
   Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

PTO/AIA/96 (11-23)
Approved for use through 11/30/2027. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# STATEMENT UNDER 37 CFR 3.73(c)

3. From: Castillo, Sianna To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

4. From: Price, Christopher P. To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

5. From: Aoyagi-Scharber, Mika To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

6. From: Daniel J. Wendt To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel 052962 , Frame 0859 , or for which a copy thereof is attached*.

[X] Additional documents in the chain of title are listed on a supplemental sheet(s).

[X] *As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

| /Katherine L. Neville/ | August 29, 2025 |
|---|---|
| Signature | Date |
| Katherine L. Neville | 53,379 |
| Printed or Typed Name | Title or Registration Number |

## STATEMENT UNDER 37 CFR 3.73(c) - Supplemental Sheet

Continuation of chain of title from the inventor(s) to the current assignee:

7. From: Sean Bell To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

8. From: Joshua R. Bliesath To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

9. From: Michel Claude Vellard To: BIOMARIN PHARMACEUTICAL INC.
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

10. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

11. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

12. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

13. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

14. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

15. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

16. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____ , Frame _____ , or for which a copy thereof is attached.

Docket Number (Optional)
30610/44556C1-R2

## REISSUE APPLICATION DECLARATION BY THE INVENTOR

I hereby declare that:
Each inventor's residence and mailing address are stated below next to their name.
I believe I am the original inventor or an original joint inventor of the subject matter which is described and claimed in patent number 8,598,121, granted December 3, 2013 and for which a reissue patent is sought on the invention titled

Variants of C-Type Natriuretic Peptide

the specification of which

☐ is attached hereto.

☒ was filed on July 11, 2017 as reissue application number 15/646,822 .

☐ As amended on _____ (if applicable)

The above-identified application was made or authorized to be made by me.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein. I acknowledge the duty to disclose information that is material to patentability as defined in 37 CFR 1.56.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by

I believe the original patent to be wholly or partly inoperative or invalid, for the reasons described below. (Check all boxes that apply.)

☐ by reason of a defective specification or drawing.

☒ by reason of the patentee claiming more or less than he had the right to claim in the patent.

☐ by reason of other errors.

At least one error upon which reissue is based is described below. If the reissue is a broadening reissue, a claim that the application seeks to broaden must be identified:

Patentee claimed less than Patentee had a right to claim in that claim 1 of U.S. Patent No. 8,598,121 is broadened by the presentation of new claim 15 in the Preliminary Amendment filed in the subject continuation reissue application, USSN 15/646,822, on July 11, 2017. New claim 15, directed to a macromolecule capable of releasing a CNP variant, is broadened to add additional, alternative amino acid sequences of the CNP variants of the macromolecule, such as, for example, the CNP variants set forth in SEQ ID NOs: 145-178.

PTO/AIA/05 (06-12)
Approved for use through 08/31/2013. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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(REISSUE APPLICATION DECLARATION BY THE INVENTOR, page 2)

Docket Number (Optional): 30610/44556C1-R2

Note: To appoint a power of attorney, use form PTO/AIA/81.

Correspondence Address: Direct all communications about the application to:

[x] The address associated with Customer Number: 90849

***OR***

[ ] Firm or Individual Name

| | | | | | |
|---|---|---|---|---|---|
| Address | | | | | |
| City | | State | | Zip | |
| Country | | | | | |
| Telephone | | Email | | | |

## WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

Legal name of sole or first inventor (*E.g.*, Given Name (first and middle (if any) and Family Name or Surname)

Sean Bell

| | | | |
|---|---|---|---|
| Inventor's Signature | | | Date (Optional) |
| Residence: City<br>San Rafael | State<br>CA | Country<br>United States of America | |
| Mailing Address<br>19 Ridge Ave. | | | |
| City<br>San Rafael | State<br>CA | Zip<br>94901 | Country<br>United States of America |

[x] Additional joint inventors are named on the 1 supplemental sheet(s) PTO/AIA/10 attached hereto.

PTO/AIA/10 (06-12)
Approved for use through 11/30/2027. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

| SUPPLEMENTAL SHEET FOR DECLARATION | ADDITIONAL INVENTOR(S)<br>Supplemental Sheet (for PTO/AIA/08,09)<br>Page 1 of 1 |
|---|---|

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)
Joshua R. Bliesath

| Inventor's Signature: DocuSigned by: Joshua Bliesath 372D022FDA90440... | | | Date (Optional) |
|---|---|---|---|
| San Diego<br>Residence: City | CA<br>State | United States of America<br>Country | |

1306 Scenic Drive
Mailing Address

| Escondido<br>City | CA<br>State | 92029<br>Zip | United States of America<br>Country |
|---|---|---|---|

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

| Inventor's Signature | | | Date (Optional) |
|---|---|---|---|
| Residence: City | State | Country | |

Mailing Address

| City | State | Zip | Country |
|---|---|---|---|

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

| Inventor's Signature | | | Date (Optional) |
|---|---|---|---|
| Residence: City | State | Country | |

Mailing Address

| City | State | Zip | Country |
|---|---|---|---|

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No.: RE48,267

Issued: October 20, 2020

For: Variants of C-Type Natriuretic Peptide

**REQUEST FOR CERTIFICATE OF CORRECTION**
**PURSUANT TO 37 C.F.R. § 1.323**

Commissioner for Patents
Office of Data Management Attention: Certificates of Correction Branch
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Upon reviewing the above-identified patent, Patentee noted an error in the application and patent:

| In the Application | | In the Patent | | Error |
|---|---|---|---|---|
| | | On the Cover Page: | | |
| **Document** | **Line** | **Field** | | |
| Supplemental Application Data Sheet dated March 7, 2018 | After Inventor No. 7:: | (72) | Line 1 | Applicant |
| | | | | |

The error to be corrected is to correct the listing of inventors. The correct list of inventors is:

Daniel J. Wendt; Shinong Long; Sianna Castillo; Christopher P. Price; Mika Aoyagi-Scharber; Michel Claude Vellard; Augustus O. Okhamafe; Sean Bell; Joshua R. Bliesath.

The error now sought to be corrected is a mistake of minor character that does not materially affect the scope or meaning of the patent and does not involve new matter or require reexamination.

Transmitted herewith is a proposed Certificate of Correction affecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

Please charge our Credit Card in the amount of $172.00 covering the fee set forth in 37 C.F.R. § 1.20(b). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 13-2855, under Order No. 30610/44556C1-R2.

Dated: August 29, 2025

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 — Issued: October 20, 2020
U.S. Serial No. 15/646,822 — Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF SIANNA CASTILLO UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Sianna Castillo, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 28-Aug-2025

DocuSigned by:
Sianna Castillo
B76A63B6350E469...
Sianna Castillo

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267
U.S. Serial No. 15/646,822

Issued: October 20, 2020
Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**CONSENT OF ASSIGNEE FOR**
**CORRECTION OF INVENTORSHIP UNDER 37 C.F.R. 1.324 (b)(2)**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The undersigned, as an authorized representative of BioMarin Pharmaceutical Inc., hereby states that:

1. BioMarin Pharmaceutical Inc. is the assignee and owner of the entire right, title and interest to the above-referenced patent;

2. BioMarin Pharmaceutical Inc. consents to the addition of Sean Bell and Joshua R. Bliesath as named inventors of the invention claimed in the above-identified patent; and

3. A statement under 37 CFR 3.73(c) specifying where documentary evidence of a chain of title from the original owner to the assignee is included herewith.

BioMarin Pharmaceutical Inc.

E2DD63391BD0497...
DocuSigned By: Luisa Bigornia

Dated: 29-Aug-2025

Name: Luisa Bigornia
Title: VP, Intellectual Property

docusign

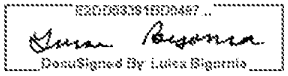
## Certificate Of Completion

Envelope Id: 70BC0C95-B5E3-4256-A3E3-65731F4ED07D
Status: Completed
Subject: Complete with Docusign: Assignee BMRN Consent for Correction of Inventorship Under 37 C.F.R. 1....
Source Envelope:
Document Pages: 1
Signatures: 1
Certificate Pages: 1
Initials: 0
AutoNav: Enabled
EnvelopeId Stamping: Disabled
Time Zone: (UTC-08:00) Pacific Time (US & Canada)

Envelope Originator:
Rhoda Jagow
105 Digital Drive
Novato, CA 94949
rjagow@bmrn.com
IP Address: 208.127.92.234

## Record Tracking

Status: Original
8/29/2025 8:56:59 AM
Holder: Rhoda Jagow
rjagow@bmrn.com
Location: DocuSign

| Signer Events | Signature | Timestamp |
|---|---|---|
| Luisa Bigornia<br>lbigornia@bmrn.com<br>Vice President, Intellectual Property<br>BioMarin Pharmaceutical Inc.<br>Security Level: Email, Account Authentication (None) |  <br>DocuSigned By: Luisa Bigornia<br>Signature Adoption: Uploaded Signature Image<br>Using IP Address: 208.127.92.234 | Sent: 8/29/2025 8:58:22 AM<br>Viewed: 8/29/2025 8:59:10 AM<br>Signed: 8/29/2025 8:59:18 AM |

Electronic Record and Signature Disclosure:
Not Offered via Docusign

| In Person Signer Events | Signature | Timestamp |
|---|---|---|

| Editor Delivery Events | Status | Timestamp |
|---|---|---|

| Agent Delivery Events | Status | Timestamp |
|---|---|---|

| Intermediary Delivery Events | Status | Timestamp |
|---|---|---|

| Certified Delivery Events | Status | Timestamp |
|---|---|---|

| Carbon Copy Events | Status | Timestamp |
|---|---|---|

| Witness Events | Signature | Timestamp |
|---|---|---|

| Notary Events | Signature | Timestamp |
|---|---|---|

| Envelope Summary Events | Status | Timestamps |
|---|---|---|
| Envelope Sent | Hashed/Encrypted | 8/29/2025 8:58:22 AM |
| Certified Delivered | Security Checked | 8/29/2025 8:59:10 AM |
| Signing Complete | Security Checked | 8/29/2025 8:59:18 AM |
| Completed | Security Checked | 8/29/2025 8:59:18 AM |

| Payment Events | Status | Timestamps |
|---|---|---|

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

# ELECTRONIC PAYMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| **15/646,822** | **08/29/2025 06:06:13 PM Z ET** | **30610/44556C1-R2** |

## Title of Invention

Variants of C-Type Natriuretic Peptide

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - Reissue | PATENT # | RE48267 |
| CONFIRMATION # | 7727 | FILED BY | Cassandra Brannon |
| PATENT CENTER # | 72085692 | AUTHORIZED BY | Katherine Neville |
| CUSTOMER # | 90849 | FILING DATE | 07/11/2017 |
| CORRESPONDENCE ADDRESS | - | FIRST NAMED INVENTOR | Daniel J. Wendt |

## Payment Information

| PAYMENT METHOD | PAYMENT TRANSACTION ID | PAYMENT AUTHORIZED BY |
|---|---|---|
| **CARD / 2617** | **E20258SI06435606** | **Cassandra Brannon** |

| FEE CODE | DESCRIPTION | ITEM PRICE($) | QUANTITY | ITEM TOTAL($) |
|---|---|---|---|---|
| 1816 | PROCESSING FEE FOR CORRECTING INVENTORSHIP IN A PATENT | 172.00 | 1 | 172.00 |
| | | | **TOTAL AMOUNT:** | **$172.00** |

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage

submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF MICHEL CLAUDE VELLARD UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Michel Claude Vellard, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 26-août-2025

Signé par :
171ADDDE7E3D4D4...

Michel Claude Vellard

**PATENT ASSIGNMENT COVER SHEET**

Assignment ID: 1251723

Electronic Version v1.1
Stylesheet Version v1.2

| | |
|---|---|
| **SUBMISSION TYPE:** | NEW ASSIGNMENT |
| **NATURE OF CONVEYANCE:** | ASSIGNMENT |

**CONVEYING PARTY DATA**

| **Name** | **Execution Date** |
|---|---|
| Sean Bell | 07/27/2010 |
| Joshua R. Bliesath | 07/21/2010 |

**RECEIVING PARTY DATA**

| | |
|---|---|
| **Name:** | BioMarin Pharmaceut ical Inc. |
| **Street Address:** | 105 Digital Drive |
| **City:** | Novato |
| **State/Country:** | CALIFORNIA UNITED STATES |
| **Postal Code:** | 94949 |

**PROPERTY NUMBERS Total:1**

| **Property Type** | **Number** |
|---|---|
| **Application Number:** | 15646822 |

**CORRESPONDENCE DATA**

**Phone:** 3124746300
**Email:** cbrannon@marshallip.com
***Correspondence will be sent to the e-mail address first; if that is unsuccessful, it will be sent using a fax number, if provided; if that is unsuccessful, it will be sent via US Mail.***
**Correspondent Name:** Marshall, Gerstein & Borun LLP
**Address Line 1:** 233 South Wacker Drive
**Address Line 2:** 6300 Willis Tower
**Address Line 4:** Chicago , ILLINOIS 60606

| | |
|---|---|
| **ATTORNEY DOCKET NUMBER:** | 30610/44556C1-R2 |
| **NAME OF SUBMITTER:** | Cassandra Brannon |
| **Signature:** | /Cassandra Brannon/ |

| | |
|---|---|
| **Date:** | 08/29/2025 |

**TOTAL ATTACHMENTS: 1**
source= Bell Bliesath assignment-pat-25094-534.pdf

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 — Issued: October 20, 2020
U.S. Serial No. 15/646,822 — Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

## AFFIDAVIT OF JOSHUA R. BLIESATH

I, Joshua R. Bliesath, hereby affirm:

I executed an assignment of my entire right, title, and interest of the invention or improvements disclosed in the U.S. Patent Application No. 15/646,822 to BioMarin Pharmaceutical Inc. on July 21, 2010.

I declare under penalty of perjury that the foregoing is true and accurate to the best of my knowledge.

Dated: 28-Aug-2025

DocuSigned by:
Joshua Bliesath
372D022FDA90440...

Joshua R. Bliesath

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 — Issued: October 20, 2020
U.S. Serial No. 15/646,822 — Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

## AFFIDAVIT OF SEAN BELL

I, Sean Bell, hereby affirm:

I executed an assignment of my entire right, title, and interest of the invention or improvements disclosed in the U.S. Patent Application No. 15/646,822 to BioMarin Pharmaceutical Inc. on July 27, 2010.

I declare under penalty of perjury that the foregoing is true and accurate to the best of my knowledge.

Dated: 28-Aug-2025

DocuSigned by:
Sean Bell
1036E9A24C214CA...

Sean Bell

**ASSIGNMENT**

Serial No: 12/744,079

Filed: May 21, 2010

Title: VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

This assignment is effective *nunc pro* tunc as May 21, 2010. For $10.00 (Ten Dollars) and other good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, we, the undersigned, Dan J. Wendt, a citizen of the United States, residing at 496 La Vista Road, Walnut Creek, CA 94588; Mika Aoyagi-Scharber, a citizen of Japan, residing at 383 Durant Way, Mill Valley, CA 94941; Sean Bell, a citizen of the United States, residing at 19 Ridge Ave., San Rafael, CA 94901; Dong Wei, a citizen of the United States, residing at 1543 22nd Ave., San Francisco, CA 94122; Joshua R Bliesath, a citizen of the United States, residing at 1306 Scenic Drive, Escondido, CA 92029 and Emil D. Kakkis, a citizen of the United States, residing at 2512 Laguna Vista Drive, Novato, CA 94949, hereby assign to BIOMARIN PHARMACEUTICAL INC., 105 Digital Drive, Novato, California 94949 (hereinafter "Assignee"), its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in the above-identified application for letters patent of the United States, executed by the undersigned, and in said application and any and all other applications, both in the United States and in other countries, which the undersigned may file or be named as an inventor, either solely or jointly with others, on said invention or improvements, and in any and all letters patent of the United States and other countries, which may be obtained on any of said applications, and in any reissue or extension thereof.

The undersigned hereby authorize and request the Commissioner for Patents to issue said letters patent to said Assignee.

The undersigned warrant themselves to be the owners of the interest herein assigned and to have the right to make this assignment and further warrant that there are no outstanding prior assignments, licenses, or other rights in the interest herein assigned except in favor of Assignee.

For said consideration the undersigned hereby agree, upon the request and at the expense of said Assignee, its successors and assigns, to execute any and all applications on said invention or improvements, and any necessary oath or affidavit relating thereto, and any application for the reissue or extension of any letters patent that may be granted upon any and all of said applications, and any and all applications and other documents for letters patent in other countries on said invention or improvements, that said Assignee, its successors or assigns, may deem necessary or expedient, and for said consideration the undersigned further agree upon the request of said Assignee, its successors or assigns, in the event of any application or letters patent assigned herein becoming involved in Interference or Opposition, to cooperate to the best of the ability of the undersigned with said Assignee, its successors or assigns, in the matters of preparing and executing the preliminary statement and giving and producing evidence in support thereof, the undersigned hereby agreeing to perform, upon request, any and all affirmative acts to obtain said letters patent, both in the United States and in other countries, and vest all rights therein hereby conveyed in said Assignee, its successors and assigns, whereby said letters patent will be held and enjoyed by said Assignee, its successors and assigns, to the full end of the term for which said letters patent

MARSHALL, GERSTEIN & BORUN LLP, 233 S. Wacker Drive, Suite 6300, Sears Tower, Chicago, Illinois 60606-6357

may be granted as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment and sale had not been made.

10/4/10 — Dan J. Wendt
Date

State of California
County of Marin

On Oct 4, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Dan J. Wendt, who proved to me on the basis of satisfactory evidence to be the person~~(s)~~ whose name~~(s)~~ is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signatures~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Mary Ann J. Flores
Signature

07/15/2010 — Mika Aoyagi-Scharber
Date

State of California
County of Marin

On July 15, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Mika Aoyagi-Scharber, who proved to me on the basis of satisfactory evidence to be the person~~(s)~~ whose name~~(s)~~ is/are subscribed to the within instrument and acknowledged to me that ~~he~~/she/~~they~~ executed the same in ~~his~~/her/~~their~~ authorized capacity~~(ies)~~, and that by ~~his~~/her/~~their~~ signatures~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Mary Ann J. Flores
Signature

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

07/27/10
Date

Sean Bell

State of California
County of Marin

On July 27, 2010 before me, Mary Ann J. Flores, Notary Public, personally appeared Sean Bell, who proved to me on the basis of satisfactory evidence to be the person~~(s)~~ whose name~~(s)~~ is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signatures~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Signature

Date

Dong Wei

State of
County of

On _____ before me, _____, Notary Public, personally appeared Dong Wei, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature

Date Sean Bell

State of _____
County of _____

On _____ before me, _____, Notary Public, personally appeared Sean Bell, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature

(seal)

7/21/10 Dong Wei
Date

State of California
County of San Mateo

On 7-21-10 before me, GOPAL C PATEL, Notary Public, personally appeared Dong Wei, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/th~~ey executed the same in his/~~her/thei~~r authorized capacity(ies), and that by his/~~her/thei~~r signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

GOPAL C. PATEL
COMM. # 1892056
NOTARY PUBLIC • CALIFORNIA
SAN MATEO COUNTY
My Comm Expires JUNE 07, 2014
JAS1 JAS1

Signature

(seal)

7-21-10
Date

Joshua R. Bliesath

State of CALIFORNIA
County of SAN DIEGO

On JULY 21, 2010 before me, IRENE ALICEA, Notary Public, personally appeared Joshua R. Bliesath, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity(~~ies~~), and that by his/~~her/their~~ signatures(~~s~~) on the instrument the person(~~s~~), or the entity upon behalf of which the person(~~s~~) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

IRENE ALICEA
Commission # 1866591
Notary Public - California
San Diego County
My Comm. Expires Oct 18, 2013

Signature

(seal)

Date

Emil D. Kakkis

State of
County of

On _____ before me, _____, Notary Public, personally appeared Emil D. Kakkis, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature

(seal)

Date

Joshua R. Bliesath

State of _____
County of _____

On _____ before me, _____, Notary Public, personally appeared Joshua R. Bliesath, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature

(seal)

July 19, 2010
Date

Emil D. Kakkis

State of California
County of Marin

On July 19, 2010 before me, Mary Ann Flores, Notary Public, personally appeared Emil D. Kakkis, who proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signatures(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

MARY ANN J. FLORES
Commission # 1769582
Notary Public - California
Marin County
My Comm. Expires Sep 23, 2011

Signature

(seal)

Docket Number (Optional)
30610/44556C1-R2

## REISSUE APPLICATION DECLARATION BY THE INVENTOR

I hereby declare that:
Each inventor's residence and mailing address are stated below next to their name.
I believe I am the original inventor or an original joint inventor of the subject matter which is described and claimed in patent number 8,598,121, granted December 3, 2013 and for which a reissue patent is sought on the invention titled

Variants of C-Type Natriuretic Peptide

the specification of which

☐ is attached hereto.

☒ was filed on July 11, 2017 as reissue application number 15/646,822 .

☐ As amended on _____ (if applicable)

The above-identified application was made or authorized to be made by me.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein. I acknowledge the duty to disclose information that is material to patentability as defined in 37 CFR 1.56.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by

I believe the original patent to be wholly or partly inoperative or invalid, for the reasons described below. (Check all boxes that apply.)

☐ by reason of a defective specification or drawing.

☒ by reason of the patentee claiming more or less than he had the right to claim in the patent.

☐ by reason of other errors.

At least one error upon which reissue is based is described below. If the reissue is a broadening reissue, a claim that the application seeks to broaden must be identified:

Patentee claimed less than Patentee had a right to claim in that claim 1 of U.S. Patent No. 8,598,121 is broadened by the presentation of new claim 15 in the Preliminary Amendment filed in the subject continuation reissue application, USSN 15/646,822, on July 11, 2017. New claim 15, directed to a macromolecule capable of releasing a CNP variant, is broadened to add additional, alternative amino acid sequences of the CNP variants of the macromolecule, such as, for example, the CNP variants set forth in SEQ ID NOs: 145-178.

PTO/AIA/05 (06-12)
Approved for use through 08/31/2013. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

| (REISSUE APPLICATION DECLARATION BY THE INVENTOR, page 2) | Docket Number (Optional) 30610/44556C1-R2 |
|---|---|

Note: To appoint a power of attorney, use form PTO/AIA/81.

Correspondence Address: Direct all communications about the application to:

[x] The address associated with Customer Number: 90849

***OR***

| [ ] Firm or Individual Name | | | | | |
|---|---|---|---|---|---|
| Address | | | | | |
| City | | State | | Zip | |
| Country | | | | | |
| Telephone | | Email | | | |

## WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

Legal name of sole or first inventor (*E.g.*, Given Name (first and middle (if any) and Family Name or Surname)
Sean Bell

| Inventor's Signature | DocuSigned by: Sean Bell 1036E9A240214CA... | Date (Optional) |
|---|---|---|
| Residence: City | State | Country |
| San Rafael | CA | United States of America |

| Mailing Address | 19 Ridge Ave. | | |
|---|---|---|---|
| City | State | Zip | Country |
| San Rafael | CA | 94901 | United States of America |

[x] Additional joint inventors are named on the 1 supplemental sheet(s) PTO/AIA/10 attached hereto.

PTO/AIA/10 (06-12)
Approved for use through 11/30/2027. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

| SUPPLEMENTAL SHEET FOR DECLARATION | ADDITIONAL INVENTOR(S)<br>Supplemental Sheet (for PTO/AIA/08,09)<br>Page 1 of 1 |
|---|---|

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)
Joshua R. Bliesath

| Inventor's Signature | Date (Optional) |
|---|---|

| Residence: City | State | Country |
|---|---|---|
| San Diego | CA | United States of America |

Mailing Address: 1306 Scenic Drive

| City | State | Zip | Country |
|---|---|---|---|
| Escondido | CA | 92029 | United States of America |

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

| Inventor's Signature | Date (Optional) |
|---|---|

| Residence: City | State | Country |
|---|---|---|

Mailing Address

| City | State | Zip | Country |
|---|---|---|---|

**Legal Name of Additional Joint Inventor, if any:**
(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

| Inventor's Signature | Date (Optional) |
|---|---|

| Residence: City | State | Country |
|---|---|---|

Mailing Address

| City | State | Zip | Country |
|---|---|---|---|

PTO/SB/44 (09-07)
Approved for use through 03/31/2026. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.
(Also Form PTO-1050)

# UNITED STATES PATENT AND TRADEMARK OFFICE
## CERTIFICATE OF CORRECTION



| | | |
|---|---|---|
| PATENT NO. | : | RE48,267 |
| APPLICATION NO. | : | 15/646,822 |
| ISSUE DATE | : | October 20, 2020 |
| INVENTOR(S) | : | Daniel J. Wendt et al. |

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Cover Page

At field (72), Inventors: "**Daniel J. Wendt**, Novato, CA (US); **Shinong Long**, Novato, CA (US); **Sianna Castillo**, Novato, CA (US); **Christopher P. Price**, Munich (DE); **Mika Aoyagi-Scharber**, Novato, CA (US); **Michel Claude Vellard**, Novato, CA (US); **Augustus O. Okhamafe**, Concord, CA (US)" should be -- **Daniel J. Wendt**, Novato, CA (US); **Shinong Long**, Novato, CA (US); **Sianna Castillo**, Novato, CA (US); **Christopher P. Price**, Munich (DE); **Mika Aoyagi-Scharber**, Novato, CA (US), **Michel Claude Vellard**, Novato, CA (US); **Augustus O. Okhamafe**, Concord, CA (US); **Sean Bell**, San Rafael, CA (US); **Joshua R. Bliesath**, San Diego, CA (US) --.

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
Katherine L. Neville
MARSHALL, GERSTEIN & BORUN LLP 1
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

# ELECTRONIC ACKNOWLEDGEMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| **15/646,822** | **08/29/2025 06:06:13 PM Z ET** | **30610/44556C1-R2** |

## Title of Invention

Variants of C-Type Natriuretic Peptide

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - Reissue | PATENT # | RE48267 |
| CONFIRMATION # | 7727 | FILED BY | Cassandra Brannon |
| PATENT CENTER # | 72085692 | FILING DATE | 07/11/2017 |
| CUSTOMER # | 90849 | FIRST NAMED INVENTOR | Daniel J. Wendt |
| CORRESPONDENCE ADDRESS | - | AUTHORIZED BY | Katherine Neville |

## Documents

**TOTAL DOCUMENTS: 19**

| DOCUMENT | PAGES | DESCRIPTION | SIZE (KB) |
|---|---|---|---|
| Application Data Sheet.pdf | 8 | Application Data Sheet | 60 KB |
| Warning: This is not a USPTO supplied ADS fillable form. Data in the form cannot be automatically loaded to other USPTO systems. | | | |
| Certificate of Correction.pdf | 1 | Request for Certificate of Correction | 20 KB |
| Joshua Bliesath Declaration_for_Reissue_Application_by_Inventor_PTO_AIA-05 _ 4929-6424-585.pdf | 3 | Reissue Oath or Declaration | 248 KB |
| Warning: The PDF file has been signed with a digital signature and the legal effect of the document will be based on the contents of the file not the digital signature. | | | |
| Sean Bell Declaration for Reissue Application by | 3 | Reissue Oath or Declaration | 239 KB |

| | | | |
|---|---|---|---|
| Inventor PTO AIA-05 4929-6424-5857 docx.pdf | | | |
| Warning: The PDF file has been signed with a digital signature and the legal effect of the document will be based on the contents of the file not the digital signature. | | | |
| Request for Correction of Inventorship Under 37 CFR 1324.pdf | 2 | Miscellaneous Incoming Letter | 17 KB |
| Request for Certificate of Correction -- Error in Application -- fee required 37 CFR 1323.pdf | 2 | Request for Certificate of Correction | 23 KB |
| Statement of Daniel J Wendt 37 CFR 1324 4918-7478-9985 - executeddocx.pdf | 1 | Miscellaneous Incoming Letter | 161 KB |
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| Statement Under 37 CFR 373 c PTO AIA-96.pdf | 3 | Miscellaneous Incoming Letter | 30 KB |
| Corrective assignment RE48267.pdf | 13 | Miscellaneous Incoming Letter | 600 KB |
| Bell Bliesath assignment-pat-25094-534.pdf | 11 | Miscellaneous Incoming Letter | 673 KB |
| Assignee BMRN Consent for Correction of Inventorship Under 37 CFR 1324 b2 - executed.pdf | 2 | Consent of Assignee accompanying the declaration | 1152 KB |

## Digest

| DOCUMENT | MESSAGE DIGEST(SHA-512) |
|---|---|
| Application Data Sheet.pdf | F2290EEE5CACC614DC2A2BB01D0F61B268639A1CF63240C89B72E1DE60FEA4D2E90FC54FF731878525383F334C383B5256AC8789AD112307A35E335CF7718AF3 |
| Certificate of Correction.pdf | E76FC8DFB55C43578CE0F5A92D2EDB0DFF5DFCDD871D4D1 |

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|---|---|
| | A3A926BC47DB33C5FFA1A14305D3ED0B673019CEC3B0C98A6BCBE1844B67A689B001819C1C74F05C8 |
| Joshua Bliesath Declaration_for_Reissue_Application_by_Inventor_ PTO_AIA-05 _ 4929-6424-585.pdf | 2D30817A1B26C74E520108C7A505CB778B27BA00B13BFA648A4066AB2E16F7069EBBD0742DE92AA64FD9D223F5D08E868252E2597D568AFF0DA8D4964B508BA1 |
| Sean Bell Declaration for Reissue Application by Inventor PTO AIA-05 4929-6424-5857 docx.pdf | 9E2D901744815D08D4A6C95157BEA426AEB7F65F043E4729D800F93E420B23E48A38731305A13E17F6D28AFAA5ED7959BB361084D64ACF0BF481015B9D8D3F12 |
| Request for Correction of Inventorship Under 37 CFR 1324.pdf | 0724B300EA6957717C4802229EEA7D5EA902C75737C239051AB5F613B82A0D6997E6C845DFBC8DEA9445704FA20F672D1E0BDF966A77548499FD0B9C1D90D8BE |
| Request for Certificate of Correction -- Error in Application -- fee required 37 CFR 1323.pdf | D10F9682F28B80901F3A29DD4CE40C86F8AE1E7CA8C81E8DD323E8A77DAE74B4D381B5969EDCEE5CEC4B3C7EB68E9AEC11B6EBBB29632306290A6C2DD90F5A28 |
| Statement of Daniel J Wendt 37 CFR 1324 4918-7478-9985 - executeddocx.pdf | D64D96EC061E2F66909563FF90D1CB129F3E6A3A7E444BFE20D0E36C055FFC363BA30798F1B9F0CAA7BCB47917BB6C6F41B3B058ED84918D4095CBA1CD53B19C |
| Statement of Shinong Long 37 CFR 1324 4900-4758-0769 - executeddocx.pdf | 13CB2D460061D30A27F6D16BFEBEF63C28902DAC67ADB44D3522C79EAD03FF870E7405AAEA104C344208D9AFB596F80994629DA12510093F4F0A6940ED85D41C |
| Statement of Sianna Castillo 37 CFR 1324 4930-8556-7585 - executeddocx.pdf | 4B97D99CC3766AE4A68F2FA0DE6AC8EDA7349310909E780105EAEC1268912F9C1444CD83F017CD9AD2E0F08CAA6661D1F524FEF03B367436979238AD0BF8713F |
| Statement of Christopher Price 37 CFR 1324 4911-5972-6689 - executeddocx.pdf | 79D4D67D596845BC5B53F82D89B8B96D6A83BF13449064CD58E1CCE5A4849A8A1FF5D1E3BD75E5F9F6B677611EEE08B7A0C62358BE93C1EF93E78087BB3AC253 |
| Statement of Mika Aoyagi-Scharber 37 CFR 1324 4933-5918-0385 - executeddocx.pdf | A650CC2D5480423F8F94BFF0915745744FCA065626276FEA30AEEFC76A996FBF785E41FCB01760795174731E465191819234158B094F1023280DDE68A931ED45 |
| Statement of Michel Claude Vellard 37 CFR 1324 4937-7979-0433 - executeddocx.pdf | 16C50C944E6975CA0615D9E09EE2B12B36C5FA74EF764029997AFD54C944AF4CB99AEA9307889CF89905A73AE5412AF43E72971878C7377AB32B71176DE4C4AF |
| Statement of Augustus O Okhamafe 37 CFR 1324 4933-2785-4177 - executeddocx.pdf | 001FB3B927B824D9D93672737415759C6D1AB78E144EACA54F40EDA1724255E310F9643F5CBCF80952EF2C1430176593CB34CF2EB6E08A69E354127412C49CB9 |
| Statement of Sean Bell 37 CFR 1324 4916-5124-6689 - executteddocx.pdf | 917B28AEC1AA2B5D46CEE0D7BC56DD6F521DE5FD624EFF39DD580ACDF4310A4091A08556FB4C1AF657350BAB43A678F26F1110DD1B4900B7894B3052AEDEDD77 |

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|---|---|
| Statement of Joshua R Bliesath 37 CFR 1324 4933-4725-2833 - executeddocx.pdf | 6A695686CCFA3EAE763D9D86E1C51E8711D304D704429D920F159520FD932DAEED226FAA580F18641F4B4F6FC9FED9C6973EB0B8D426FC6B17A22C0AA9C89260 |
| Statement Under 37 CFR 373 c PTO AIA-96.pdf | EE4D7DD5DEF00921EFC357962CAF5E9017239C53233A41E6B0A49882893B69C6607EE21AC4E8E913A5ED3B80717BB7ACAE5AB7C366F4C3A4A1A9A13204C6A2A3 |
| Corrective assignment RE48267.pdf | C5735AD7CCBAA6EEBFC031A4E260B160AC0C93EBF8668EF8156EE68B112D08365BA2A2F285B67B62B92A6D0721C28FFCBAEA6DA22E18B0B67B5E996F550328F9 |
| Bell Bliesath assignment-pat-25094-534.pdf | D07AC498C319B9123D596D57BCCD2DC84A333A711FD2477909321143B5A5A31C937E02B4A56B0DBC3906C1118455A79125036F29142476B009B1306391CDB60E |
| Assignee BMRN Consent for Correction of Inventorship Under 37 CFR 1324 b2 - executed.pdf | 9B9C31A3F9FA33789D2DCA8D783E9B506EAEFB293871E303871534708CB2A6C4FC60D83CA806FDB30F8B2FBBE00E7974CBBC4FC177A1AA7154E0CFED412BBB95 |

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**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

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Docket No.: 30610/44556C1-R2
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Letters Patent of:
Daniel J. Wendt et al.

Patent No. RE48,267 Issued: October 20, 2020
U.S. Serial No. 15/646,822 Filed: July 11, 2017

For: Variants of C-Type Natriuretic Peptide

**STATEMENT OF DANIEL J. WENDT UNDER 37 C.F.R. 1.324**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Daniel J. Wendt, hereby state:

I understand that a request to correct the inventorship of the above-identified patent will be made to name as *additional* inventors Sean Bell and Joshua R. Bliesath. I understand further that, upon a grant of the request, the named inventors of the invention claimed in the application/patent shall be:

Daniel J. Wendt
Shinong Long
Sianna Castillo
Christopher P. Price
Mika Aoyagi-Scharber
Michel Claude Vellard
Augustus O. Okhamafe
Sean Bell
Joshua R. Bliesath

I hereby agree to the requested change.

Dated: 27-Aug-2025

DocuSigned by:
Daniel Wendt
F6047FF0F70546F...

Daniel J. Wendt

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/646,822 | 07/11/2017 | Daniel J. Wendt | 30610/44556C1-R2 | 7727 |

90849 7590 11/04/2025

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

| EXAMINER | |
|---|---|
| CAMPELL, BRUCE R | |

| ART UNIT | PAPER NUMBER |
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| 3991 | |

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| 11/04/2025 | ELECTRONIC |

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| APPLICATION NO. | ISSUE DATE | PATENT NO. |
|---|---|---|
| 15/646,822 | 04-Nov-2025 | RE048267 |

Marshall, Gerstein & Borun LLP (Biomarin)
Chicago, IL 60606

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