[0073] Examples of the amino acid sequences of the VH includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu is introduced in the amino acid sequence represented by SEQ ID NO:24.

[0074] Specific examples of the amino acid sequence of VH in which ten modifications are introduced include an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0075] Specific examples of the amino acid sequence of VH in which nine modifications are introduced include the following amino acid sequences (1) to (10):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(2) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(3) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(5) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(6) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(7) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(8) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(9) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,

(10) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0076] Specific examples of the amino acid sequence of VH in which eight modifications are introduced include the following amino acid sequences (1) to (17):

(1) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at

position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(9) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(10) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(11) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(12) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(13) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(14) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(15) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(16) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Tyr at position 95 with Phe, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(17) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Val at position 112 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0077] Specific examples of the amino acid sequence of VH in which seven modifications are introduced include the following amino acid sequences (1) to (8):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0078] Specific examples of the amino acid sequence of VH in which six modifications are introduced include the following amino acid sequences (1) to (13):

(1) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which substitutions of Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(9) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,

(10) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(11) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(12) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(13) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0079] Specific examples of the amino acid sequence of VH in which five modifications are introduced include the following amino acid sequences (1) to (5):

(1) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0080] Specific examples of the amino acid sequence of VH in which four modifications are introduced include the following amino acid sequences (1) to (5):

(1) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, Met at position 48 with Ile, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0081] Specific examples of the amino acid sequence of VH in which three modifications are introduced include the following amino acid sequences (1) to (12):

(1) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 67 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,

(3) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Lys at position 12 with Val, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Met at position 48 with Ile, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Val at position 68 with Ala, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Ile at position 70 with Leu, and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which substitutions of Ala at position 9 with Pro, Arg at position 38 with Lys, and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO:24,
(9) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO:24,
(10) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO:24,
(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO:24,
(12) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0082] Specific examples of the amino acid sequence of VH in which two modifications are introduced include the following amino acid sequences (1) to (18):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which substitutions of Met at position 48 with Ile and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which substitutions of Ala at position 9 with Pro and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which substitutions of Lys at position 12 with Val and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(9) an amino acid sequence in which substitutions of Ala at position 9 with Pro and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO:24,
(10) an amino acid sequence in which substitutions of Lys at position 12 with Val and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO:24,
(11) an amino acid sequence in which substitutions of Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO:24,
(12) an amino acid sequence in which substitutions of Met at position 48 with Ile and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO:24,
(13) an amino acid sequence in which substitutions of Met at position 48 with Ile and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO:24,
(14) an amino acid sequence in which substitutions of Ala at position 9 with Pro and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(15) an amino acid sequence in which substitutions of Lys at position 12 with Val and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(16) an amino acid sequence in which substitutions of Arg at position 67 with Lys and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,
(17) an amino acid sequence in which substitutions of Val at position 68 with Ala and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24,

(18) an amino acid sequence in which substitutions of Ile at position 70 with Leu and Tyr at position 95 with Phe are introduced in the amino acid sequence represented by SEQ ID NO:24.

[0083] Specific examples of the amino acid sequence of VH in which one modification is introduced include the following amino acid sequences (1) to (10):

(1) an amino acid sequence in which Ala at position 9 is substituted with Pro in the amino acid sequence represented by SEQ ID NO:24,
(2) an amino acid sequence in which Lys at position 12 is substituted with Val in the amino acid sequence represented by SEQ ID NO:24,
(3) an amino acid sequence in which Val at position 20 is substituted with Ile in the amino acid sequence represented by SEQ ID NO:24,
(4) an amino acid sequence in which Arg at position 38 is substituted with Lys in the amino acid sequence represented by SEQ ID NO:24,
(5) an amino acid sequence in which Met at position 48 is substituted with Ile in the amino acid sequence represented by SEQ ID NO:24,
(6) an amino acid sequence in which Arg at position 67 is substituted with Lys in the amino acid sequence represented by SEQ ID NO:24,
(7) an amino acid sequence in which Val at position 68 is substituted with Ala in the amino acid sequence represented by SEQ ID NO:24,
(8) an amino acid sequence in which Ile at position 70 is substituted with Leu in the amino acid sequence represented by SEQ ID NO:24,
(9) an amino acid sequence in which Tyr at position 95 is substituted with Phe in the amino acid sequence represented by SEQ ID NO:24,
(10) an amino acid sequence in which Val at position 112 is substituted with Leu in the amino acid sequence represented by SEQ ID NO:24.

[0084] Regarding VL which is included in a humanized antibody of the present invention, the following (1) to (4) are preferable:

(1) VL which comprises an amino acid sequence in which Leu at position 15, Ala at position 19, Ile at position 21, and Leu at position 84 in the amino acid sequence represented by SEQ ID NO:26 are substituted with other amino acid residues,
(2) VL which comprises an amino acid sequence in which Leu at position 15, Ala at position 19, and Leu at position 84 in the amino acid sequence represented by SEQ ID NO:26 are substituted with other amino acid residues,
(3) VL which comprises an amino acid sequence in which Ala at position 19, Ile at position 21, and Leu at position 84 in the amino acid sequence represented by SEQ ID NO:26 are substituted with other amino acid residues,
(4) VL which comprises an amino acid sequence in which Ala at position 19 and Leu at position 84 in the amino acid sequence represented by SEQ ID NO:26 are substituted with other amino acid residues.

[0085] Example of the amino acid sequences of the VL includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Leu at position 15 with Ala, Ala at position 19 with Val, Ile at position 21 with Met, and Leu at position 84 with Val is introduced in the amino acid sequence represented by SEQ ID NO:26.

[0086] Specific examples of the amino acid sequences of VL in which four modifications are introduced include an amino acid sequence in which substitutions of Leu at position 15 with Ala, Ala at position 19 with Val, Ile at position 21 with Met, and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26.

[0087] Specific examples of the amino acid sequence of VL in which three modifications are introduced include the following amino acid sequences (1) to (4):

(1) an amino acid sequence in which substitutions of Ala at position 19 with Val, Ile at position 21 with Met, and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(2) an amino acid sequence in which substitutions of Leu at position 15 with Ala, Ala Ile at position 21 with Met, and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(3) an amino acid sequence in which substitutions of Leu at position 15 with Ala, Ala at position 19 with Val, and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(4) an amino acid sequence in which substitutions of Leu at position 15 with Ala, Ala at position 19 with Val, and Ile at position 21 with Met are introduced in the amino acid sequence represented by SEQ ID NO:26.

[0088] Specific examples of the amino acid sequence of VL in which two modifications are introduced include the following amino acid sequences (1) to (6):

(1) an amino acid sequence in which substitutions of Leu at position 15 with Ala and Ala at position 19 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(2) an amino acid sequence in which substitutions of Leu at position 15 with Ala and Ile at position 21 with Met are introduced in the amino acid sequence represented by SEQ ID NO:26,
(3) an amino acid sequence in which substitutions of Leu at position 15 with Ala and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(4) an amino acid sequence in which substitutions of Ala at position 19 with Val and Ile at position 21 with Met are introduced in the amino acid sequence represented by SEQ ID NO:26,
(5) an amino acid sequence in which substitutions of Ala at position 19 with Val and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26,
(6) an amino acid sequence in which substitutions of Ile at position 21 with Met and Leu at position 84 with Val are introduced in the amino acid sequence represented by SEQ ID NO:26.

[0089] Specific examples of the amino acid sequence of VL in which one modification is introduced include the following amino acid sequences (1) to (4):

(1) an amino acid sequence in which Leu at position 15 with Ala is substituted in the amino acid sequence represented by SEQ ID NO:26,
(2) an amino acid sequence in which Ala at position 19 with Val is substituted in the amino acid sequence represented by SEQ ID NO:26,
(3) an amino acid sequence in which Ile at position 21 with Met is substituted in the amino acid sequence represented by SEQ ID NO:26,
(4) an amino acid sequence in which Leu at position 84 with Val is substituted in the amino acid sequence represented by SEQ ID NO:26.

[0090] Specific examples of a humanized antibody of the present invention include the following humanized antibodies (1) to (3):

(1) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:24, and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:26,
(2) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:24, and VL of the antibody comprising any one amino acid sequence among amino acid sequences shown in Fig. 13,
(3) a humanized antibody which comprises at least one of VH of the antibody comprising any one amino acid sequence among amino acid sequences shown in Fig. 12, and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:26.

[0091] In addition, examples of the humanized antibody of the present invention include a humanized antibody which competes with the above humanized antibody and a humanized antibody which binds to the same epitope as that bound by the above humanized antibody.

[0092] A human antibody means an antibody naturally existing in the human body, and also includes an antibody obtained from a human antibody phage library or a human antibody-producing transgenic animal, which is prepared based on the recent advanced techniques in genetic engineering, cell engineering and developmental engineering.

[0093] The antibody naturally existing in the human body can be prepared, for example by isolating a human peripheral blood lymphocyte, immortalizing it by infecting with EB virus or the like, and then cloning it to thereby obtain lymphocytes capable of producing the antibody, culturing the lymphocytes thus obtained, and purifying the antibody from the supernatant of the culture.

[0094] The human antibody phage library is a library in which antibody fragments such as Fab and scFv are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment on the cell surface having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the index. The antibody fragment can be converted further into a human antibody molecule consisting of two full H chains and two full L chains by genetic engineering techniques.

[0095] A human antibody-producing transgenic animal means an animal in which a human antibody gene is integrated

into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a gene encoding a human antibody into a mouse ES cell, grafting the ES cell into an early stage embryo of other mouse and then developing it into a complete animal. A human antibody derived from the human antibody-producing transgenic non-human animal can be prepared by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in non-human animals, culturing the obtained hybridoma and producing and accumulating the human antibody in the supernatant of the culture.

[0096] An antibody or antibody fragment thereof in which one or more amino acids are deleted, substituted, inserted or added in the amino acid sequence constituting the above antibody or antibody fragment, having activity similar to the above antibody or antibody fragment is also included in the antibody or antibody fragment of the present invention.

[0097] The number of amino acid residues which are deleted, substituted, inserted and/or added is one or more, and is not specifically limited, but it is within the range where deletion, substitution or addition is possible by known methods such as the site-directed mutagenesis described in Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory Press (1989); Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997); Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci. USA, 79, 6409 (1982); Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985); Proc. Natl. Acad. Sci. USA, 82, 488 (1985) or the like. For example, the number is 1 to dozens, preferably 1 to 20, more preferably 1 to 10, and most preferably 1 to 5.

[0098] Deleting, substituting, inserting and/or adding one or more amino acid residue(s) in the amino acid sequence of the above antibody means the followings. That is, it means there is deletion, substitution, insertion or addition of one or plural amino acid residues at any positions in one or plural amino acid sequences in a single sequence. Also, the deletion, substitution, insertion or addition may exist at the same case and the amino acid which is substituted, inserted or added may be either a natural type or a non-natural type.

[0099] The natural type amino acid includes L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-cysteine and the like.

[0100] Preferable examples of mutually substitutable amino acids are shown below. The amino acids in the same group are mutually substitutable.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine

Group B: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid

Group C: asparagine, glutamine

Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid

Group E: proline, 3-hydroxyproline, 4-hydroxyproline

Group F: serine, threonine, homoserine

Group G: phenylalanine, tyrosine

[0101] The antibody fragment of the present invention includes Fab, $F(ab')_2$, Fab', scFv, diabody, dsFv, a peptide comprising CDR and the like.

[0102] An Fab is an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating an IgG antibody molecule with a protease, papain (cleaved at an amino acid residue at position 224 of the H chain), are bound together through a disulfide bond.

[0103] The Fab of the present invention can be obtained by treating the monoclonal antibody of the present invention with papain. Also, the Fab can be produced by inserting DNA encoding Fab of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or an eukaryote to express the Fab.

[0104] An $F(ab')_2$ is an antibody fragment having a molecular weight of about 100,000 and having antigen binding activity and comprising two Fab regions which are bound in the hinge portion obtained by digesting the lower part of two disulfide bonds in the hinge region of IgG with an enzyme, pepsin.

[0105] The $F(ab')_2$ of the present invention can be obtained by treating the monoclonal antibody of the present invention with pepsin. Also, the $F(ab')_2$ can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

[0106] An Fab' is an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, which is obtained by cleaving a disulfide bond at the hinge region of the above $F(ab')_2$. The Fab' of the present invention can be obtained by treating $F(ab')_2$ of the present invention with a reducing agent, such as dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to express the Fab'.

[0107] An scFv is a VH-P-VL or VL-P-VH polypeptide in which a VH and a VL are linked using an appropriate peptide linker (hereinafter referred to as "P") and is an antibody fragment having antigen binding activity.

[0108] The scFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody, constructing a DNA encoding the scFv, inserting the DNA into an expression vector for prokaryote or an

expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv.

[0109] A diabody is an antibody fragment wherein scFv is dimerized, is an antibody fragment having divalent antigen binding activity. In the divalent antigen binding activity, two antigens may be the same or different. The diabody of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention, constructing DNA encoding scFv so that the length of the amino acid sequence of the peptide linker is 8 or less residues, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the diabody.

[0110] A dsFv is obtained by binding polypeptides in which one amino acid residue of each of VH and VL is substituted with a cysteine residue via a disulfide bond between the cysteine residues. The amino acid residue to be substituted with a cysteine residue can be selected based on a conformation prediction of the antibody in accordance with a known methods [Protein Engineering, 7, 697 (1994)].

[0111] The dsFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention, constructing DNA encoding dsFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the dsFv.

[0112] A peptide comprising CDR is constituted by including one or more regions of CDRs of VH or VL. Peptides comprising plural CDRs can be connected directly or via an appropriate peptide linker.

[0113] The peptide comprising CDR of the present invention can be produced by constructing DNAs encoding CDRs of VH and VL of the monoclonal antibody of the present invention, inserting the DNAs into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the peptide. The peptide comprising CDRs can also be produced by a chemical synthesis method such as Fmoc method or tBoc method.

[0114] The present invention includes an antibody derivative in which the monoclonal antibody of the present invention is chemically or genetically bound to a radioisotope, a small-molecular weight agent, an high-molecular weight agent, a protein, a therapeutic antibody or the like.

[0115] The antibody derivative of the present invention can be produced by conjugating a radioisotope, a low-molecular-weight agent, a high-molecular-weight agent, an immunostimulator, a protein, a therapeutic antibody or the like to the N-terminal side or C-terminal side of an H chain or an L chain of the monoclonal antibody of the present invention and the antibody fragment thereof using chemical methods [Antibody Engineering Handbook, published by Chijin Shokan (1994)].

[0116] Also, the antibody derivative of the present invention can be genetically produced by linking a DNA encoding the monoclonal antibody of the present invention to other DNA encoding a protein or a therapeutic antibody to be conjugated, inserting the DNA into an expression vector, and introducing the expression vector into an appropriate host cell.

[0117] The radioisotopes include ^{131}I, ^{125}I, ^{90}Y, ^{64}Cu, ^{199}Tc, ^{77}Lu, ^{211}At and the like. The radioisotope can be directly conjugated with the antibody by Chloramine-T method or the like. Also, a substance chelating the radioisotope can be conjugated with the antibody. The chelating agent includes 1-isothiocyanatobenzyl-3-methyldiethylene-triaminepentaacetic acid (MX-DTPA) and the like.

[0118] The low-molecular-weight agents include an anti-tumor agent such as an alkylating agent, a nitrosourea agent, a metabolism antagonist, an antibiotic substance, a plant alkaloid, a topoisomerase inhibitor, a hormonotherapeutic agent, a hormone antagonist, an aromatase inhibitor, a P-glycoprotein inhibitor, a platinum complex derivative, an M-phase inhibitor and a kinase inhibitor [Rinsho Syuyo-gaku (Clinical Oncology), Gan to Kagaguryoho-Sha (1996)], a steroid agent such as hydrocortisone and prednisone, a nonsteroidal agent such as aspirin and indomethacin, immune-regulating agent such as aurothiomalate, penicillamine, immune-suppressing agent such as cyclophosphamide and azathioprine, anti-inflammatory agent such as anti-histamine agent, for example, chlorpheniramine maleate and clemastine /Ensho to Kouensho-Ryoho (Inflammation and Anti-inflammation Therapy), Ishiyaku Shuppann (1982)], and the like.

[0119] Examples of the antitumor agent include amifostine (Ethyol), cisplatin, dacarbazine (DTIC), dactinomycin, mecloretamin (nitrogen mustard), streptozocin, cyclophosphamide, iphosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), epirubicin, gemcitabine (Gemsal), daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, fluorouracil, vinblastine, vincristine, bleomycin, daunomycin, peplomycin, estramustine, paclitaxel (Taxol), docetaxel (Taxotea), aldesleukin, asparaginase, busulfan, carboplatin, oxaliplatin, nedaplatin, cladribine, camptothecin, 10-hydroxy-7-ethylcamptothecin (SN38), floxuridine, fludarabine, hydroxyurea, iphosphamide, idarubicin, mesna, irinotecan (CPT-11), nogitecan, mitoxantrone, topotecan, leuprolide, megestrol, melfalan, mercaptopurine, hydroxycarbamide, plicamycin, mitotane, pegasparagase, pentostatin, pipobroman, streptozocin, tamoxifen, goserelin, leuprorelin, flutamide, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil, hydrocortisone, prednisolone, methylprednisolone, vindesine, nimustine, semustine, capecitabine, Tomudex, aza-

cytidine, UFT, oxaliplatin, gefitinib (Iressa), imatinib (STI 571), elrotinib, FMS-like tyrosine kinase 3 (Flt3) inhibitor, vascular endothelial growth facotr receptor (VEGFR) inhibitor, fibroblast growth factor receptor (FGFR) inhibitor, epidermal growth factor receptor (EGFR) inhibitor such as Iressa and Tarceva, radicicol, 17-allylamino-17-demethoxygeldanamycin, rapamycin, amsacrine, all-trans-retinoic acid, thalidomide, anastrozole, fadrozole, letrozole, exemestane, gold thiomalate, D-penicillamine, bucillamine, azathioprine, mizoribine, cyclosporine, rapamycin, hydrocortisone, bexarotene (Targretin), tamoxifen, dexamethasone, progestin substances, estrogen substances, anastrozole (Arimidex), Leuplin, aspirin, indomethacin, celecoxib, azathioprine, penicillamine, gold thiomalate, chlorpheniramine maleate, chlorpheniramine, clemastine, tretinoin, bexarotene, arsenic, voltezomib, allopurinol, calicheamicin, ibritumomab tiuxetan, Targretin, ozogamine, clarithromycin, leucovorin, ifosfamide, ketoconazole, aminoglutethimide, suramin, methotrexate, maytansinoid and derivatives thereof.

[0120] The method for conjugating the low-molecular-weight agent with the antibody include a method in which the agent and an amino group of the antibody are conjugated via glutaraldehyde, a method in which an amino group of the agent and a carboxyl group of the antibody are conjugated via water-soluble carbodiimide, and the like.

[0121] The high-molecular-weight agents include polyethylene glycol (hereinafter referred to as "PEG"), albumin, dextran, polyoxyethylene, styrene-maleic acid copolymer, polyvinylpyrrolidone, pyran copolymer, hydroxypropylmethacrylamide, and the like.

[0122] By binding these high-molecular-weight compaunds to the antibody or antibody fragment, the following effects are expected: (1) improvement of stability against various chemical, physical or biological factors, (2) remarkable prolongation of half life in blood, (3) depletion of immunogenicity or suppression of antibody production, and the like *[*Bioconjugate Drug, Hirokawa Shoten (1993)].

[0123] Examples of the methods for conjugating PEG to the antibody include a method for reacting an antibody with a PEG-modifying reagent [Bioconjugate Drug, Hirokawa Shoten (1993)]. Examples of the PEG-modifying reagents include a modifying agent for ε-amino group of lysine (Japanese Published Unexamined Patent Application No. 178926/86), a modifying agent for a carboxyl group of aspartic acid and glutamic acid (Japanese Published Unexamined Patent Application No. 23587/81), a modifying agent for a guanidino group of arginine (Japanese Published Unexamined Patent Application No. 117920/90) and the like.

[0124] The immunostimulator may be any natural products known as immunoadjuvants. Examples of the agents enhancing immunity include β(1→3)glucan (lentinan, schizophyllan), α-galactosylceramide and the like.

[0125] The proteins include a cytokine or a growth factor which activates a immunocompetent cell, such as NK cell, macrophage or neutrophil, a toxic protein, and the like.

[0126] Examples of the cytokines or the growth factors include interferon (hereinafter referred to as "INF")-α, INF-β, INF-γ, interleukin (hereinafter referred to as "IL")-2, IL-12, IL-15, IL-18, IL-21, IL-23, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) and the like.

[0127] The toxic proteins include ricin, diphtheria toxin, ONTAK and the like, and also includes a toxic protein wherein mutation is introduced into a protein in order to control the toxicity.

[0128] The therapeutic antibody include an antibody against an antigen which induces apoptosis by binding of the antibody, an antibody against an antigen which participates in formation of tumor condition, an antibody against an antigen which regulates immunological function and an antibody against an antigen which relate to angiogenesis in the pathologic region.

[0129] The antigens which induces apoptosis by binding of the antibody includes cluster of differentiation (hereinafter "CD") 19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85, CD86 (B7.2), human leukocyte antigen (HLA)-Class II, epidermal growth factor receptor (EGFR) and the like.

[0130] The antigens participating in formation of tumor condition or the antigens for the antibody which regulates immunological function include CD4, CD40, CD40 ligand, B7 family molecule (CD80, CD86, CD274, B7-DC, B7-H2, B7-H3, B7-H4), ligand of B7 family molecule (CD28, CTLA-4, ICOS, PD-1, BTLA), OX-40, OX-40 ligand, CD137, tumor necrosis factor (TNF) receptor family molecule (DR4, DR5, TNFR1, TNFR2), TNF-related apoptosis-inducing ligand receptor (TRAIL) family molecule, receptor family of TRAIL family molecule (TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4), receptor activator of nuclear factor kappa B ligand (RANK), RANK ligand, CD25, folic acid receptor 4, cytokine [IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, transforming growth factor (TGF) β, TNFα, *etc.*], receptors of these cytokines, chemokine (SLC, ELC, I-309, TARC, MDC, CTACK, *etc.*) and receptors of these chemokines.

[0131] The antigens for the antibody which inhibits angiogenesis in the pathologic region include vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), EGF, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), erythropoietin (EPO), TGFβ, IL-8, Ephilin, SDF-1, receptors thereof and the like.

[0132] A fusion antibody with a protein or therapeutic antibody can be produced by constructing a DNA encoding the fusion antibody in which a cDNA encoding the protein is linked to a cDNA encoding a monoclonal antibody or antibody fragment thereof, inserting the DNA into an expression vector for prokaryote or eukaryote, and then introducing the

expression vector into a prokaryote or eukaryote to express.

[0133] In the case where the above antibody derivative is used for a detection method, a quantification method, a detection reagent, a quantification reagent or a diagnostic agent in the present invention, examples of the agents for conjugation to the monoclonal antibody of the present invention include a label used in general immunological detection or measuring method.

[0134] The labels include enzymes such as alkaline phosphatase, peroxidase and luciferase, luminescent materials such as acridinium ester and lophine, fluorescent materials such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (RITC), and the like.

[0135] Furthermore, the present invention includes an agent for treating a disease relating to a Trop-2 positive cell, comprising the monoclonal antibody of the present invention or the antibody fragment thereof as an active ingredient.

[0136] The disease relating to the Trop-2 positive cell is not limited, so long as it is a disease relating to a Trop-2-expressing cell, such as cancer.

[0137] The cancers include blood cancer, breast cancer, uterine cancer, colorectal cancer, esophageal cancer, gastric cancer, ovarian cancer, lung cancer, renal cancer, rectal cancer, thyroid cancer, uterine cervix cancer, small intestinal cancer, prostate cancer and pancreatic cancer. Preferable examples of the cancer include blood cancer, esophageal cancer, gastric cancer, colorectal cancer, liver cancer and prostate cancer.

[0138] The therapeutic agent comprising the monoclonal antibody of the present invention or the antibody fragment thereof, or the derivative thereof may comprise only the antibody or the antibody fragment thereof, or the derivative thereof as an active ingredient. It is generally preferred that the therapeutic agent is provided as a pharmaceutical formulation produced by mixing it with one or more pharmaceutically acceptable carriers and manufacturing by an appropriate method well known in the technical field of pharmaceutics.

[0139] It is preferred that the route of administrarion is most effective for the treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration and intravenous administration is preferred.

[0140] The therapeutic agent may be in the form of spray, capsules, tablets, powder, granules, syrup, emulsion, suppository, injection, ointment, tape, and the like.

[0141] Although the dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 μg/kg to 10 mg/kg per day and per adult.

[0142] Further, the present invention relates to a method for immunologically detecting or measuring Trop-2, a reagent for immunologically detecting or measuring Trop-2, a method for immunologically detecting or measuring a Trop-2-expressing cell, and a diagnostic agent for a disease relating to a Trop-2 positive cell, comprising the monoclonal antibody of the present invention or the antibody fragment thereof as an active ingredient.

[0143] In the present invention, the method for detecting or measuring the amount of Trop-2 may be any known method. For example, it includes an immunological detecting or measuring method.

[0144] The immunological detecting or measuring method means a method for detecting and measuring an antibody amount or an antigen amount using a labeled antigen or antibody. Examples of the immunological detecting or measuring method include radioimmunoassay (RIA), enzyme immunoassay (EIA or ELISA), fluorescent immunoassay (FIA), luminescent immunoassay, Western blotting method, physico-chemical method and the like.

[0145] The disease relating to Trop-2 can be diagnosed by detecting or measuring a Trop-2-expressing cell by using the monoclonal antibody or antibody fragment of the present invention.

[0146] For the detection of the Trop-2-expressing cell, known immunological detection methods can be used, and an immunoprecipitation method, a fluorescent cell staining method, an immune tissue staining method, immunofluorescence method and the like are preferably used. Also, a fluorescent antibody staining method using FMAT 8100 HTS system (Applied Biosystem) and the like can be used.

[0147] In the present invention, the living body sample to be used for detecting or measuring Trop-2 is not particularly limited, so long as it has a possibility of containing a Trop-2-expressing cell, such as tissue cells, blood, blood plasma, serum, pancreatic fluid, urine, fecal matter, tissue fluid or culture fluid.

[0148] The diagnostic agent containing the monoclonal antibody of the present invention or the antibody fragment thereof, or the derivative thereof may further contain a reagent for carrying out an antigen-antibody reaction or a reagent for detection of the reaction depending on the desired diagnostic method. The reagent for carrying out the antigen-antibody reaction includes a buffer, a salt, and the like.

[0149] The detection reagents include a reagent generally used for the immunological detecting or measuring method, such as labeled secondary antibody which recognizes the monoclonal antibody, antibody fragment thereof or derivative thereof and substrate corresponding to the labeling.

[0150] A process for producing the monoclonal antibody of the present invention, a method for treating the disease and a method for diagnosing the disease are specifically described below.

1. Preparation method of monoclonal antibodies

(1) Preparation of antigens

[0151] Trop-2 as an antigen or a Trop-2-expressing cell can be obtained by introducing an expression vector comprising cDNA encoding a full length of Trop-2 or a partial length thereof is introduced into *Escherichia coli,* yeast, an insect cell, an animal cell or the like. In addition, Trop-2 can be purified from various human tumor cell lines, human tissue and the like which express a large amount of Trop-2. The whole tumor cell line and the whole tissue can be used as antigens. Furthermore, a synthetic peptide having a partial sequence of the Trop-2 can be prepared by a chemical synthesis method such as Fmoc method or tBoc method and used as an antigen.

[0152] Trop-2 used in the present invention can be produced, for example, by expressing a DNA encoding Trop-2 in a host cell using a method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) or the like according to the following method.

[0153] Firstly, a recombinant vector is prepared by inserting a full length cDNA comprising the region encoding Trop-2 into downstream of a promoter of an appropriate expression vector. At this time, if necessary, a DNA fragment having an appropriate length containing a region encoding the polypeptide, which is prepared based on the full length cDNA, may be used instead of the above full length cDNA. Next, a transformant producing polypeptides can be obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

[0154] The expression vector may be any one, so long as it is autonomously replicable or abele to integrate into a chromosome of the host cell to be used, and comprises an appropriate promoter at a position that the DNA encoding the polypeptide can be transcribed.

[0155] The host cell may be any one, so long as it can express the objective gene. Examples include a microorganism which belongs to the genera *Escherichia,* such as *Escherichia coli,* a yeast, an insect cell, an animal cell and the like.

[0156] When a prokaryote such as *Escherichia coli* is used as the host cell, it is preferred that the recombinant vector used in the present invention is autonomously replicable in the prokaryote and comprising a promoter, a ribosome binding sequence, the DNA encoding Trop-2 and a transcription termination sequence. The recombinant vector is not necessary to have a transcription termination sequence, but a transcription termination sequence is preferably set just below the structural gene. The recombinant vector may further comprise a gene regulating the promoter.

[0157] Also, the above recombinant vector is preferably a plasmid in which the space between Shine-Dalgarno sequence, which is the ribosome binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides).

[0158] Furthermore, the nucleotide sequence of the DNA encoding Trop-2 can be substituted with another base so as to be a suitable codon for expressing in a host cell, thereby improve the productivity of the objective Trop-2.

[0159] Any expression vector can be used, so long as it can function in the host cell to be used. Examples of the expression vectors include pBTrp2, pBTac1, pBTac2 (all manufactured by Roche Diagnostics), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK(-) (manufactured by Stratagene), pTrs30 [prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)], pTrs32 [prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)], pGHA2 [prepared from *Escherichia coli* IGHA2 (FERM BP-400), Japanese Published Unexamined Patent Application No. 221091/85], pGKA2 [prepared from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/85], pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (manufactured by Pharmacia), pET system (manufactured by Novagen), pME 18 SFL3 and the like.

[0160] Any promoter can be used, so long as it can function in the host cell to be used. Examples include promoters derived from *Escherichia coli,* phage and the like, such as trp promoter (Ptrp), lac promoter, PL promoter, PR promoter and T7 promoter. Also, artificially designed and modified promoters, such as a promoter in which two Ptrp are linked in tandem, tac promoter, lac T7 promoter and let I promoter, can be used.

[0161] Examples of host cell include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* DH5α and the like.

[0162] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA into the host cell, and examples include a method using a calcium ion described in Proc. Natl. Acad. Sci. USA, 69, 2110 (1972), Gene, 17,107 (1982) and Molecular & General Genetics, 168, 111 (1979) and the like.

[0163] When an animal cell is used as the host cell, any expression vector can be used, so long as it can function in the animal cell. Examples include pcDNAI, pcDM8 (manufactured by Funakoshi), pAGE107 [Japanese Published Un-

examined Patent Application No. 22979/91; Cytotechnology, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [Nature, 329, 840,(1987)], pcDNAI/Amp (manufactured by Invitrogen), pcDNA3.1 (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J. Biochemistry, 101, 1307 (1987)], pAGE210, pME18SFL3, pKANTEX93 (WO 97/10354) and the like.

[0164] Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of immediate early (IE) gene of cytomegalovirus (CMV), SV40 early promoter, a promoter of retrovirus, a metallothionein promoter, a heat shock promoter, SRα promoter, Molony murine leukemia virus promoter or enhancer, and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

[0165] The host cell includes human Burkitt's lymphoma cell Namalwa, monkey kidney cell COS, Chinese hamster ovary cell CHO (Journal of Experimental Medicine, 108, 945 (1958); Proc .Natl. Acad. Sci. USA, 60 ,1275 (1968); Genetics, 55, 513 (1968); Chromosoma,41,129 (1973); Methods in Cell Science,18,115 (1996); Radiation Research, 148, 260 (1997); Proc. Natl. Acad. Sci. USA, 77, 4216 (1980); Proc. Natl. Acad. Sci.,60,1275 (1968); Cell, 6, 121 (1975); Molecular Cell Genetics, Appendix I, II (pp.883-900);), CHO/DG44, CHO-K1 (ATCC CCL-61), DUKXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S (Life Technologies, Cat # 11619), Pro-3, rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also referred to as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14, Syrian hamster kidney-derived BHK cell or human leukemic cell HBT5637 (Japanese Published Unexamined Patent Application No. 299/88) and the like.

[0166] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA into an animal cell, and examples include electroporation [Cytotechnology, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the like.

[0167] Trop-2 can be produced by culturing the transformant derived from a microorganism, an animal cell or the like having a recombinant vector comprising the DNA encoding Trop-2 in a medium to form and accumulate Trop-2 in the culture, and recovering it from the culture. The method for culturing the transformant in the medium is carried out according to the usual method used in culturing of the hosts.

[0168] When Trop-2 is expressed in a cell derived from an eukaryote, Trop-2 with addition of sugars or sugar chains can be obtained.

[0169] When a microorganism transformed with a recombinant vector containing an inducible promoter is cultured, an inducer can be added to the medium, if necessary. For example, isopropyl-β-D-thiogalactopyranoside or the like can be added to the medium when a microorganism transformed with a recombinant vector using *lac* promoter is cultured; or indoleacrylic acid or the like can be added thereto when a microorganism transformed with a recombinant vector using trp promoter is cultured.

[0170] The media for culturing a transformant obtained using an animal cell as the host cell, the medium include generally used RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], Dulbecco's modified MEM medium [Virology, 8, 396 (1959)] and 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)], Iscoove's modified Dulbecco's medium (IMDM), the media to which fetal calf serum, *etc.* is added, and the like. The culturing is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO_2. If necessary, an antibiotic such as kanamycin or penicillin can be added to the medium during the culturing.

[0171] As the expression method for the gene encoding Trop-2, in addition to direct expression, secretory production, fusion protein expression and the like can be carried out according to the method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

[0172] The process for producing Trop-2 includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, a method of producing on a host cell membrane outer envelope, and the like. The appropriate method can be selected by changing the host cell used and the structure of the Trop-2 produced.

[0173] When the Trop-2 is produced in a host cell or on a host cell membrane outer envelope, Trop-2 can be positively secreted extracellularly in accordance with the method of Paulson et al. [J. Biol. Chem., 264, 17619 (1989)], the method of Lowe et al. [Proc. Natl. Acad. Sci. USA, 86, 8227 (1989), Genes Develop., 4, 1288 (1990)], the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and WO 94/23021, and the like.

[0174] Also, the production amount of Trop-2 can be increased in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 utilizing a gene amplification system using a dihydrofolate reductase gene.

[0175] The resulting Trop-2 can be isolated and purified, for example, as follows.

[0176] When Trop-2 is intracellularly expressed as a soluble form, the post-cultured cells are recovered by centrifugation, suspended in an aqueous buffer and then homogenated using ultrasonicator, French press, Manton Gaulin homogenizer, dynomill or the like to obtain a cell-free extract.

[0177] Using a supernatant derived by centrifugation of the cell-free extract, a purified preparation can be obtained by a general protein isolation and purification techniques such as solvent extraction; salting out with ammonium sulfate

etc.; desalting; organic solvent precipitation; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical); cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia); hydrophobic chromatography using a resin such as butyl-Sepharose or phenyl-Sepharose; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing; and the like which may be used alone or in combination.

[0178] When Trop-2 is expressed intracellularly by forming an inclusion body, the cells are recovered, homogenated and centrifuged in the same manner as the described above, and the inclusion body of Trop-2 are recovered as a precipitation fraction. The recovered inclusion body of Trop-2 is solubilized with a protein denaturing agent. The Trop-2 is turned back into a normal conformation by diluting or dialyzing the solubilized solution, and then a purified preparation of Trop-2 is obtained by the same isolation purification method as above.

[0179] When Trop-2 or the derivative thereof such as a glycosylated product is secreted extracellularly, Trop-2 or the derivative such as a glycosylated product can be recovered from the culture supernatant. A purified preparation of Trop-2 can be obtained from the culture supernatant by preparing a soluble fraction using a method such as centrifugation in the same manner as described above and applying the same isolation purification method as described above.

[0180] Also, Trop-2 used in the present invention can be produced by a chemical synthesis method, such as Fmoc method or tBoc method. Also, it can be chemically synthesized using a peptide synthesizer manufactured by Advanced ChemTech, Perkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corporation, or the like.

(2) Immunization of animal and preparation of antibody-producing cells for fusion

[0181] A mouse, rat or hamster 3 to 20 weeks old is immunized with the antigen prepared in the above (1), and antibody-producing cells are collected from the spleen, lymph node or peripheral blood of the animal. Also, when the increase of a sufficient titer in the above animal is not found due to low immunogenecity, a Trop-2 knockout mouse may be used as an animal to be immunized.

[0182] The immunization is carried out by administering the antigen to the animal through subcutaneous, intravenous or intraperitoneal injection together with an appropriate adjuvant (such as complete Freund's adjuvant, combination of aluminum hydroxide gel with pertussis vaccine). When a partial peptide is used as the antigen, a conjugate with a carrier protein such as BSA (bovine serum albumin), KLH (keyhole limpet hemocyanin) or the like is produced to use as an immunogen.

[0183] The administration of the antigen is carried out 5 to 10 times every one week or every two weeks after the first administration. On the 3rd to 7th day after each administration, a blood sample is collected from the eyeground venous plexus to determine antibody titer of the serum by enzyme immunoassay [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like. An animal showing a sufficient antibody titer in their sera against the antigen used for the immunization is used as a donor of antibody-producing cells for fusion.

[0184] Three to seven days after final administration of the antigen, tissue containing the antibody-producing cells such as the spleen from the immunized animal is excised to collect the antibody-producing cells. When spleen cells are used, the spleen is cut out and loosened, followed by centrifuged, and then erythrocytes are removed to obtain antibody-producing cells for fusion.

(3) Preparation of myeloma cells

[0185] Cell lines established from a mouse are used as myeloma cells. Examples include 8-azaguanine-resistant mouse (derived from BALB/c) myeloma cell line P3-X63Ag8-U1 (P3-U1) [Current Topics in Microbiology and Immunology, 18, 1 (1978)], P3-NS1/1-Ag41 (NS-1) [European J. Immunology, 6, 511 (1976)], SP2/0-Ag14 (SP-2) *(*Nature, 276, 269 (1978)], P3-X63-Ag8653 (653) [J. Immunology, 123, 1548 (1979)], P3-X63-Ag8 (X63) [Nature, 256,495 (1975)] and the like.

[0186] The myeloma cells are subcultured in a normal medium [RPMI1640 medium containing glutamine, 2-mercaptoethanol, gentamicin, FBS and 8-azaguanine] and then cultured in the normal medium 3 or 4 days before cell fusion to ensure the cell number of 2×10^7 or more on the day for fusion.

(4) Cell fusion and preparation of hybridomas for producing monoclonal antibodies

[0187] The antibody-producing cells for fusion obtained by the above (2) and the myeloma cells obtained by the above (3) were thoroughly washed with a minimum essential medium (MEM) or PBS (1.83 g of disodium hydrogen phosphate, 0.21 g of potassium dihydrogen phosphate, 7.65 g of sodium chloride, 1 liter of distilled water, pH 7.2) and mixed to give a ratio of the antibody-producing cells: the myeloma cells = 5 to 10: 1, followed by centrifugation, and then, the supernatant is discarded.

[0188] After thoroughly loosening the precipitated cell clumps, the mixture of polyethylene glycol-1000 (PEG-1000), MEM and dimethylsulfoxide is added to the cell under stirring at 37°C. In addition, 1 to 2 mL of MEM medium is added several times every one or two minutes, and MEM is added to give a total amount of 50 mL. After centrifugation, the supernatant is discarded. After the cell clumps are gently loosen, the cells are gently suspended in HAT medium [the normal medium containing hypoxanthine, thymidine and aminopterin]. The suspension is cultured in a 5% CO_2 incubator for 7 to 14 days at 37°C.

[0189] After the culturing, a portion of the culture supernatant is sampled and cell lines which is reactive to an antigen with Trop-2 and is not reactive to an antigen without Trop-2 is selected by binding assay as described below or the like. Then, cloning is carried out twice by a limiting dilution method [Firstly, HT medium (in the first round, HAT medium without aminopterin) is used, and sin the second round, the normal medium is used], and a hybridoma which shows a stably high antibody titer is selected as the monoclonal antibody-producing hybridoma.

(5) Preparation of purified monoclonal antibodies

[0190] The hybridoma cells producing a monoclonal antibody obtained by the above (4) are administered by intraperitoneal injection into 8- to 10-week-old mice or nude mice pre-treated with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane (Pristane) is intraperitoneally administered, followed by feeding for 2 weeks). The hybridoma forms ascites tumor in 10 to 21 days. The ascitic fluid is collected from the mice, centrifuged to remove solids, subjected to salting out with 40 to 50% ammonium sulfate and then precipitated by caprylic acid, passed through a DEAE-Sepharose column, a protein A column or a gel filtration column to collect IgG or IgM fractions as purified monoclonal antibodies.

[0191] Furthermore, after a monoclonal antibody-producing hybridoma obtained by the above (4) is cultured in RPMI1640 medium containing 10% FBS or the like, the supernatant is removed by centrifugation. The precipitated cells are suspended in Hybridoma SFM medium and cultured for 3 to 7 days. The purified monoclonal antibody can be obtained by centrifusing the obtained cell suspension, followed by purifying the resulting supernatant with Protein A colomun or Protein G colomn to collect the IgG fractions. Hybridoma SFM medium may contain 5% DIGO GF21.

[0192] The subclass of the antibody can be determined using a subclass typing kit by enzyme immunoassay. The amount of the protein can be calculated by the Lowry method or from the absorbance at 280 nm.

(6) Selection of monoclonal antibodies

[0193] Selection of monoclonal antibody is carried out by the following binding assay using an enzyme immunoassay method, and a kinetic analysis using Biacore. Other than these methods, a target antigen can be identified by a publically-known method (The Prostane, 67, 1163 (2007)).

(6-a) Binding assay

[0194] As the antigen, a gene-introduced cell or a recombinant protein obtained by introducing an expression vector containing a cDNA encoding Trop-2 obtained in (1) into *Escherichia coli,* yeast, an insect cell, an animal cell or the like, or a purified polypeptide or a partial peptide obtained from a human tissue is used. When a partial peptide is used as the antigen, a conjugate with a carrier protein such as BSA or KLH is prepared to be used.

[0195] After immobilizing these antigens as a solid layer by dispensing in a 96-well plate, a test substance such as serum, a culture supernatant of a hybridoma or a purified monoclonal antibody is dispensed therein as the primary antibody and allowed to react. After thoroughly washing with PBS or PBS-Tween, an anti-immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent material, a radiation compound or the like is dispensed therein as the secondary antibody and allowed to react. After thoroughly washing with PBS-Tween, the reaction depending on the label of the secondary antibody is carried out to select a monoclonal antibody which specifically reacts with the antigen.

[0196] In addition, the antibody which competes with the monoclonal antibody of the present invention can be obtained by adding a test antibody to the above-mentioned binding assay system and carrying out reaction. That is, a monoclonal antibody which competes for binding to an extracellular region of Trop-2 with the obtained monoclonal antibody can be prepared by screening an antibody which inhibits the binding of the monoclonal antibody when the test antibody is added.

[0197] Furthermore, an antibody which binds to an epitope which is the same as the epitope recognized by the monoclonal antibody of the present invention can be obtained by identifying the epitope of the antibody obtained in the above binding assay, and preparing a partial synthetic peptide, a synthetic peptide mimicking the conformation of the epitope or the like, followed by immunization.

(6-b) Kinetic analysis with Biacore

[0198] The kinetics between an antigen and a test substance is measured using Biacore T100 and then the obtained

results are analyzed using analysis software accompanied with the apparatus. After anti-IgG mouse antibody is immobilized onto a CM5 sensor chip by an amine coupling method, a test substance such as culture supernatant of a hybridoma, a purified antibody is allowed to flow, bind at an appropriate amount, and further flow an antigen at plural known concentrations, followed by measuring the binding and dissociation.

[0199] Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using the 1:1 binding model to obtain various parameters. Otherwise, after human Trop-2 is immobilized onto the sensor chip by an amine coupling method or the like, a purified monoclonal antibody is allowed to flow at plural known concentrations followed by measuring the binding and dissociation. Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using bivalent binding model to obtain various parameters.

2. Preparation of recombinant antibodies

[0200] As preparation examples of recombinant antibodies, the methods for producing a human chimeric antibody and a humanized antibody are shown below.

(1) Construction of an expression vector of a recombinant antibody

[0201] An expression vector of a recombinant antibody is an expression vector for animal cell into which DNAs encoding CH and CL of a human antibody have been inserted, and can be constructed by cloning each of DNAs encoding CH and CL of a human antibody into an expression vector for animal cell.

[0202] The C region of a human antibody may be CH and CL of any human antibody. Examples include CH belonging to γ1 subclass, CL belonging to κ class, and the like. As the DNAs encoding CH and CL of a human antibody, the cDNA may be generally used and a chromosomal DNA comprising an exon and an intron can be also used.

[0203] As the expression vector for animal cell, any expression vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [Cytotechnol., 3, 133 (1990)], pAGE103 [J. Biochem., 101, 1307 (1987)], pHSG274 [Gene, 27, 223 (1984)], pKCR *[*Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)], pSG1bd2-4 [Cytotechnol., 4, 173 (1990)], pSE1UK1Sed1-3 [Cytotechnol., 13, 79 (1993)] and the like.

[0204] Examples of promoters and enhancers used for expression vectors for animal cells include an SV40 early promoter [J. Biochem., 101, 1307 (1987)], a Moloney mouse leukemia virus LTR [Biochem. Biophys. Res. Commun., 149, 960 (1987)], an immunoglobulin H chain promoter [Cell, 41, 479 (1985)] and enhancer [Cell, 33, 717 (1983)] and the like.

[0205] The exoression vector of a recombinant antibody may be either of a type in which a gene encoding an antibody H chain and a gene encoding an antibody L chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of an expression vector for of recombinant antibody, easiness of introduction into animal cells, and balance between the expression amounts of antibody H and L chains in animal cells, a tandem type of the expression vector of a recombinant antibody is more preferred [J. Immunol. Methods, 167, 271 (1994)]. Examples of the tandem type of the expression vector of a recombinant antibody include pKANTEX93 (WO 97/10354), pEE18 [Hybridoma, 17, 559 (1998)], and the like.

(2) Obtaining of cDNAs encoding V regions of an antibody derived from a non-human animal and analysis of amino acid sequences

[0206] Obtaining of cDNAs encoding VH and VL of a non-human animal antibody and analysis of amino acid sequence are carried out as follows. mRNA is extracted from hybridoma cells producing a non-human animal antibody to synthesize cDNA. The synthesized cDNA is cloned into a vector such as a phage or a plasmid, to prepare a cDNA library.

[0207] Each of a recombinant phage or recombinant plasmid containing cDNA encoding VH or VL is isolated from the library using DNA encoding a part of the C region or V region of a mouse antibody as a probe. The full length of the objected nucleotide sequences of VH and VL of a mouse antibody on the recombinant phage or recombinant plasmid are determined, and the full length of the amino acid sequences of VH and VL are deduced from the nucleotide sequences, respectively.

[0208] As the non-human animal for preparing a hybridoma cell which produces a non-human antibody, mouse, rat, hamster, rabbit or the like can be used. Any animals can be used so long as a hybridoma cell can be produced therefrom.

[0209] For preparing total RNA from a hybridoma cell, a guanidine thiocyanate-cesium trifluoroacetate method [Methods in Enzymol., 154, 3 (1987)], the use of a kit such as RNA easy kit (manufactured by Qiagen) or the like may be used.

[0210] For preparing mRNA from total RNA, an oligo (dT) immobilized cellulose column method [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)], a kit such as Oligo-dT30 <Super> mRNA Purification Kit (manufactured by Takara Bio) or the like may be used.

[0211] Also, mRNA can be prepared from a hybridoma cell using Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

[0212] For synthesizing cDNA and preparing a cDNA library, publically-known methods [Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Lab. Press (1989); Current Protocols in Molecular Biology, Supplement 1, John Wiley & Sons (1987-1997)]; a kit such as Super Script Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL), ZAP-cDNA Kit (manufactured by Stratagene), *etc.;* or the like may be used.

[0213] As the vector for insertion of the synthesized cDNA templated by mRNA extracted from a hybridoma cell, any vector can be used for preparing a cDNA library may be any vector, so long as the cDNA can be inserted. Examples include ZAP Express [Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], λzapII (manufactured by Stratagene), λgt10 and λgt11 [DNA Cloning: A Practical Approach, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell and pT7T3 18U (manufactured by Pharmacia), pcD2 [Mol. Cell. Biol., 3, 280 (1983)], pUC18 [Gene, 33, 103 (1985)], and the like.

[0214] Any *Escherichia coli* for introducing the cDNA library constructed by a phage or plasmid vector may be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF^t [Strategies, 5, 81 (1992)], C600 [Genetics, 39, 440 (1954)], Y1088 and Y1090 [Science, 222: 778 (1983)], NM522 [J. Mol. Biol., 166, 1 (1983)], K802 [J. Mol. Biol., 16, 118 (1966)], JM105 [Gene, 38, 275 (1985)], and the like.

[0215] A colony hybridization or plaque hybridization method using an isotope- or fluorescence-labeled probe may be used for selecting cDNA clones encoding VH or VL of a non-human antibody or the like from the cDNA library [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)].

[0216] Also, the cDNAs encoding VH or VL can be prepared by synthesizing primers and performing polymerase chain reaction (hereinafter referred to as "PCR"; Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989); Current Protocols in Molecular Biology, Supplement 1, John Wiley & Sons (1987-1997)) using cDNAs synthesized from mRNAs or a cDNA library as a template.

[0217] The nucleotide sequence of the cDNA can be determined by digesting the selected cDNA with appropriate restriction enzymes or the like, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), carrying out a genarally-used method of nucleotide sequence analysis. As a method for nucleotide sequence analysis, an automatic nucleotide sequence analyzer such as ABI PRISM3700 (manufactured by PE Biosystems), A.L.F. DNA sequencer (manufactured by Pharmacia) or the like may be used after a reaction such as the dideoxy method [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)].

[0218] Whether the obtained cDNAs encode the complete amino acid sequences of VH and VL of the antibody containing a secretory signal sequence can be confirmed by estimating the whole amino acid sequences of VH and VL from the determined nucleotide sequence and comparing them with the whole amino acid sequences of VH and VL of known antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)].

[0219] Regarding the complete amino acid sequences of VL and VH of the antibody comprising a secretory signal sequence, the length of the secretory signal sequence and N-terminal amino acid sequence can be deduced by comparing the whole amino acid sequences of VH and VL of the antibody with the whole amino acid sequences of VH and VL of known antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and further the subgroup to which they belong can also be known. Furthermore, the amino acid sequence of each of CDRs of VH and VL can be identified by comparing with amino acid sequences of VH and VL of known antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)].

[0220] Moreover, the novelty of the complete amino acid sequence of VH and VL can be confirmed by carrying out a homology search such as BLAST method [J. Mol. Biol., 215, 403 (1990)] with the obtained complete amino acid sequences of VH and VL in arbitrary database such as SWISS-PROT and PIR-Protein.

(3) Construction of an expression vector of a human chimeric antibody

[0221] cDNAs encoding each of VH and VL of a non-human animal antibody are cloned in the upstream of genes encoding CH and CL of human antibody of an expression vector of the recombinant antibody mentioned in the above (1) to thereby construct an expression vector of a human chimeric antibody.

[0222] In order to ligate a 3'-terminal of cDNA encoding VH or VL of a non-human animal antibody and a 5'-terminal of CH or CL of a human antibody, each cDNA encoding VH and VL of of a non-human animal antibody is constructed so that a nucleotide sequence of a linkage portion would encode appropriate amino acids and have an appropriate recognition sequence of a restriction enzyme.

[0223] An expression vector for human chimeric antibody is constructed by cloning the obtained cDNAs encoding VH and VL respectively in the upstream of gene encoding CH or CL of a human antibody of the expression vector for human CDR-grafted antibody mentioned in the above (1) so that each of them is expressed in an appropriate form.

[0224] In addition, cDNA encoding VH and VL of a non-human antibody is amplified respectively by PCR using a synthetic DNA having a recognition sequence of an appropriate restriction enzyme at both ends and each of them is

cloned to the expression vector of the recombinant antibody obtained in the above (1).

(4) Construction of cDNAs encoding V regions of a humanized antibody

[0225] A cDNA encoding VH or VL of a humanized antibody can be obtained as follows.

[0226] Amino acid sequences of framework regions (hereinafter referred to as "FR") in VH or VL of a human antibody, to which amino acid sequences of CDRs in VH or VL of an antibody derived from a non-human antibody are grafted are respectively selected. Any amino acid sequences of FRs of a human antibody can be used, so long as they are derived from human.

[0227] Examples include amino acid sequences of FRs of human antibodies registered in database such as Protein Data Bank or the like, and amino acid sequences common to subgroups of FRs of human antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the like.

[0228] In order to reduce the loss in the binding activity of the antibody, amino acid sequences having high homology (at least 60% or more) with the amino acid sequence of FR in VH or VL of the original antibody is selected.

[0229] Then, amino acid sequences of CDRs of the original antibody are grafted to the selected amino acid sequence of FRs in VH and VL of the human antibody, respectively, to design each amino acid sequence of VH and VL of a human CDR-grafted antibody. The designed amino acid sequences are converted to DNA sequences by considering the frequency of codon usage found in nucleotide sequences of genes of antibodies [Sequence of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the DNA sequences encoding the amino acid sequences of VH and VL of a human CDR-grafted antibody are designed, respectively.

[0230] Based on the designed nucleotide sequences, several synthetic DNAs consisting of a length of about 100 nucleotides are synthesized, and PCR is carried out using them. In this case, it is preferred that 6 synthetic DNAs per each of the VH and the VL are designed in view of the reaction efficiency of PCR and the DNA lengths possibly synthesized. Furthermore, the cDNA encoding VH or VL of a human CDR-grafted antibody can be easily cloned into the expression vector of human CDR-grafted antibody constructed in (1) by introducing the recognition sequence of an appropriate restriction enzyme to each 5' terminal of the synthetic DNAs existing on the both ends.

[0231] After the PCR, each amplified product is cloned into a plasmid such as pBluescript SK (-) (manufactured by Stratagene) or the like, and the nucleotide sequence is determined according to a method similar to the method described in (2) to obtain a plasmid comprising a DNA sequence encoding the amino acid sequence of VH or VL of a desired human CDR-grafted antibody.

[0232] Otherwise, based on the designed DNA sequence, a synthetic DNA as one DNA encoding each of the full-length VH and the full-length VL can be used instead of the above PCR amplification products. Furthermore, the cDNA encoding VH or VL of a human CDR-grafted antibody can be easily cloned into the expression vector of human CDR-grafted antibody constructed in (1) by introducing the recognition sequence of an appropriate restriction enzyme to the both ends of the synthetic DNAs.

(5) Modification of amino acid sequences of V regions of a human CDR-grafted antibody

[0233] It is known that by just grafting only CDRs in VH and VL of a non-human antibody into FRs of VH and VL of a human antibody, the antigen binding activity decreases compared to the original non-human antibody [BIO/TECHNOLOGY, 9, 266 (1991)].

[0234] In human CDR-grafted antibodies, among the amino acid sequences of FRs in VH and VL of a human antibody, amino acid residues which directly relate to binding to an antigen, amino acid residues which interact with amino acid residues in CDRs, and amino acid residues which maintain the conformation of an antibody and indirectly relate to binding to an antigen are identified and modified to an amino acid residues which are found in the original non-human antibody to thereby increase the antigen binding activity which has been decreased.

[0235] In order to identify the amino acid residues relating to the antigen binding activity in FRs, the three-dimensional structure of an antibody can be constructed and analyzed by X-ray crystallography [J. Mol. Biol., 112, 535 (1977)], computer-modeling *[*Protein Engineering, 7, 1501 (1994)] or the like.

[0236] In addition, a modified human CDR-grafted antibody having a sufficient antibody-binding activity can be obtained by repetition of various attempts for producing several modified antibodies of each antibody and examining the correlation between each of the modified antibodies and its antibody binding activity.

[0237] The amino acid sequences of FRs in VH and VL of a human antibody can be modified using various synthetic DNA for modification by PCR as described in (4). The nucleotide sequence of the amplified product obtained by the PCR is determined according to the method as described in (2) so as to confirm that the designed modification has been generated.

(6) Construction of an expression vector of a human CDR-grafted antibody

[0238] An expression vector for of a human CDR-grafted antibody can be constructed by cloning each cDNA encoding VH or VL of a constructed recombinant antibody into upstream of each gene encoding CH or CL of the human antibody in the expression vector of the recombinant antibody as described in (1).

[0239] For example, by introducing a recognition sequences of appropriate restriction enzymes on the 5'-terminals of synthetic DNAs, which are selected to be positioned at both ends among synthetic DNAs used for the construction of VH or VL of the human CDR-grafted antibody in (4) and (5), VH and VL can be cloned into the upstream of each gene encoding CH or CL of the human antibody in the expression vector of the human CDR-grafted antibody as described in (1), so that they are expressed in appropriate forms.

(7) Transient expression of a recombinant antibody

[0240] The antigen binding activity of various human CDR-grafted antibodies produced can be efficiently evaluated by transiently expressing the recombinant antibodies using the expression vector of the recombinant antibody as described in (3) and (6) or the modified expression vector thereof.

[0241] Any cell can be used as a host cell, so long as the host cell can express a recombinant antibody. Examples include COS-7 cell (ATCC CRL1651) [Methods in Nucleic Acids Res., CRC Press, 283 (1991)].

[0242] Examples of the method for introducing the expression vector into COS-7 cell include a DEAE-dextran method [Methods in Nucleic Acids Res., CRC Press, 283 (1991)], a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the like.

[0243] After introduction of the expression vector, the expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be determined by the enzyme immunoassay [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988), Monoclonal Antibody Experiment Manual, Kodansha Scientific (1987)] and the like.

(8) Obtaining transformants which stably expresses a recombinant antibody and preparation of a recombinant antibody

[0244] A transformant which stably expresses a recombinant antibody can be obtained by introducing the expression vector of a recombinant antibody described in (3) and (6) into an appropriate host cell.

[0245] Examples of the methods for introducing the expression vector into a host cell include electroporation [Japanese Published Unexamined Patent Application No. 257891/90, Cytotechnology, 3, 133 (1990)] and the like.

[0246] As the host cell for introduction of an expression vector of a recombinant antbody, any cell can be used, so long as it is a host cell which can produce the recombinant antibody. Examples include Chinese hamster ovary cell CHO-K1 (ATCC CCL-61), DUKXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S (Life Technologies, Cat # 11619), rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also referred to as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14 (ATCC No. CRL1581), mouse myeloma P3X63-Ag8.653 cell (ATCC No. CRC1580), CHO cell deficient in dihydrofolate reductase gene (hereinafter referred to as "dhfr") [Proc. Natl. Acad. Sci. U.S.A., 77, 4216 (1980)], and the like.

[0247] In addition, host cells in which activity of a protein such as an enzyme relating to synthesis of GDP-fucose, which is an intracellular sugar nucleotide, a protein such as an enzyme relating to the modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α-bond in a complex type N-glycoside-linked sugar chain, or a protein relating to transport of GDP-fucose, which is an intracellular sugar nucleotide, to the Golgi body are introduced is decreased or deleted, such as CHO cell deficient in α1,6-fucosyltransferase gene (WO05/35586, WO02/31140), Lec13 acquiring a lectin resistance [Somatic Cell and Molecular genetics, 12, 55 (1986)] or the like, can also be used.

[0248] After introduction of the expression vector, transformants which stably express a recombinant antibody are selected by culturing in an animal cell culture medium containing an agent such as G418 sulfate (hereinafter referred to as "G418") or the like (Japanese Published Unexamined Patent Application No. 257891/90).

[0249] Examples of the animal cell culture media include RPMI1640 medium (manufactured by Invitrogen), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL301 medium (manufactured by JRH), IMDM medium (manufactured by Invitrogen), Hybridoma-SFM medium (manufactured by Invitrogen), media thereby obtaining various additives such as fetal calf serum (hereinafter referred to as "FCS"), and the like.

[0250] The recombinant antibody can be produced and accumulated in a culture supernatant by culturing the obtained transformants in a medium. The expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be measured by ELISA or the like. Also, in the transformant, the expression amount of the recombinant antibody can be increased by DHFR amplification system (Japanese Published Unexamined Patent Application No. 257891/90) or the like.

[0251] The recombinant antibody can be purified from the culture supernatant of the transformant by a protein A column [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)]. In addition, a method for use in protein purification such as gel filtration, ion-exchange chromatography, ultrafiltration can be combimed.

[0252] The molecular weight of the H chain or the L chain of the purified recombinant antibody or of the whole antibody molecule is determined by polyacrylamide gel electrophoresis *[*Nature, 227, 680 (1970)], Western blotting [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], and the like.

3. Activity evaluation of the monoclonal antibodies or antibody fragments

[0253] The activity of the purified monoclonal antibody or antibody fragment of the present invention can be evaluated in the following manner. The binding activity to Trop-2-expressing cell is evaluated by the binding assay described in the above 1-(6-a) and a surface plasmon resonance method using such as Biacore system described in the above (6-b).

[0254] Furthermore, the binding activity can be measured by fluorescent antibody technique [Cancer Immunol. Immunother., 36, 373 (1993)] or the like. In addition, CDC activity or ADCC activity against an antigen positive cultured cell line is evaluated by a known method [Cancer Immunol. Immunother., 36, 373 (1993)].

4. Method of controlling effector activity of antibodies

[0255] As a method for controlling an effector activity of the monoclonal antibody of the present invention, a method for controlling an amount of fucose (hereinafter, referred to also as "core fucose") which is bound in α-1,6 linkage to N-acetylglucosamine (GlcNAc) present in a reducing end of a complex type N-linked sugar chain which is bound to asparagine (Asn) at position 297 of an Fragment, crystalizable region (hereinafter reffered to as Fc region) of an antibody (WO2005/035586, WO2002/31140, and WO00/61739), a method for controlling an effector activity of a monoclonal antibody by modifying amino acid group(s) of an Fc region of the antibody, and the like are known. The effector activity of the monoclonal antibody of the present invention can be controlled by using any of the methods.

[0256] The effector activity means an antibody-dependent activity which is induced via an Fc region of an antibody. As the effector activity, an antibody-dependent cellular cytotoxicity (ADCC activity), a complement-dependent cytotoxicity (CDC activity), an antibody-dependent phagocytosis (ADP activity) by phagocytic cells such as macrophages or dendritic cells, and the like are known.

[0257] By controlling a content of core fucose of a complex type N-linked sugar chain of Fc, an effector activity of the antibody can be increased or decreased. As a method for reducing a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of the antibody, an antibody to which fucose is not bound can be obtained by the expression of an antibody using a CHO cell which is deficient in α1,6-fucosyltransferase gene. The antibody to which fucose is not bound has a high ADCC activity.

[0258] On the other hand, as a method for increasing a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of an antibody, an antibody to which fucose is bound can be obtained by the expression of an antibody using a host cell into with introduction of α1,6-fucosyltransferase gene is introduced. The antibody to which fucose is bound has a lower ADCC activity than the antibody to which fucose is not bound.

[0259] Further, by modifying amino acid residue(s) in an Fc region of an antibody, the ADCC activity or CDC activity can be increased or decreased. For example, the CDC activity to an antibody can be increased by using the amino acid sequence of the Fc region described in US2007/0148165. Further, the ADCC activity or CDC activity can be increased or decreased by modifying the amino acid as described in US Patent Nos. 6,737,056, 7,297,775 or 7,317,091.

[0260] Furthermore, an antibody in which the effector actibity is controlled can be obtained by combining the above method.

5. Method for treating disease using the monoclonal antibody or antibody fragment of the present invention

[0261] The monoclonal antibody or an antibody fragment thereof of the present invention can be used for treating a disease relating to a Trop-2 positive cell.

[0262] The therapeutic agent comprising the antibody or antibody fragment of the present invention or derivatives thereof may contain only the antibody or antibody fragment or derivatives thereof as an active ingredient, and is typically supplied as a pharmaceutical preparation produced by an publically-known method in the technical field of pharmaceutics, by mixing it with one or more pharmaceutically acceptable carriers.

[0263] Examples of a route of administration include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration. Examples of the dosage form includes sprays, capsules, tablets, powder, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

[0264] The pharmaceutical preparation suitable for oral administration includes emulsions, syrups, capsules, tablets, powders, granules and the like.

[0265] Liquid preparations such as emulsions and syrups can be produced using, as additives, water; sugars such as sucrose, sorbitol and fructose; glycols such as polyethylene glycol and propylene glycol; oils such as sesame oil, olive oil and soybean oil; antiseptics such as p-hydroxybenzoic acid esters; flavors such as strawberry flavor and peppermint; and the like.

[0266] Capsules, tablets, powders, granules and the like can be produced using, as additives, excipients such as lactose, glucose, sucrose and mannitol; disintegrating agents such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose and gelatin; surfactants such as fatty acid ester; plasticizers such as glycerin; and the like.

[0267] The pharmaceutical preparation suitable for parenteral administration includes injections, suppositories, sprays and the like.

[0268] Injections can be prepared using a carrier such as a salt solution, a glucose solution or a mixture of both thereof.

[0269] Suppositories can be prepared using a carrier such as cacao butter, hydrogenated fat or carboxylic acid.

[0270] Sprays can be prepared using the antibody or antibody fragment as such or using it together with a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the compound by dispersing it as fine particles. The carrier includes lactose, glycerol and the like. It is possible to produce pharmaceutical preparations such as aerosols and dry powders

[0271] In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

6. Method for diagnosing diseases using the monoclonal antibody or antibody fragment of the present invention

[0272] The disease relating to Trop-2 can be diagnosed by detecting or determining Trop-2 or a Trop-2-expressing cell using the monoclonal antibody or antibody fragment of the present invention.

[0273] A diagnosis of cancer which is one of the diseases relating to Trop-2 can be carried out by, such as the following detection or measurement of Trop-2. The diagnosis can be carried out by detecting Trop-2 expressing on the cancer cell in a patient's body by an immunological method such as a flow cytometry.

[0274] An immunological method means a method in which an antibody amount or an antigen amount is detected or determined using a labeled antigen or antibody. Examples of the immunological method include radioimmunoassays, enzyme immunoassay, fluorescent immunoassay, luminescent immunoassay, Western blotting method, physico-chemical meathod, and the like.

[0275] Examples of the radioimmunoassays include a method, in which the antibody or the antibody fragment of the present invention is allowed to react with an antigen, an antigen-expressing cell or the like, then a radiolabeling anti-immunoglobulin antibody or a binding fragment thereof is allowed to react therewith, followed by determination using a scintillation counter or the like.

[0276] Examples of the enzyme immunoassays include a method, in which the antibody or antibody fragment of the present invention is allowed to react with an antigen, an antigen-expressing cell or the like, then a labeling anti-immunoglobulin antibody or an binding fragment thereof is allowed to react therewith and the chromogenic pigment is measured by a spectrophotometer, and, for example, sandwich ELISA may be used. As a label used in the enzyme immunoassay, any known enzyme label [Enzyme Immunoassay, published by Igaku Shoin, (1987)] can be used as described already. Examples include alkaline phosphatase labeling, peroxidase labeling, luciferase labeling, biotin labeling and the like.

[0277] Sandwich ELISA is a method in which an antibody is bound to a solid phase, a target antigen for detection or measurement is trapped and another antibody is allowed to react with the trapped antigen. In the ELISA, after two kinds of antibodies, which recognize the target antigen for detection or measurement and has difference in a recognizing site, or the antibody fragment thereof are prepared and the first antibody or antibody fragment is previously fixed on a plate (such as a 96-well plate) and the second antibody or antibody fragment is labeled with a fluorescent substance such as FITC, an enzyme such as peroxidase, a biotin or the like.

[0278] The plate to which the above antibody is fixed is allowed to react with the cell separated from living body or homogenate solution thereof, tissue or homogenate solution thereof, cultured cell supernatant, serum, pleural effusion, ascites, eye solution or the like, then allowed to react with a labeled monoclonal antibody or an antibody fragment and a detection reaction corresponding to the labeled substance is carried out. The antigen concentration in the sample to be tested can be calculated from a calibration curve prepared by a stepwise dilution of antigen of known concentration.

[0279] As antibodies used for sandwich ELISA, either polyclonal antibodies or monoclonal antibodies may be used. Also, antibody fragments such as Fab, Fab' and $F(ab)_2$ may be used. As a combination of two kinds of antibodies used in sandwich ELISA, a combination of monoclonal antibodies or antibody fragments recognizing different epitopes or a combination of a polyclonal antibody with a monoclonal antibody or a antibody fragment may be used.

[0280] Examples of fluorescent immunoassays include a method described in the literatures [Monoclonal Antibodies

- Principles and practice, Third Edition, Academic Press (1996); Manual for Monoclonal Antibody Experiments, Kodansha Scientific (1987)] and the like. As a label for the fluorescent immunoassay, any of known fluorescent labels [Fluorescent Immunoassay, by Akira Kawao, Soft Science, (1983)] may be used as described already. Examples of the labels include FITC, RITC and the like.

[0281] The luminescent immunoassay can be carried out using the methods described in the literature [Bioluminescence and Chemical Luminescence, Rinsho Kensa, 42, Hirokawa Shoten (1998)] and the like. As a label for luminescent immunoassay, any of known luminescent labels can be included. Examples include acridinium ester, lophine or the like may be used.

[0282] Western blotting can be carried out using a method in which an antigen or an antigen-expressing cell is fractionated by SDS-polyacrylamide gel electrophoresis [Antibodies-A Laboratory Manual (Cold Spring Harbor Laboratory, 1988)], the gel is blotted onto PVDF membrane or nitrocellulose membrane, the membrane is allowed to react with an antibody or antibody fragment which can recognize the antigen, further allowed to react with an anti-mouse IgG antibody or antibody fragment which is labeled with a fluorescent substance such as FITC, an enzyme label such as peroxidase, a biotin labeling, and the label is visualized to confirm the reaction. An example thereof is described below.

[0283] Cells or tissues expressing a polypeptide having the amino acid sequence represented by SEQ ID NO: is expressed are dissolved in a solution and, 0.1 to 30 μg of protein per lane is electrophoresed under reducing conditions by an SDS-PAGE method. The electrophoresed protein is transferred to a PVDF membrane and allowed to react with PBS containing 1 to 10% of BSA (hereinafter referred to as "BSA-PBS") at room temperature for 30 minutes for blocking.

[0284] Here, the monoclonal antibody of the present invention is allowed to react therewith, washed with PBS containing 0.05 to 0.1% Tween 20 (hereinafter referred to as "Tween-PBS") and allowed to react with a peroxydase-labeled goat anti-mouse IgG at room temperature for 2 hours. After washing with Tween-PBS, a band bound by the monoclonal antibody is detected using ECL Western Blotting Detection Reagents (manufactured by Amersham) or the like to thereby detect a polypeptide having the amino acid sequence represented by SEQ ID NO:1. As an antibody used for the detection in Western blotting, an antibody which can be bound to a polypeptide having a native conformation is used.

[0285] The physicochemical method can be carried out, for example, by reacting Trop-2 as the antigen with the antibody or antibody fragment of the present invention to form an aggregate, and detecting this aggregate. Other examples of the physicochemical methods include a capillary method, a one-dimensional immunodiffusion method, an immunoturbidimetry, a latex immunoturbidimetry [Handbook of Clinical Test Methods, Kanehara Shuppan, (1998)] and the like.

[0286] For example, in a latex immunodiffusion method, a carrier such as polystyrene latex having a particle size of about of 0.1 to 1 μm sensitized with antibody or antigen may be used and when an antigen-antibody reaction is carried out using the corresponding antigen or antibody, scattered light in the reaction solution increases while transmitted light decreases. When such a change is detected as absorbance or integral sphere turbidity, it is now possible to measure antigen concentration, etc. in the sample to be tested.

[0287] In addition, for the detection of the Trop-2-expressing cell, known immunological detection methods can be used, and an immunoprecipitation method, an immuno cell staining method, an immune tissue staining method, a fluorescent antibody staining method and the like are preferably used.

[0288] An immunoprecipitation method can be carried out using a method in which a cell expressing Trop-2 is allowed to react with the monoclonal antibody or antibody fragment thereof of the present invention and then a carrier having specific binding ability to immunoglobulin such as protein G-Sepharose is added so that an antigen-antibody complex is precipitated. Also, the following method can be carried out. The above-described antibody or antibody fragment of the present invention is immobilized on a 96-well plate for ELISA and then blocked with BSA-PBS.

[0289] When the antibody is in a non-purified state such as a culture supernatant of hybridoma, anti-mouse immunoglobulin or anti-rat immunoglobulin or protein A or Protein G or the like is previously immobilized to a 96-well plate for ELISA and blocked with BSA-PBS and a culture supernatant of hybridoma cell is dispensed thereto for binding. After BSA-PBS is discarded and the residue is sufficiently washed with PBS, reaction is carried out with a dissolved solution of cells or tissues expressing Trop-2. An immune precipitate is extracted from the well-washed plate with a sample buffer for SDS-PAGE and detected by the above-described Western blotting.

[0290] An immune cell staining method or an immune tissue staining method are a method where antigen-expressing cells or tissues are treated, if necessary, with a surfactant, methanol or the like to increase permeability of an antibody to the cells or tissues, then the monoclonal antibody of the present invention is allowed to react therewith, then further allowed to react with an anti-immunoglobulin antibody or binding fragment thereof labeled by a fluorescent substrate such as FITC, an enzyme label such as peroxidase, a biotin or the like, and the label is visualized and observed under a microscope.

[0291] In addition, detection can be carried out by an immunofluorescent staining method [Monoclonal Antibodies - Principles and practice, Third Edition, Academic Press (1996), Manual for Experiments of Monoclonal Antibodies, Kodansha Scientific (1987)] in which cells are allowed to react with a fluorescence-labeled antibody and analyzed by a flow cytometer. Particularly, the monoclonal antibody or antibody fragment of the present invention which binds to an extracellular region of the Trop-2 can detect a cell expressing the polypeptide maintaining a native conformation.

[0292] In addition, in the case of using FMAT8100HTS system (manufactured by Applied Biosystems) and the like among fluorescent antibody staining methods, the antigen amount or antibody amount can be measured without separating the formed antibody-antigen complex and the free antibody or antigen which does not participate in the formation of the antibody-antigen complex.

The present invention can provide a monoclonal antibody or an antibody fragment thereof, which binds to the extracellular region of human Trop-2 with high affinity, and exhibits high antibody-dependent cellular cytotoxicity (hereinafter referred to as "ADCC activity") and high antitumor activity; a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtainable by introducing the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a therapeutic agent or a diagnostic agent using the antibody or the antibody fragment thereof.

EXAMPLE

[0293] Hereinafter, exemplary embodiments of the present invention will be described specifically. However, the present invention is not limited to the following examples

[Example 1]

[0294] Obtaining of an anti-Trop-2 mouse monoclonal antibody

(1) Preparation of antigen

[0295] The cancer cell line Pc1 was established from a human prostate cancer and used as an antigen.

(2) Immunization of animal and preparation of antibody-producing cells

[0296] Balb/c mice were immunized by administering with 1×10^6 Pc1 cells 5 times every two weeks and spleens were isolated on three days after the last administration. Spleens were cut out in an RPMI1640 (hereinafter referred as "RPMI medium") and crushed out and centrifuged (1,200 rpm, 5 minutes). Then, a Tris-ammonium chloride buffer (pH 7.6) was added to the obtained precipitate fraction and treated for 1 minute at 37°C to remove erythrocytes. After washing the precipitate fraction (the cell fraction) 3 times with the RPMI medium, the obtained cells was used for cell fusion.

(3) Preparation of mouse myeloma cells

[0297] An 8-azaguanine-resistant mouse myeloma cell line P3X63Ag8U.1(P3-U1) was cultured in RPMI1640 (manufactured by Invitrogen) supplemented with 10% fetal calf serum and used as a parental cell line for cell fusion.

(4) Preparation of adenovirus vector

[0298] Ax3CAZ3-FZ33 in which β-galactosidase gene was introduced in a modified adenovirus type 5 containing a Z33 motif of protein A which has a binding ability IgG in the HI loop was used for the introduction into hybridomas.

(5) Preparation of hybridoma

[0299] The mouse spleen cells obtained in Example 1(2) and the myeloma cells obtained in Example 1(3) were mixed at a ratio of 5:1 and centrifuged (1,200 rpm, 5 minutes). After thoroughly loosening a group of cells in the thus obtained precipitate fraction, 1 ml of polyethyleneglycol-4000 (PEG-4000) was added thereto over 1 minute under stirring, 1 ml of the RPMI medium was added to the cell solution several times every one minute, and then the total volume was adjusted to 50 ml by adding the RPMI medium. The cell suspension was centrifuged (900 rpm, 5 minutes), cells of the obtained precipitation fraction were mildly loosened, and then the cells were gently suspended in 100 ml of HAT medium [MI1640 medium supplemented with 10% fetal bovine serum and HAT Media Supplement]. The suspension was dispensed at 200μ l/well into a 96-well culture plate and cultured in a 5% CO_2 incubator at 37°C until the cells reached 50% confluence.

[0300] Meanwhile, the culture medium was removed from the Pc1 cells cultured in a 96-well culture plate, and the culture supernatant of the hybridoma was added thereto. After the cells were incubated at 4°C for 1 hour, the culture supernatant was removed and the cells were washed twice with PBS. Then, Ax3CAZ3-FZ33 prepared in Example 1(4) was added into the cells so as to give a multiplicity of infection (MOI) of 1,000. After the cells were incubated again at 4°C for 1 hour, the virus solution was removed and the cells were washed twice with PBS.

[0301] β-Galactosidase reporter assay was carried out using β-Gal Reporter Gene Assay, chemiluminescent kit (manufactured by Roche Diagnostics), and wells of which activity was high were selected. Cloning the cells of the selected wells by the limiting dilution method was repeated twice, and an antibody which reacts with Pc1 cell-producing a hybridoma which produced an antibody which reacts with Pc1 cell was isolated [The Prostate, 67, 1163 (2007)].

(6) Determination of target antigen

[0302] A target antigen of the hybridoma established in Example 1(5) was determined by a known method [The Prostate, 67, 1163 (2007)] and antibody KM4097 which recognized Trop-2 was found among the hybridomas. The isotype of the antibody was determined as IgG1 and κ by using Iso Strip mouse monoclonal antibody isotyping kit (manufactured by Roche Applied Science).

[Example 2]

Preparation of IgG1 type chimeric antibody and defucose type antibody of anti-Trop-2 mouse antibody AR47A6.4.2

(1) Construction of vectors for expression of AR47A6.4.2-derived human chimeric antibody

[0303] For the purpose of the comparison with the activity of an anti-Trop-2 monoclonal antibody of the present invention, a chimeric antibody (hereinafter referred as "cAR47A6.4.2") comprising the variable regions of known anti-Trop-2 mouse monoclonal antibody AR47A6.4.2 and the Fc region of human IgG1 was prepared.

[0304] Based on the sequence information disclosed in the specification of U.S. Patent No 7,420,040, plasmids in which the DNA sequence represented by SEQ ID NO:3 and the DNA sequence represented by SEQ ID NO:4 were inserted into a pZErO-2 vector as a gene encoding VH and a gene encoding VL of AR47A6.4.2, respectively, were made in custom synthesis (offered by IDT).

[0305] The plasmid comprising VH of AR47A6.4.2 was treated with restriction enzymes *Apa*I (manufactured by New England Biolabs) and *Not*I (manufactured by New England Biolabs) to obtain, the *Not*I-*Apa*I fragment comprising VH was obtained. In addition, the plasmid comprising VL of AR47A6.4.2 was obtained with restriction enzymes *Bsi*WI (manufactured by New England Biolabs) and *Eco*RI (manufactured by New England Biolabs) to obtain, the *Eco*RI-*BsiW*I fragment.

[0306] Meanwhile, an antibody expression vector pKANTEX93 was treated with two kinds of combinations of restriction enzymes *Not*I and *Apa*I, or *Eco*RI and *Bsi*WI. Two kinds of combinations of the *Not*I-*Apa*I fragment comprising VH and the *Not*I-*Apa*I fragment derived from pKANTEX93, and the *Eco*RI-*BsiW*I fragment comprising VL and the *Eco*RI-*BsiW*I fragment derived from pKANTEX93 were ligated using Ligation high (manufactured by Toyobo Co., Ltd.).

[0307] *Escherichia coli* DH5α (manufactured by Toyobo Co., Ltd.) was transformed using the obtained ligation reaction solution. Plasmid DNAs were prepared from the transformed clone, and pKANTEX93 vectors in which a cDNA encoding VH or VL of AR47A6.4.2 was cloned were obtained.

[0308] After treating each pKANTEX93 vector comprising VH or VL of AR47A6.4.2 with restriction enzymes *Eco*RI and *Not*I, the *Eco*RI-*Not*I fragment comprising VL and the *Not*I- *Eco*RI fragment comprising VH were obtained. The two kinds of obtained fragments were ligated using Ligation high (manufactured by Toyobo Co., Ltd.).

[0309] *Escherichia coli* DH5α (manufactured by Toyobo Co., Ltd.) was transformed using the thus obtained ligation reaction solution. A plasmid DNA was prepared from the transformed clone, and an anti-Trop-2 human chimeric antibody expression vector comprising cDNA encoding VH and VL of AR47A6.4.2 was obtained.

(2) Expression of AR47A6.4.2-derived human chimeric antibody using animal cells

[0310] The antibody expression vector obtained in Example 2(1) was introduced into an animal cell line by a conventional method [Antibody Engineering, A Practical Guide, W. H. Freeman and Company (1992)] to obtain a transformant which produces anti-Trop-2 human chimeric antibody. For a host animal cell line, a CHO/DG44 cell line and a CHO/DG44 cell in which α1,6 fucosyltransferase (FUT8) gene is knocked out (hereinafter referred as "FUT8 knockout CHO/DG44 cell) were used.

(3) Obtaining of AR47A6.4.2 purified chimeric antibody

[0311] The cell suspensions of each transformant obtained in Example 2(2) were centrifuged for 20 minutes under conditions of 3,000 rpm at 4°C to recover the culture supernatants, and then the culture supernatants were filtration-sterilized using Millex GV filter having a pore size of 0.22 μm (manufactured by Millipore). Each anti-Trop-2 human chimeric antibody was purified from the thus obtained culture supernatant using Protein A High-capacity Resin (manufactured

by Millipore). The antibody obtained from the transformant derived from a CHO/DG44 cell and the antibody obtained from the transformant derived from FUT8 knockout CHO/DG44 cell were called as cAR47A6.4.2 and cAR47A6.4.2-P, respectively.

(4) Determination of fucose content in AR47A6.4.2 chimeric antibody

[0312] In accordance with the method described in WO2002/31140, a ratio of a sugar chain in which fucose was not bound to the complex-type N-linked sugar chains present in cAR47A6.4.2 and cAR47A6.4.2-P was examined. The results are shown in Table 1.

[Table 1]

Fucose content of anti-Trop-2 antibody	
cAR47A6.4.2	100 %
cAR47A6.4.2-P	0 %

[0313] As the results shown in Table 1, it was confirmed that fucose was not added to cAR47A6.4.2-P.

[Example 3]

Evaluation of antigen binding activity of anti-Trop-2 mouse monoclonal antibody KM4097

(1) Reactivity of KM4097 to human Trop-2-positive cell line by flow cytometry (hereinafter referred as "FCM")

[0314] In PBS containing 1% of BSA (hereinafter referred as "BSA-PBS"), each of human pancreatic carcinoma cell line BxPC-3, human breast cancer cell line MCF-7, ovarian cancer cell line CaOV-3 and colon cancer cell line Colo205 were suspended at a density of 1 to 2 $\times$ 10^5 cells for blocking.

[0315] Then, anti-Trop-2 monoclonal antibody KM4097 was diluted appropriately with BSA-PBS and added to the cells to give a total volume of 100 μl. After the cell suspension was allowed to incubate on ice for 60 minutes, the cells were washed with BSA-PBS. Then, 100 μl of a FITC-labeled goat anti-mouse IgG antibody (manufactured by Dako Japan Co., Ltd.) which was diluted with BSA-PBS was added to the cells to react on ice for 30 minutes. After washing the cells with BSA-PBS, the cells were suspended in PBS. The fluorescence intensity was measured by FCM (manufactured by Beckman Coulter Inc.).

[0316] Histograms and the mean fluorescence intensity (MFI value) in cases where 10 μg/ml of KM4097 was allowed to react are shown in Fig. 1(a) to Fig. 1(d) and Table 2.

[0317]

[Table 2]

MFI value of anti-Trop-2 monoclonal antibody KM4097	
BxPC-3	124
CaOV-3	146
MCF-7	92.8
Colo205	54.8

[0318] Based on the results shown in Fig. 1(a) to Fig. 1(d) and Table 2, it was found that KM4097 could bind to any of the Trop-2-positive cell lines in an antibody concentration-dependent manner.

(2) Binding activity of KM4097 to recombinant human Trop-2 using Biacore

[0319] In order to analyze the binding activity of each anti-Trop-2 mouse monoclonal antibody to recombinant human Trop-2 in view of the reaction kinetics, the binding activity was measured by a surface plasmon resonance method (SPR method). In addition, the binding activity of 77220 (manufactured by R&D) and MOv16 (manufactured by Alexis Biomedical) which was known anti-Trop-2 monoclonal antibodies were also analyzed as a control.

[0320] All the operations described below were carried out using Biacore T100 (manufactured by GE Healthcare Bio-sciences). Tetra-His antibody (manufactured by Qiagen) was immobilized so as to be 12,000 RU (resonance unit) on the CM5 sensor 77 chip (manufactured by GE Healthcare Bio-sciences) using Amine Coupling Kit (manufactured by

GE Healthcare Bio-sciences). After recombinant Trop-2/Fc/His fusion proteins (manufactured by R&D) were allowed to be captured on the sensor chip, anti-Trop-2 antibodies which were diluted in five-fold steps, starting from a concentration of 5,000ng/ml were flowed at 30μL/min, followed by obtaining sensorgrams at each concentration.

[0321] Using the software for analysis accompanied with the equipment, the results were analyzed based on Bivalent Analyte model to obtain the binding rate constant and the dissociation rate constant for binding of each antibody to a recombinant human Trop-2/Fc/His fusion protein.

[0322] The binding rate constants (hereinafter referred as "ka"), the dissociation rate constants (hereinafter referred as "kd"), and the dissociation constants K_D (kd/ka) of each antibody were shown in Table 3 (the mean value of duplicate experiments).

[Table 3]

	kd (1/Ms)	ka (1/s)	K_D (nM)
KM4097	3.89×10^{5}	1.01×10^{-4}	0.261
77220	5.55×10^{5}	6.10×10^{-4}	1.10
MOv16	1.83×10^{5}	1.84×10^{-4}	0.929

[0323] As shown in Table 3, KM4097 exhibited higher affinity than conventional antibodies.

(3) Evaluation of competition binding activities between anti-Trop-2 monoclonal antibodies using FCM

[0324] In BSA-PBS, human pancreatic carcinoma cell line BxPC-3 was suspended at a density of 1 to 2×10^{5}cells for blocking. Meanwhile, the concentration of cAR47A6.4.2 was made to be 0.3 μg/ml with BSA-PBS and KM4097 or 77220 was serially diluted with BSA-PBS. Each prepared antibody solution was added into the cell suspension to give a total volume of 100 μl.

[0325] After the cell suspension was allowed to react on ice for 60 minutes, the cells were washed with BSA-PBS. Then, 100 μl of a FITC-labeled goat anti-mouse IgG antibody (manufactured by Jackson Immuno research Laboratories) which was diluted with BSA-PBS was added to the cells to allow to react on ice for 30 minutes. After washing the cells with BSA-PBS, the cells were suspended in PBS. The fluorescence intensity was measured by FCM (manufactured by Beckman Coulter Inc.).

[0326] Fig.2 shows the mean fluorescence intensity (MFI value) of the binding activity of cAR47A6.4.2 to BxPC-3 cells when the concentration of KM4097 or 77220 was changed. It was found that, while 77220 inhibited the binding of cAR47A6.4.2 to cells in a concentration-dependent manner, KM4097 did not inhibit the binding of cAR47A6.4.2 to BxPC-3 cells.

[0327] Subsequently, BxPC-3 cells were blocked with BSA-PBS in the same manner as described the above. Meanwhile, the concentration of KM4097 or 77220 was made to be 1 μg/ml with BSA-PBS and cAR47A6.4.2 was serially diluted with BSA-PBS. The prepared antibody solution was added into the cell suspension and then the total volume was adjusted to 100 μl.

[0328] After the cell suspension was allowed to react on ice for 60 minutes and the cells were washed with BSA-PBS. Then, 100 μl of a FITC-labeled goat anti-mouse IgG antibody (manufactured by Dako Japan Co., Ltd.) which was diluted with BSA-PBS was added to the cells to carry out the reaction on ice for 30 minutes. After washing the cells with BSA-PBS, the cells were suspended in PBS and then the fluorescence intensity was measured by FCM (manufactured by Beckman Coulter Inc.).

[0329] Fig. 3 shows that the mean fluorescence intensity (MFI values) of the binding ability of KM4097 or 77220 to BxPC-3 cells when the concentration of cAR47A6.4.2 was changed. As shown in Fig. 3, cAR47A6.4.2 was found not to inhibit the binding of KM4097 to BxPC-3 cells.

[0330] Based on the above results, it was shown that KM4097 recognizes an epitope different from cAR47A6.4.2.

(4) Evaluation of competitive activities between anti-Trop-2 monoclonal antibodies using binding ELISA of Trop-2

[0331] Into a 96-well ELISA plate (manufactured by Greiner), 2 μg/ml of a recombinant human Trop-2/Fc/His fusion protein (manufactured by R&D) was dispensed at 50 μl/well, and allowed to stand at 4°C overnight for immobilization. After washing the plate with PBS, BSA-PBS was added thereto at 100 μl/well and allowed to react at room temperature for 1 hour for blocking.

[0332] After removing BSA-PBS from the plate, BSA-PBS dilution solution of 0.3 μg/ml of KM4097 (mouse IgG1 subclass) as the primary antibody and serial dilution solutions of a competing antibody (subclass of the antibody is other than mouse IgG1) with BSA-PBS were dispensed to give a total volume of 50 μl/well and allowed to stand for 2 hours.

[0333] After washing the plate with PBS containing 0.05% polyoxyethylene (20) sorbitan monolaurate manufactured by Wako Pure Chemical Industries, Ltd.; equivalent to ICI Inc.'s trademark Tween 20)(hereinafter referred as "Tween-PBS"), a peroxidase-labeled rabbit anti-mouse IgG1 antibody (manufactured by Zymed) as the second antibody was added at 50 μl/well and allowed to stand at room temperature for 1 hour.

[0334] After washing the plate with Tween-PBS, the ABTS [2,2-Azino-bis(3-ethyl benzothiazol-6-sulfonic acid)ammonium)] substrate solution [1 mmol/l ABTS, 0.1 mol/l citric acid buffer (pH 4.2), 0.1% H_2O_2] was added at 50 μl/well for color development, and the chromogenic reaction was stopped by adding a 5% SDS (sodium lauryl sulfate) solution at a volume of 50 μl/well. Thereafter, the value of the absorbance of OD415 nm was measured by a plate reader Emax (manufactured by Molecular Devices, LLC.).

[0335] Fig. 4 shows the binding of KM4097 to a Trop-2/Fc/His fusion protein when the concentration of 77220 was changed. As shown in Fig. 4, it was found that 77220 did not inhibit the binding of KM4097 to a Trop-2/Fc/His fusion protein.

[0336] Next, in the same manner as described in the experiment before, a recombinant human Trop-2/Fc/His fusion protein was immobilized onto a 96-well ELISA plate, washed with PBS, and blocked with BSA-PBS. In the plate, BSA-PBS dilution solution of 0.3 μg/ml of 77220 (mouse IgG2a subclass) as the primary antibody and BSA-PBS serial dilution solutions of a competing antibody (subclass of the antibody is other than mouse IgG2a) were dispensed to give a total volume of 50 μl/well and allowed to stand for 2 hours.

[0337] After washing the plate with Tween-PBS, a peroxidase-labeled rabbit anti-mouse IgG2a antibody (manufactured by Zymed) as the second antibody was added at 50 μl/well and allowed to stand at room temperature for 1 hour. After washing the plate with Tween-PBS, the ABTS substrate solution was added at 50 μl/well for color development, and the chromogenic reaction was stopped by adding a 5% SDS solution at 50 μl/well. Then, the value of the absorbance of OD415 nm was measured using a plate reader Emax (manufactured by Molecular Devices, LLC.).

[0338] Fig. 5 shows the binding of 77220 to a Trop-2/Fc/His chimeric protein when the concentrations of KM4097 and MOv16 were changed. As shown in Fig. 5, it was found that MOv16 inhibited the binding of 77220 to a Trop-2/Fc/His chimeric protein, but KM4097 did not inhibit the binding of 77220 to a Trop-2/Fc/His chimeric protein.

[0339] Based on the above results, it was found that KM4097 recognized the epitope which was different from those of 77220 and MOv16.

[Example 4]

Isolation of cDNA encoding the variable regions of anti-Trop-2 mouse monoclonal antibody KM4097

(1) Preparation of mRNA from KM4097 producing hybridoma cells

[0340] From 5×10^7 to 1×10^8 cells of the hybridoma cells obtained in Example 1, mRNA was prepared using RNAeasy Maxi Kit (manufactured by Qiagen) and Oligotex™-dT30<Super>mRNA Purification Kit (manufactured by Takara Bio Inc.).

(2) Gene cloning of H chain variable regions and L chain variable regions of KM4097

[0341] Using SMART RACE cDNA Amplification Kit (manufactured by Clontech), a cDNA was obtained from the mRNA of KM4097 obtained in Example 4(1). A cDNA fragment of VH of each antibody was amplified by carrying out PCR using the thus obtained cDNA as the template and using a universal primer Amix (attached to the kit) and the mouse IgG1-specific primers (SEQ ID NOs:5 and 6). AcDNA fragment of VL of each antibody was amplified by carrying out PCR using the mouse Ig(κ)-specific primers (SEQ ID NOs:7 and 8) instead of primers specific to subclass of each antibody. In the every PCRs, using PTC-200 DNA Engine (manufactured by Bio-Rad Laboratories), after heating at 94°C for 2 minutes, reaction was carried out by 30 cycles, each cycle consisting of reaction at 94°C for 15 seconds and reaction at 68°C for 1 minutes.

[0342] The thus obtained PCR products were separated by agarose gel electrophoresis, and a DNA fragment comprising VH and a DNA fragment comprising VL were obtained. Subsequently, after deoxyadenines were attached to both ends of the obtained each DNA fragment using Target Clone Plus (manufactured by Toyobo Co., Ltd.), each of the DNA fragments was ligated to a pTA2 vector. Each of the obtained vectors was introduced into *Escherichia coli* DH5α, and each of the plasmids was extracted from the obtained transformed clone. The nucleotide sequence of each PCR product obtained by cloning was analyzed by BigDye Terminator Cycle sequencing FS Ready Reaction Kit (manufactured by PE Biosystems) and a sequencer manufactured by the same company, ABI PRISM 3700. As a result, it was determined that a plasmid comprising complete length cDNA of VH and a plasmid comprising complete length cDNA of VL in which an ATG sequence considered to be the initiation codon was present in the 5' terminal of the cDNA, were obtained.

(3) Analysis of V region amino acid sequences of KM4097

[0343] Complete nucleotide sequence of VH of KM4097 contained in the plasmid obtained in Example 4(2) was shown as SEQ ID NO:9, complete amino acid sequence of VH including a signal sequence deduced from the nucleotide sequence was shown as SEQ ID NO:10, the complete nucleotide sequence of VL contained in the plasmid is represented by SEQ ID NO:11, and the complete amino acid sequence of VL including a signal sequence deduced from the nucleotide sequence was shown as SEQ ID NO:12.

[0344] Based on the comparison with the known sequence data of mouse antibodies [SEQUENCES of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], it was found that the isolated each cDNA was a complete length cDNA encoding the variable region (hereinafter referred as "V region") including a secretion signal sequence of KM4097, and the sequence at positions 1 to 19 of the amino acid sequence represented by SEQ ID NO: 10 is the secretion signal sequence of the H chain of KM4097, and the sequence at positions 1 to 20 of the amino acid sequence represented by SEQ ID NO:12 is the secretion signal sequence of the L chain of KM4097.

[0345] Next, the novelty of the amino acid sequences of the VH and the VL of KM4097 was examined. Using GCG Package (version 9.1, Genetics Computer Group) as the sequence analyzing system, amino acid sequence data base of the known proteins was retrieved by the BLASTP method *[*Nucleic Acids Res., 25, 3389 (1997)]. As a result, since completely identical amino acid sequences were not found for both of the VH and VL in the data base, it was found that the VH and the VL of KM4097 have novel amino acid sequences.

[0346] CDRs of the VH and VL of KM4097 were identified by comparing with known amino acid sequences of antibodies. The amino acid sequences of CDR1, CDR2 and CDR3 of the VH of KM4097 were represented by SEQ ID NOs:13, 14 and 15, respectively, and the amino acid sequences of the CDR1, CDR2 and CDR3 of the VL were represented by SEQ ID NOs:16, 17 and 18, respectively.

[Example 5]

Preparation of human chimeric antibody KM4097

(1) Construction of vector for expression of human chimeric antibody KM4097

[0347] A chimeric antibody prepared in the present invention is a chimeric antibody in which the H chain constant region of Fc region of human IgG1 and human κ type L chain constant region were ligated to the variable region of the H chain and the variable region of the L chain of KM4097 obtained in Example 4(2), respectively. A vector for expression of a human chimeric antibody KM4097 was constructed in the following manner using the vector for expression of antibody pKANTEX93 and each pTA2 vector comprising VH or VL obtained in Example 4(2).

[0348] PCR was carried out by preparing 15 μl of a reaction solution containing 100 ng of the pTA2 vector having the VH or VL of KM4097 as the template, 1.5 μl of 10×ExTaq buffer, 1.2 μl of 2.5 mmol/l dNTP, 1 μl of ExTaq DNA polymerase (manufactured by Takara Bio Inc.), and 0.75 μl of 10 μmol/l of each of the primers which are specific for the VH or VL. The primers of the VH of KM4097 are represented by SEQ ID NOs:19 and 20 and the primers of the VL of KM4097 are represented by SEQ ID NOs:21 and 22. The PCR was carried out by, after heat treatment at 94°C for 2 minutes, repeating 30 cycles, each cycle consisting of reaction at 94°C for 15 seconds, reaction at 55°C for 30 seconds, and reaction 72°C for 1 minute, followed by reaction at 72°C for 10 minutes.

[0349] Respective PCR reaction products were separated by agarose gel electrophoresis, and the DNA fragment comprising VH and the DNA fragment comprising VL were obtained. Next, using TOPO TA cloning kit for sequencing (manufactured by Invitrogen), the each DNA fragment was inserted into a pCR4-TOPO vector.

[0350] After treating the plasmid comprising the VH of KM4097 with restriction enzymes *Apa*I (manufactured by New England Biolabs) and *Not*I (manufactured by New England Biolabs), the *Not*I-*Apa*I fragment comprising the VH was obtained. In addition, after treating the plasmid comprising the VL of KM4097 with restriction enzymes *Bsi*WI (manufactured by New England Biolabs) and *Eco*RI (manufactured by New England Biolabs), the *Eco*RI-*Bsi*WI fragment was obtained.

[0351] Meanwhile, the antibody expression vector pKANTEX93 was treated with two kinds of combinations of restriction enzymes *Not*I and *Apa*I, or *Eco*RI and *Bsi*WI. Two kinds of combinations of the *Not*I-*Apa*I fragment comprising the VH and the *Not*I-*Apa*I fragment derived from pKANTEX93, and the *Eco*RI-*Bsi*WI fragment comprising the VL and the *Eco*RI-*Bsi*WI fragment derived from pKANTEX93 were ligated using Ligation high (manufactured by Toyobo Co., Ltd.).

[0352] *Escherichia coli* DH5α (manufactured by Toyobo Co., Ltd.) was transformed using the thus obtained ligation reaction solution. Plasmid DNAs were prepared from the transformed clone, and pKANTEX93 vectors were obtained in which a cDNA encoding the VH or a cDNA encoding the VL of KM4097 were cloned, respectively.

[0353] After treating each pKANTEX93 vector comprising the VH or VL of KM4097 with restriction enzymes *Eco*RI and *Not*I, the *Eco*RI-*Not*I fragment comprising the VL and the *Not*I- *Eco*RI fragment comprising the VH were obtained.

The two kinds of fragments obtained were ligated using Ligation high (manufactured by Toyobo Co., Ltd.). *Escherichia coli* DH5α (manufactured by Toyobo Co., Ltd.) was transformed using the thus obtained ligation reaction solution. A plasmid DNA was prepared from the transformed clone, and anti-Trop-2 human chimeric antibody expression vectors which had a cDNA encoding the VH and a cDNA encoding the VL of KM4097 were obtained.

(2) Obtaining KM4097 human chimeric antibody using animal cells

[0354] In the same manner described in Example 2, the vector for expression of anti-Trop-2 chimeric antibody obtained in Example 5(1) was introduced into a CHO/DG44 cell line and FUT8 knockout CHO/DG44 cell line, and from the obtained each transformant, anti-Trop-2 human chimeric antibodies KM4590 and KM4591 were obtained.

(3) Determination of fucose content in KM4097 chimeric antibodies

[0355] In accordance with the method described in W02002/31140, a ratio of a sugar chain in which fucose was not bound to the complex-type N-linked sugar chains present in KM4590 and KM4591 was examined. The results are shown in Table 4.

[Table 4]

Fucose content of anti-Trop-2 antibody	
KM4590	100 %
KM4591	0 %

[0356] As the results shown in Table 4, it was demonstrated that fucose was not added to human chimeric antibody KM4591 prepared in Example 5(2).

[Example 6]

Activity evaluation of anti-Trop-2 human chimeric antibodies KM4590 and KM4591

[0357] Activity of KM4590 and KM4591 obtained in Example 5 and cAR47A6.4.2 and cAR47A6.4.2-P obtained in Example 2 were evaluated.

(1) Binding activity of anti-Trop-2 human chimeric antibody to recombinant human Trop-2 using Biacore

[0358] In the same manner described in Example 3, the affinity of KM4591 and cAR47A6.4.2-P to human Trop-2/Fc/His fusion proteins (manufactured by R&D) were measured. ka, kd, and K_D (kd/ka) of each antibody are shown in Table 5 (the mean values of duplicate experiments).

[Table 5]

	kd (1/Ms)	ka (1/s)	K_D (nM)
KM4591	4.16×10^{5}	5.80×10^{-5}	0.143
cAR47A6.4.2-P	2.21×10^{5}	2.18×10^{-4}	1.01

[0359] As shown in Table 5, KM4591 exhibited higher affinity than cAR47A6.4.2-P.

(2) Evaluation of ADCC activity of anti-Trop-2 human chimeric antibodies toward human cancer cell lines

[0360] ADCC activity of KM4590 and cAR47A6.4.2-1 on human cancer cell lines was measured in the following manner.

(2)-1 Preparation of target cell suspension

[0361] After washing with PBS, human cancer cell lines MCF-7 and Colo205 were washed using phenol red-free RPMI1640 medium (manufactured by Invitrogen) containing 5% fetal bovine serum (FBS, manufactured by Invitrogen) (hereinafter referred as "ADCC activity measuring medium"), and then the cell density was adjusted appropriately and used as the target cell suspension.

(2)-2 Preparation of effector cell solution

[0362] According to the following manner, peripheral blood mononuclear cells (PMBC) were separated from peripheral blood of healthy subjects. Using a syringe containing 0.5 ml of heparin sodium (manufactured by Ajinomoto), 50 ml of healthy normal peripheral blood was collected from a healthy normal person. The equal volume of saline (manufactured by Otsuka Pharmaceutical Co., Ltd.) was added to the collected peripheral blood and the blood was diluted and thoroughly mixed.

[0363] In 15 ml tubes, 10 ml of diluted peripheral blood was carefully overlayered onto 4.5 ml of Lymphoprep (manufactured by Axis-Shield) dispensed and centrifuged at 2,000 rpm for 20 minutes at room temperature with no brake. Then, a PBMC fraction was separated. The thus separated PBMC fraction was washed twice with the ADCC activity measuring medium and then the cell density was adjusted appropriately with the same medium and used as the effector cell suspension.

(2)-3 Measurement of ADCC activity

[0364] Into each well of a 96-well U bottom plate (manufactured by Falcon), the antibody solution in which each antibody was serially ten-fold diluted starting from 3 μg/ml, was dispensed at 50 μl. Next, the target cell solution prepared in the above-mentioned (2)-1 was dispensed at 1×10^4 cells/50 μl/well and finally, the effector cell solution prepared in the above-mentioned (2)-2 was dispensed at 2.5×10^5cells/50 μl/well into the 96-well U bottom plate. After adjusting the total volume to 150 μl, allowed to react at 37°C for 4 hours. The ratio of the effector cell (E) and target cell (T) was set to be 25:1 in the present system. After the reaction, each color development in the supernatant was measured using LDH Cytotoxic Test (manufactured by Wako Pure Chemical Industries, Ltd.).

[0365] The ADCC activity was calculated by the following formula. Those results are shown in Fig. 6A and Fig. 6B, Fig. 7A and Fig. 7B, Fig. 8A and Fig. 8B, and Fig. 9A and Fig. 9B.

[0366]

(Formula)

ADCC activity (%) = {([absorbance of sample]-[absorbance of target cell spontaneous release]-[absorbance of effector cell spontaneous release])/([absorbance of target cell total release]-[absorbance of target cell spontaneous release])}×100

[0367] As shown in Fig. 6A and Fig. 6B and Fig. 7A and Fig. 7B, KM4590 shows the ADCC activity for human cancer cell lines MCF-7 and Colo205, and the activity was higher than that of a known anti-Trop-2 human chimeric antibody cAR47A6.4.2. As shown in Fig. 8A and Fig. 8B, and Fig. 9A and Fig. 9B, KM4591 had higher ADCC activity on human cancer cell lines MCF-7 and Colo205 than KM4590.

(3) Evaluation of antitumor activity of human chimeric antibody anti-Trop-2 on xenograft mouse of BxPC-3 cell line

[0368] BxPC-3 cells were prepared with PBS to give a density of 5×10^7 cells/ml and injected under the right armpit of a 6 week-old male SCID mouse (manufactured by CLEA Japan) at a dose of 100 μl Six days after injection, tumor diameter was measured and animals were divided into groups so as to be the average tumor volume [tumor volume was calculated by length ×(breadth)^2 ×0.5] of each group of 100 mm^3 (six animals per group). PBS or anti-Trop-2 chimeric antibodies (KM4591 or cAR47A6.4.2-P) was administered twice a week for 4 weeks, and the antitumor activity was evaluated.

[0369] The results are shown in Fig. 10. As shown in Fig. 10, KM4591 showed significant antitumor activity and the activity was higher than that of cAR47A6.4.2-P.

(4) Evaluation of antitumor activity on anti-Trop-2 chimeric antibody on xenograft mouse of Colo205 cell line

[0370] Colo205 cells were prepared with PBS to give a density of 5×10^7cells/ml and injected under the right armpit of a 5 week-old male SCID mouse (manufactured by CLEA Japan) at a dose of 100 μl. Five days after injection, tumor diameter was measured and animals were divided into groups with average tumor volume [tumor volume was calculated by length ×(breadth)^2 ×0.5] of each group of 100 mm^3 (five animals per group). PBS or anti-Trop-2 chimeric antibodies (KM4591 or cAR47A6.4.2-P) was administered twice a week for 4 weeks, and the antitumor activity was evaluated.

[0371] The results are shown in Fig. 11. As shown in Fig. 11, KM4591 showed significant antitumor activity and the

activity was higher than that of cAR47A6.4.2-P.

[Example 7]

Epitope analysis of anti-Trop-2 human chimeric antibody KM4590

(1) Evaluation of competitive activities for various anti-Trop-2 monoclonal antibodies using binding ELISA of Trop-2

[0372] Into a 96-well ELISA plate (manufactured by Greiner), 2 μg/ml of a recombinant human Trop-2/Fc/His chimeric protein (manufactured by R&D) was dispensed at 50 μl/well, and allowed to stand at 4°C overnight for immobilization. After washing the plate with PBS, BSA-PBS was added thereto at 100 μl/well and allowed to stand at room temperature for 1 hour for blocking the active groups.

[0373] Then, BSA-PBS was removed from the plate, BSA-PBS dilution solution of 0.3 μg/ml of various mouse anti-Trop-2 antibodies [77220 (manufactured by R&D), MOv16 (manufactured by ALEXIS BIOCHEMICALS), MM0588-49D6 (manufactured by Angio-Proteomie), YY-01 (manufactured by Santa Cruz) or 162-46 (manufactured by BD Pharmigen)] as the primary antibody and serial dilution solutions of a competing chimeric antibody (KM4590 or cAR47A6.4.2) were dispensed at 50 μl/well and allowed to stand for 2 hours.

[0374] After washing the plate with Tween-PBS, a peroxidase-labeled rabbit anti-human IgG antibody (manufactured by American Qualex) was added at 50 μl/well as the second antibody and allowed to stand at room temperature for 1 hour. After washing the plate with Tween-PBS, the ABTS [2,2-Azino-bis(3-Ethyl benzothiazol-6-Sulfonic Acid)ammonium)] substrate solution [1 mmol/l ABTS, 0.1mol/l citric acid buffer (pH 4.2), 0.1% H_2O_2] was added at 50 μl/well for color development, and the chromogenic reaction was stopped by adding a 5% SDS (Sodium lauryl sulfate) solution at 50 μl/well. Thereafter, the value of the absorbance of OD415 nm was measured by a plate reader Emax (manufactured by Molecular Devices, LLC.).

[0375] The bindings of various anti-Trop-2 mouse monoclonal antibodies to Trop-2/Fc/His chimeric proteins when the concentrations of KM4590 and cAR47A6.4.2 were changed were shown in Fig. 14(a) to Fig. 14(f).

[0376] As shown in Fig. 14(a) to Fig. 14(f), it was found that the binding of anti-Trop-2 mouse monoclonal antibodies other than KM4097 to Trop-2/Fc/His chimeric proteins were not inhibited by KM4590. Meanwhile, it was found that the bindings of anti-Trop-2 mouse monoclonal antibodies other than KM4097 to Trop-2/Fc/His chimeric proteins were inhibited by cAR47A6.4.2.

[0377] Based on the above results, it was found that KM4097 and its chimeric antibody, KM4590, were recognized epitopes which were different from those of other anti-Trop-2 antibodies.

(2) Production of Trop-2 extracellular region protein and the domain deletion mutant protein

[0378] An extracellular region of Trop-2 and a domain deletion mutant were produced in order to determine an epitope of an anti-Trop-2 monoclonal antibody of the present invention [Fig. 15]. First, a vector (INPEP4-FLAG-Fc) wherein the DNA (SEQ ID NO::39) in which FLAG tag-encoding DNA and Fc region-encoding DNA were arranged in order from 5' terminal to 3' terminal was inserted into a INPEP4 vector (manufactured by Biogen-IDEC) was produced.

[0379] Next, by inserting the DNA (SEQ ID NO:40) encoding a protein in which the secretion signal sequence was added to the Trop-2 extracellular region to the 5' terminal of FLAG tag sequence of INPEP4-FLAG-Fc vector, a vector for expressing a fusion protein of Trop-2 extracellular region, FLAG tag and Fc (referred as "Trop-2/FLAG/Fc") was constructed.

[0380] In the same manner, using DNAs (SEQ ID NOs:41, 42, and 43) encoding three types of proteins (referred to as Mutant A, Mutant B, and Mutant C, respectively) which lacked only domain I; domain I and domain II; and full length of domain I and domain II and part of domain III, respectively in the extracellular region of Trop-2, respectively, expression vectors of fusion proteins of the extracellular region of Trop-2, FLAG tag and Fc were constructed. The amino acid sequences of Trop-2/FLAG/FC, Mutant A, Mutant B or Mutant C are represented by SEQ ID NOs:44 to 47, respectively.

[0381] Gene introduction and protein expression were carried out using HEK293F as a host cell and using FreeStyle™ 293 Expression system (manufactured by Invitrogen). First, 30 μg of the expression vector plasmid was mixed with OPTI-MEM I (manufactured by Invitrogen) to give a total volume of 900 μl. Next, 30 μl of 293 fectin™ (manufactured by Invitrogen) was mixed with OPTI-MEM I to give a total volume of 900 μl and then allow to stand at room temperature for 5 minutes.

[0382] The 293 fectin™ solution was mixed with the expression vector plasmid solution and allowed to stand at room temperature for 20 to 30 minutes. After all the mixed plasmid solution was added to 30 ml of HEK293F at a cell density of 1.1×10^6cells/ml which was cultured with FreeStyle™ 293 SFM (manufactured by Invitrogen), the cells were cultured in suspension at 37°C, 5% CO_2, on an orbital shaker at 125 rpm for four days.

[0383] After culture, the cell suspension was centrifuged for 20 minutes under conditions of 3,000 rpm at 4°C to recover

the culture supernatant, and then the culture supernatant was filtration-sterilized using Millex GV filter having a pore size of 0.22 μm.

[0384] Econo-Pac column was packed with 1 ml of MabSelect (manufactured by GE Healthcare) and the thus recovered culture supernatant was loaded onto the column, and the column was washed with 10 ml of PBS. After washing, the antibody adsorbed to a carrier was eluted using 0.1mol/l citric acid buffer (pH 3.4).

[0385] The eluted fraction was neutralized with 1mol/l Tris buffer (pH 8.5). Next, the fractions in which the elution of target proteins was confirmed by SDS-PAGE analysis were gathered, and the thus gathered fraction was concentrated with Amicon Ultra-10K (manufactured by Millipore) and filtration-sterilized using Millex GV filter having a pore size of 0.22 μm. The absorbance at 280 nm (OD 280 nm) of the concentrated solution was measured using an absorption spectrophotometer (Nanodrop 2000 manufactured by ThermoScientific) and the concentration of each purified protein was calculated.

(3) Epitope analysis by Western blotting

[0386] Each protein of Trop-2/FLAG/Fc, Mutant A, Mutant B, or Mutant C prepared in this Example 2 was electrophoresed under reducing condition (treatment of samples in the presence of 10 mmol/l DTT at 95°C for 5 minutes) or non-reducing condition (treatment of samples in the absence of DTT at room temperature for 10 minutes) by an SDS-PAGE method. The proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membrane at 200 mA for 1 hour using a semi-dry method.

[0387] After the blocking of the PVDF membrane was carried out using PBS containing 5% BSA at room temperature for 1 hour, the PVDF membrane was soaked in the primary antibody solution which was prepared with TBST [an aqueous solution which was prepared by diluting Tris buffered saline (10× concentration) (pH 7.4) manufactured by Nakarai Tesque Inc.) ten-fold and further adding 0.1% concentration of 0.05% polyoxyethylene (20) sorbitan monolaurate (manufactured by Wako Pure Chemical Industries, Ltd., equivalent to ICI Inc.'s trademark Tween 20)] comprising 0.1% BSA to react for 1 hour at room temperature or overnight at 4°C.

[0388] After washing the PVDF membrane 3 to 4 times with TBST, a peroxidase-labeled goat anti-mouse IgG antibody (manufactured by GE Healthcare) prepared with TBST containing 0.1% BSA or a peroxidase-labeled goat anti-human κ chain antibody (manufactured by Southern Biotech) as a secondary antibody was allowed to react therewith at room temperature for 1 hour. After washing the PVDF membrane 3 to 4 times with TBST, the chemical luminescent reaction was carried out by adding SuperSignal West Pico Chemiluminescent Substrate (manufactured by ThermoScientific) to the PVDF membrane.

[0389] Using the imaging system LAS4000 mini (manufactured by Fujifilm), the luminescence images were obtained. Meanwhile, for the purpose of analyzing the amount of each FLAG/Fc fusion protein on the PVDF membrane, the experiment was also performed in which a peroxidase-labeled rabbit anti-human IgG antibody (manufactured by American Qualex) which recognized human Fc was allowed to react with the PVDF membrane without the primary antibody reaction for one hour, followed by the chemical luminescent reaction.

[0390] Fig. 16(a) shows the result of the experiment in which KM4590 was reacted as a primary antibody in Western blotting. In addition, Fig. 16(b) shows the result of the experiment in which cAR47A6.4.2 was reacted as a primary antibody in Western blotting.

[0391] As shown in Fig. 16(a), in both cases of non-reducing condition and reducing condition, the reactivity of KM4590 for Mutant A and Mutant B in which domain I of Trop-2 was deleted lowered. On the other hand, as shown in Fig. 16(b), the reactivity of cAR47A6.4.2 lowered only to Mutant C in which a region to a part of domain III was deleted.

[0392] Based on the above results, it was found out that epitopes of chimeric antibody KM4590 and its mouse monoclonal antibody KM4097 were present in domain I and an epitope of cAR47A6.4.2 was present in domain III.

[0393] Next, the reactivity of KM4590 and cAR47A6.4.2 in non-reducing condition and reducing condition of Trop-2/FLAG/Fc proteins was compared. The results of the experiments are shown in Fig. 17(a), Fig. 17(b) and Fig. 18.

[0394] From the PVDF membrane to which each of KM4590 and cAR47A6.4.2 was allowed to react by Western blot, the antibodies were detached using Restore™ PLUS Western Blot Stripping Buffer (manufactured by Thermo Scientific) and the blocking was carried out in the same manner as before. By allowing a peroxidase-labeled rabbit anti-human IgG antibody to react with the PVDF membrane, the amount of Trop-2/FLAG/Fc protein was evaluated.

[0395] As a result, there were no significant differences in the contained amount of Trop-2/FLAG/Fc protein between two PVDF membranes as shown in Fig. 17(b). In addition, it was found that KM4590 showed very weak reactivity to Trop-2/FLAG/Fc protein under reducing condition while cAR47A6.4.2 showed strong reactivity to Trop-2/FLAG/Fc protein under reducing condition.

[0396] Fig. 18 showed results obtained by analyzing and then quantifying the signal intensity of the band, using the image interpretation software, Multi Gauge Ver. 3.0 (manufactured by Fujifilm). Based on the above results, it was shown that KM4590 recognized the structure comprising a disulfide bond in domain I of a Trop-2 protein.

[0397] Based on the above results, it was obvious that KM4590 and its mouse antibody KM4097 recognized domain

l of the extracellular region of Trop-2 which was different from epitopes of known antibodies.

[Example 8]

Preparation of anti-Trop-2 humanized antibody

(1) Design of amino acid sequences of VH and VL of KM4097 humanized antibody

[0398] The amino acid sequence of VH of KM4097 humanized antibody was designed in the following manner. The amino acid sequence of FR of VH of a human antibody for grafting the amino acid sequences of CDR1 to CDR3 represented by SEQ ID NOs:13 to 15, respectively, of KM4097VH was selected in the following manner.

[0399] Kabat *et al.* classified the VH of known various human antibodies into three subgroups (HSG I to III) based on the homology of their amino acid sequences and reported on the consensus sequences of each of the subgroups *[*Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)]. Accordingly, homology search between the amino acid sequence of FR in the consensus sequences of the subgroups I to III of the VH of human antibodies and the amino acid sequence of FR of KM4097VH was carried out.

[0400] As a result of the homology search, the homologies with HSG I, HSG II and HSG III were 74.7%, 55.2% and 59.8%, respectively. Accordingly, the amino acid sequence of FR of KM4097VH had the highest homology with the subgroup I.

[0401] Based on the above result, the amino acid sequences (SEQ ID NOs:13 to 15, respectively) of CDRs of the KM4097VH were grafted to an appropriate position of the amino acid sequence of FR in the consensus sequence of subgroup I of VH of a human antibody. In this manner, the amino acid sequence KM4097HV0 of VH of anti-Trop-2 KM4097 humanized antibody represented by SEQ ID NO:24 was designed.

[0402] Next, the amino acid sequence of VL of KM4097 humanized antibody was designed in the following manner. The amino acid sequences of FR of VL of a human antibody which were suitable for grafting the amino acid sequences of CDR1 to CDR3 (SEQ ID NOs:16 to 18, respectively) of KM4097VL were selected in the following manner.

[0403] Kabat *et al.* classified the VL of known various human antibodies into four subgroups (HSG I to IV) based on the homology of their amino acid sequences and further reported on the consensus sequences for each of the subgroups *[*Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)]. Accordingly, homology search among the amino acid sequences of FR in the consensus sequences of the subgroups I to IV of the VL of human antibodies and the amino acid sequence of FR of KM4097VL was performed.

[0404] As a result of the homology search, the homologies with HSG I, HSG II, HSG III and HSG IV were 78.8%, 73.8%, 72.5% and 83.8%, respectively. Accordingly, the amino acid sequence of FR of KM4097VL had the highest homology with the subgroup IV.

[0405] Based on the above result, the amino acid sequences (SEQ ID NOs:16 to 18) of CDR of the KM4097VL were grafted to an appropriate position of the amino acid sequence of FR of the consensus sequence of subgroup IV of human antibody VL. However, since Leu at position 110 in the amino acid sequence of KM4097VL (SEQ ID NO:12) was not the amino acid residue having highest usage frequency cited by Kabat *et al.,* but was an amino acid residue which is used at a relatively high frequency, the above-mentioned amino acid residue which is recognized in the amino acid sequence of KM4097VL was used.

[0406] In this manner, the amino acid sequence KM4097LV0 of VL of anti-Trop-2 KM4097 humanized antibody (SEQ ID NO:26) was designed.

[0407] The amino acid sequence KM4097HV0 of VH and the amino acid sequence KM4097LV0 of VL of KM4097 humanized antibody designed in the above are sequences in which merely the amino acid sequences of CDRs of mouse monoclonal antibody KM4097 were grafted to the amino acid sequence of FR of the selected human antibody.

[0408] However, in general, when a humanized antibody is prepared, its binding activity is frequently lowered in the case where merely the amino acid sequences of CDRs of a mouse antibody are grafted to the amino acid sequence of FR of a human antibody. In order to avoid lowering of the binding activity, modification of the amino acid residues considered to have influence upon the binding activity, among the amino acid residues in FR which are different between human antibodies and mouse antibodies, is carried out together with the grafting of the amino acid sequences of CDRs.

[0409] Thus, the amino acid residues of FR which were considered to have influence upon the binding activity were identified and modified in this Example as follows.

[0410] Firstly, three-dimensional structure of an antibody V region (hereinafter referred to as HVOLV0) consisting of the amino acid sequence KM4097HV0 ofVH and the amino acid sequence KM4097LV0 of VL of KM4097 humanized antibody designed in the above was constructed using a computer modeling technique. For the preparation of the three dimensional structure coordinates and display of the three-dimensional structure, Discovery Studio (manufactured by Accelrys) was used. In addition, a computer model of the three-dimensional structure of V region of KM4097 was also constructed in the same manner.

[0411] By selecting amino acid residues which were different from those of KM4097 in the amino acid sequences of FR of VH and VL of HV0LV0 and then preparing the amino acid sequence in which the selected amino acid residues were substituted with the amino acid residues of KM4097, a three-dimensional structure model was constructed in the same manner. By comparing three-dimensional structures of the V regions of KM4097, HV0LV0 and modified product, the amino acid residues which were considered to have influence upon the binding activity were identified.

[0412] As a result, as the amino acid residues among amino acid residues of FR of HV0LV0, which are considered to change three-dimensional structure of the antigen binding region and have influence upon the binding activity, Ala at position 9, Lys at position 12, Val at position 20, Arg at position 38, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Tyr at position 95 and Val at position 112 in the KM4097HV0 and Leu at position 15, Ala at position 19, Ile at position 21 and Leu at position 84 in the KM4097LV0, respectively, were selected.

[0413] By modifying at least one or more of these selected amino acid residues into the amino acid residues which were present at the same positions of the amino acid sequence of KM4097, VH and VL of humanized antibody having various modifications were designed.

[0414] Specifically, regarding VH, at least one modification was introduced among amino acid modifications for substituting Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe and Val at position 112 with Leu in the amino acid sequence represented by SEQ ID NO:24.

[0415] In addition, regarding VL, at least one modification was introduced among amino acid modifications for substituting Leu at position 15 with Ala, Ala at position 19 with Val, Ile at position 21 with Met and Leu at position 84 with Val in the amino acid sequence represented by SEQ ID NO:26.

[0416] As an antibody V region of KM4097 humanized antibody in which at least one amino acid residue in HV0LV0 in FR was substituted, HV0LV0, HV0LV2, HV0LV3a, HV0LV3b, HV0LV4, HV2LV0, HV2LV2, HV2LV3a, HV2LV3b, HV2LV4, HV3aLV0, HV3aLV2, HV3aLV3a, HV3aLV3b, HV3aLV4, HV3bLV0, HV3bLV2, HV3bLV3a, HV3bLV3b, HV3bLV4, HV4LV0, HV4LV2, HV4LV3a, HV4LV3b, HV4LV4, HV5aLV0, HV5aLV2, HV5aLV3a, HV5aLV3b, HV5aLV4, HV5bLV0, HV5bLV2, HV5bLV3a, HV5bLV3b, HV5bLV4, HV5cLV0, HV5cLV2, HV5cLV3a, HV5cLV3b, HV5cLV4, HV6LV0, HV6LV2, HV6LV3a, HV6LV3b, HV6LV4, HV8LV0, HV8LV2, HV8LV3a, HV8LV3b, HV8LV4, HV9LV0, HV9LV2, HV9LV3a, HV9LV3b, HV9LV4, HV10LV0, HV10LV2, HV10LV3a, HV10LV3b and HV10LV4 were designed.

[0417] The amino acid sequences of H chain variable regions HV2, HV3a, HV3b, HV4, HV5a, HV5b, HV5c, HV6, HV8, HV9, HV10 and the amino acid sequences of L chain variable regions LV2, LV3a, LV3b and LV4, are shown in Fig. 12 and Fig. 13, respectively.

(2) Design of cDNA sequences of VH and VL of KM4097 humanized antibody

[0418] A DNA encoding the amino acid sequence of a variable region of KM4097 humanized antibody was designed using codons which were used in DNAs (SEQ ID NOs:23 and 25) encoding amino acid sequences of KM4097VH and KM4097VL, respectively. When the amino acid modification was carried out, the DNA was designed and prepared using codons which was used in high frequency in mammalian cells. Using these DNA sequences, antibody expression vectors were constructed and humanized antibodies were expressed.

(3) Construction of cDNA encoding VH of KM4097 humanized antibody

[0419] cDNAs (SEQ ID NOs:23, 27, 29, 31 and 33) encoding the amino acid sequence KM4097HV0 (SEQ ID NO:24) of VH of KM4097 humanized antibody designed in Example 1; and HV3a (SEQ ID NO:28), HV4 (SEQ ID NO:30), HV5 (SEQ ID NO:32) and HV10 (SEQ ID NO:34) which were designed in Example 1 and were shown in Fig. 12, respectively, were prepared by full-length synthesis.

(4) Construction of cDNA encoding VL of KM4097 humanized antibody

[0420] cDNAs (SEQ ID NOs:25, 35 and 37) encoding the amino acid sequence LV0 (SEQ ID NO:26) of VL of KM4097 humanized antibody which were designed in Example 1; and LV2 (SEQ ID NO:36) and LV4 (SEQ ID NO:38) which were designed in Example 1 and shown in Fig. 13, respectively, were prepared in full-length synthesis.

(5) Construction of vector for expression of KM4097 humanized antibody

[0421] Various vectors for expression of KM4097 humanized antibody were constructed by inserting cDNA encoding any one of HV0, HV3a, HV4, HV5 and HV10 and cDNA encoding any one of LV0, LV2 and LV4 obtained in (3) and (4) of this Example into appropriate sites of the vector for expression of humanized antibody pKANTEX93 described in WO97/10354.

(6) Expression of KM4097 humanized antibody using animal cells

[0422] A cell line (FUT8 knockout CHO cell) in which α1,6-fucosyltransferase (FUT8) gene is knocked out in a CHO-K1 cell (The Institute of Physical and Chemical Research Cell Bank, Accession No. RCB0403) was used for a gene introduction of the antibody expression vector for an animal cell which was constructed in Example 5.

[0423] Using a FUT8 knockout CHO cell as a host cell, a series of step of a gene introduction to the expression of antibody was carried out based on the instructions of FreeStyle™ MAX CHO Expression System (manufactured by Invitrogen). With OptiPro™ SFM (manufactured by Invitrogen), 312.5 μg of the expression vector plasmid was mixed and the total volume was adjusted to 5 ml.

[0424] With OptiPro™ SFM, 312.5 μl of FreeStyle™ MAX Transfection Reagent (manufactured by Invitrogen) was mixed and the total volume was adjusted to 5 ml. The expression vector plasmid solution and the FreeStyle™ MAX Transfection Reagent were mixed and allowed to stand at room temperature for 10 minutes. Then, all the mixed vector solution was added to 250 ml of FUT8 knockout CHO cells with a cell density of 1.0×10^6 cells/ml cultured with FreeStyle™ CHO Expression Medium (manufactured by Invitrogen) and cultured in suspension at 37°C, 8% CO_2, on an orbital shaker at 135 rpm for 5 to 7 days.

[0425] After culture, the cell suspension was centrifuged for 20 minutes under conditions of 3,000 rpm at 4°C to recover the culture supernatant and then the culture supernatant was filtration-sterilized using Millex GV filter having a pore size of 0.22 μm.

(7) Purification of KM4097 humanized antibody

[0426] A column having a diameter of 0.8 cm was filled with 0.5 ml of MabSelect SuRe (manufactured by GE Healthcare) and the carrier was equilibrated by loading 3.0 ml of purified water, 2.0 ml of 0.1 M citric acid buffer (pH 3.5) and 1.5 ml of 150 mM NaCl, 0.2 M sodium borate buffer (pH 7.5) in order. Next, the supernatant recovered in this Example 6 was loaded onto the column and the column was washed with 5 ml of 150 mM NaCl, 0.2 M sodium borate buffer (pH 7.5). After washing, the antibody adsorbed to the carrier was eluted using 2 ml of 0.1 M citric acid buffer (pH 3.5).

[0427] The elution was carried out to obtain four fractions of 500 μl per fraction. Next, through SDS-PAGE analysis of the thus purified fraction, fractions in which the elution of the antibody was confirmed were gathered. Then, the gathered fractions were dialyzed with 150 mM NaCl, 10 mM sodium citrate solution (pH 6.0) overnight at 4°C. After dialysis, a solution of anti-Trop-2 KM4097 humanized antibody was recovered. After filtration sterilization using Millex GV filter having a pore size of 0.22 μm (manufactured by Millipore), the absorbance at 280 nm (OD 280 nm) was measured by an absorptiometer (SHIMADZU UV-1700) and the concentration of each purified KM4097 humanized antibody was calculated.

[0428] Based on the above, six kinds of KM4097 humanized antibodies, HV0LV0 in which VH and VL of the antibody were HV0 and LV0, respectively; HV3aLV0 in which VH and VL of the antibody were HV3a and LV0, respectively; HV4LV0 in which VH and VL of the antibody were HV4 and LV0, respectively; HV5LV0 in which VH and VL of the antibody were HV5 and LV0, respectively; HV10LV2 in which VH and VL of the antibody were HV10 and LV2, respectively; and HV10LV4 in which VH and VL of the antibody were HV10 and LV4, respectively, were prepared.

(8) Determination of fucose content in KM4097 humanized antibody

[0429] In accordance with the method described in W02002/31140, a ratio of a sugar chain in which fucose was not bound to the complex-type N-linked sugar chains present in six kinds of KM4097 humanized antibodies prepared in this Example 7 was examined. As a result, it was found that fucose was not attached to the six kinds of KM4097 humanized antibodies prepared in Example 7.

[Example 9]

Activity evaluation of KM4097 humanized antibodies

[0430] The activities of KM4591 obtained in Example 5 and six kinds of KM4097 humanized antibodies (HV0LV0, HV3aLV0, HV4LV0, HV5LV0, HV10LV2 and HV10LV4) obtained in Example 8 were evaluated.

(1) Evaluation of Binding activity on KM4097 humanized antibody to recombinant human Trop-2 using Biacore

[0431] In the same manner described in Example 3(2), the affinity of KM4591 and six kinds of KM4097 humanized antibodies (HV0LV0, HV3aLV0, HV4LV0, HV5LV0, HV10LV2 and HV10LV4) obtained in Example 8 to human Trop-2/Fc/His fusion proteins (manufactured by R&D) were evaluated.

[0432] ka, kd and K_D (kd/ka) of each antibody are shown in Table 6 (the mean value of duplicate experiments).

	kd (1/Ms)	ka (1/s)	K_D (nM)
KM4591	3.23×10^{5}	1.13×10^{-4}	0.36
HV0LV0	3.30×10^{5}	1.60×10^{-4}	0.53
HV3aLV0	5.06×10^{5}	1.60×10^{-4}	0.30
HV4LV0	3.35×10^{5}	1.26×10^{-4}	0.42
HV5LV0	3.83×10^{5}	1.90×10^{-4}	0.49
HV10LV2	3.75×10^{5}	1.48×10^{-4}	0.40
HV10LV4	3.42×10^{5}	1.16×10^{-4}	0.37

[0433] As shown in Table 6, all the anti-Trop-2 KM4097 humanized antibodies (HV0LV0, HV3aLV0, HV4LV0, HV5LV0, HV10LV2 and HV10LV4) exhibited high affinity value (K_D) of 1×10^{-9} M or less. Among them, five kinds of KM4097 humanized antibodies (HV3aLV0, HV4LV0, HV5LV0, HV10LV2 and HV10LV4) exhibited high K_D value of 5×10^{-10} M or less.

(2) Evaluation of the ADCC activity on anti-Trop-2 humanized antibody on human Trop-2 expression cell line

(2)-1 Preparation of target cell suspension

[0434] A tag-added human Trop-2 expression vector was constructed by inserting the DNA (SEQ ID NO:49) encoding the sequence (SEQ ID NO:48) in which myc tag and His tag were added to the C-terminus of human Trop-2 into the appropriate site of pKANTEX93. The transformant which was obtained by introducing the expression vector into the CHO/DG44 cells was used for the target cell of the present evaluation. After washing with PBS, the cells were washed with phenol red-free RPMI1640 medium comprising 5% fetal bovine serum (FBS, manufactured by Invitrogen) (hereinafter referred as "the ADCC activity measuring medium", manufactured by Invitrogen) and the cells were adjusted to be optimum with the same medium and used as the target cell suspension.

(2)-2 Preparation of effector cell suspension

[0435] Peripheral blood mononuclear cells (PBMC, manufactured by AllCells) stored in frozen state were thawed and suspended in RPMI medium comprising DNase I (manufactured by Roche Applied Science) and 10% FBS and then centrifuged at 1,500 rpm for 10 minutes. After removing the supernatant, the cells were suspended in RPMI medium comprising DNase I and 10% FBS and incubated at 37(C for 20 minutes. After the suspension was centrifuged at 1,500 rpm for 10 minutes, the supernatant was removed and the thus suspension was washed twice with the ADCC activity measuring medium. Then the cell density was adjusted to be optimum with the same medium and used as the effector cell suspension.

(2)-3 Measurement of ADCC activity

[0436] Into each of wells of a 96-well U bottom plate (manufactured by Falcon), the antibody solution in which each antibody was serially ten-fold diluted, starting from 3 μg/ml was dispensed at 50 μl. Next, the target cell solution prepared in the above-mentioned (2)-1 was dispensed at 1×104 cells/50 μl/well. After the effector cell solution prepared in the above-mentioned (2)-2 was further dispensed at 2.5×105 cells/50 μl/well into the 96-well U bottom plate to give a total volume of 150 μl, the mixed solution was allowed to react at 37°C for 24 hours. The ratio of the effector cell (E) and target cell (T) was set to be 25:1 in the present system. After the reaction, color development in each supernatant was measured using LDH Cytotoxic Test (manufactured by Wako Pure Chemical Industries, Ltd.).

[0437] The ADCC activity was calculated by the following formula. Those results are shown in Fig. 19.

[0438]

(Formula)

ADCC activity (%) = {([absorbance of sample]-[absorbance of target cell spontaneous release]-[absorbance of effector cell spontaneous release])/([absorbance of target cell total release]-[absorbance of target cell spontaneous release])}×100

[0439] As shown in Fig. 19, the prepared humanized antibodies HV3aLV0 exhibited the same ADCC activity as KM4591.

[0440] While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. This application is based on U.S. provisional application No. 61/353,430, filed on June 10, 2010, the entire contents of which are incorporated hereinto by reference. All references cited herein are incorporated in their entirety.

Industrial Applicability

[0441] According to the present invention, a monoclonal antibody or an antibody fragment thereof, which binds to the extracellular region of human Trop-2 with high affinity and exhibits high antibody-dependent cellular cytotoxicity (hereinafter referred to as "ADCC activity") and high antitumor activity; a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtainable by introducing the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a therapeutic agent or a diagnostic agent using the antibody or the antibody fragment thereof can be provided.

Free Text of Sequence Listing

[0442]

SEQ ID NO:1 : Human Trop-2 amino acid sequence
SEQ ID NO:2 : Human Trop-2 nucleotide sequence
SEQ ID NO:3 : AR47A6.4.2 VH nucleotide sequence
SEQ ID NO:4 : AR47A6.4.2 VL nucleotide sequence
SEQ ID NO:5 : Mouse IgG1 primer mG1a1 nucleotide sequence
SEQ ID NO:6 : Mouse IgG1 primer mG1a2 nucleotide sequence
SEQ ID NO:7 : Mouse κ chain primer Ka1 nucleotide sequence
SEQ ID NO:8 : Mouse κ chain primer Ka2 nucleotide sequence
SEQ ID NO:9 : KM4097 VH nucleotide sequence
SEQ ID NO:10 : KM4097 VH amino acid sequence
SEQ ID NO:11 : KM4097 VL nucleotide sequence
SEQ ID NO:12 : KM4097 VL amino acid sequence
SEQ ID NO:13 : KM4097 VHCDR1 amino acid sequence
SEQ ID NO:14 : KM4097 VHCDR2 amino acid sequence
SEQ ID NO:15 : KM4097 VHCDR3 amino acid sequence
SEQ ID NO:16 : KM4097 VLCDR1 amino acid sequence
SEQ ID NO:17 : KM4097 VLCDR2 amino acid sequence
SEQ ID NO:18 : KM4097 VLCDR3 amino acid sequence
SEQ ID NO:19 : Nucleotide sequence of a forward primer for amplification of KM4097 VH chain to construct KM4097 chimeric antibody
SEQ ID NO:20 : Nucleotide sequence of a reverse primer for amplification of KM4097 VH chain to construct KM4097 chimeric antibody
SEQ ID NO:21 : Nucleotide sequence of a forward primer for amplification of KM4097 VL chain to construct KM4097 chimeric antibody
SEQ ID NO:22 : Nucleotide sequence of a reverse primer for amplification of KM4097 VL chain to construct KM4097 chimeric antibody
SEQ ID NO:23 : KM4097 HV0 variable region nucleotide sequence
SEQ ID NO:24 : KM4097 HV0 variable region amino acid sequence
SEQ ID NO:25 : KM4097 LV0 variable region nucleotide sequence
SEQ ID NO:26 : KM4097 LV0 variable region amino acid sequence
SEQ ID NO:27 : KM4097 HV3a variable region nucleotide sequence
SEQ ID NO:28 : KM4097 HV3a variable region amino acid sequence
SEQ ID NO:29 : KM4097 HV4 variable region nucleotide sequence
SEQ ID NO:30 : KM4097 HV4 variable region amino acid sequence
SEQ ID NO:31 : KM4097 HV5 variable region nucleotide sequence
SEQ ID NO:32 : KM4097 HV5 variable region amino acid sequence
SEQ ID NO:33 : KM4097 HV10 variable region nucleotide sequence
SEQ ID NO:34 : KM4097 HV10 variable region amino acid sequence

SEQ ID NO:35 : KM4097 LV2 variable region nucleotide sequence
SEQ ID NO:36 : KM4097 LV2 variable region amino acid sequence
SEQ ID NO:37 : KM4097 LV4 variable region nucleotide sequence
SEQ ID NO:38 : KM4097 LV4 variable region amino acid sequence
SEQ ID NO:39 : Nucleotide sequence of FLAG tag and Fc
SEQ ID NO:40 : Nucleotide sequence in which signal sequence was added to the extracellular region of Trop-2
SEQ ID NO:41 : Nucleotide sequence in which FLAG tag and Fc region were removed from Mutant A
SEQ ID NO:42 : Nucleotide sequence in which FLAG tag and Fc region were removed from Mutant B
SEQ ID NO:43 : Nucleotide sequence in which FLAG tag and Fc region were removed from Mutant C
SEQ ID NO:44 : Trop-2/FLAG/Fc amino acid sequence
SEQ ID NO:45 : Mutant A amino acid sequence
SEQ ID NO:46 : Mutant B amino acid sequence
SEQ ID NO:47 : Mutant C amino acid sequence
SEQ ID NO:48 : C-terminal tagged human Trop-2 amino acid sequence
SEQ ID NO:49 : C-terminal tagged human Trop-2 nucleotide sequence

SEQUENCE LISTING

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        35                  40                  45
Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly Met Ala Val
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Asp Cys Ser Thr Leu Thr Ser Lys Cys Leu Leu Leu Lys Ala Arg Met
65                  70                  75                  80
Ser Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala
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Leu Val Asp Asn Asp Gly Leu Tyr Asp Pro Asp Cys Asp Pro Glu Gly
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  1               5                  10                  15

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Leu Leu Leu Val Leu Ala Ala Val Thr Gly His Thr Ala Ala Gln Asp
             20                  25                  30

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         35                  40                  45

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tgc gat gag ctg gtg cgc acc cac cac atc ctc att gac ctg cgc cac       480
Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
145                 150                 155                 160

cgc ccc acc gcc ggc gcc ttc aac cac tca gac ctg gac gcc gag ctg       528
Arg Pro Thr Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu
                165                 170                 175

agg cgg ctc ttc cgc gag cgc tat cgg ctg cac ccc aag ttc gtg gcg       576
Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val Ala
            180                 185                 190

gcc gtg cac tac gag cag ccc acc atc cag atc gag ctg cgg cag aac       624
Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
        195                 200                 205
```

```
acg tct cag aag gcc gcc ggt gac gtg gat atc ggc gat gcc gcc tac       672
Thr Ser Gln Lys Ala Ala Gly Asp Val Asp Ile Gly Asp Ala Ala Tyr
    210                 215                 220

tac ttc gag agg gac atc aag ggc gag tct cta ttc cag ggc cgc ggc       720
Tyr Phe Glu Arg Asp Ile Lys Gly Glu Ser Leu Phe Gln Gly Arg Gly
225                 230                 235                 240

ggc ctg gac ttg cgc gtg cgc gga gaa ccc ctg cag gtg gag cgc acg       768
Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg Thr
                245                 250                 255

ctc atc tat tac ctg gac gag att ccc ccg aag ttc tcc atg aag cgc       816
Leu Ile Tyr Tyr Leu Asp Glu Ile Pro Pro Lys Phe Ser Met Lys Arg
            260                 265                 270

ctc acc gcc ggc ctc atc gcc gtc atc gtg gtg gtc gtg gtg gcc ctc       864
Leu Thr Ala Gly Leu Ile Ala Val Ile Val Val Val Val Val Ala Leu
        275                 280                 285

gtc gcc ggc atg gcc gtc ctg gtg atc acc aac cgg aga aag tcg ggg       912
Val Ala Gly Met Ala Val Leu Val Ile Thr Asn Arg Arg Lys Ser Gly
    290                 295                 300

aag tac aag aag gtg gag atc aag gaa ctg ggg gag ttg aga aag gaa       960
Lys Tyr Lys Lys Val Glu Ile Lys Glu Leu Gly Glu Leu Arg Lys Glu
305                 310                 315                 320

ccg agc ttg tag                                                      972
Pro Ser Leu

<210> 3
<211> 476
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(476)

<400> 3
aaggaaaaaa gcggccgctg aacacactga ctctaacc atg aga gtg ctg att ctt      56
                                          Met Arg Val Leu Ile Leu
                                            1               5

ttg tgg ctg ttc aca gcc ttt cct ggt atc ctg tct cag atc cag ttg       104
Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile Leu Ser Gln Ile Gln Leu
            10                  15                  20

gtg cag tct gga cct gag ctg aag aag cct gga gag aca gtc aag atc       152
Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu Thr Val Lys Ile
        25                  30                  35

tcc tgc aag gct tct ggg tat acc ttc aca aac tat gga atg aac tgg       200
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn Trp
    40                  45                  50

gtg aag cag gct cca gga aag ggt tta aag tgg atg ggc tgg ata aac       248
Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met Gly Trp Ile Asn
55                  60                  65                  70

acc aaa act gga gag cca aca tat gct gaa gag ttc aag gga cgg ttt       296
Thr Lys Thr Gly Glu Pro Thr Tyr Ala Glu Glu Phe Lys Gly Arg Phe
```

```
                    75                  80                  85

gcc ttc tct ttg gaa acc tct gcc agc act gcc tat ttg cag atc aac       344
Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr Leu Gln Ile Asn
             90                  95                  100

aac ctc aaa aaa gag gac acg gct aca tat ttc tgt gga aga ggg ggc       392
Asn Leu Lys Lys Glu Asp Thr Ala Thr Tyr Phe Cys Gly Arg Gly Gly
        105                 110                 115

tac ggt agt agc tac tgg tac ttc gat gtc tgg ggc gca ggg acc acg       440
Tyr Gly Ser Ser Tyr Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr
    120                 125                 130

gtc acc gtc tcc tca gcc tcc acc aag ggc cca tcg                       476
Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
135                 140                 145

<210> 4
<211> 428
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(428)

<400> 4
ccggaattca gacaggcagg ggaagcaag atg cat ttt caa gtg cag att ttc         53
                                Met His Phe Gln Val Gln Ile Phe
                                  1               5

agc ttc ctg cta atc agt gcc tca gtc ata atg tcc aga gga gac att       101
Ser Phe Leu Leu Ile Ser Ala Ser Val Ile Met Ser Arg Gly Asp Ile
     10                  15                  20

gtg atg acc cag tct cac aaa ttc atg tcc aca tca gta gga gac agg       149
Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly Asp Arg
 25                  30                  35                  40

gtc agc atc acc tgc aag gcc agt cag gat gtg agt att gct gta gcc       197
Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Ala Val Ala
                 45                  50                  55

tgg tat caa cag aaa cca gga caa tct cct aaa gtg ctg att tac tcg       245
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Val Leu Ile Tyr Ser
             60                  65                  70

gca tcc tac cgg tac act gga gtc cct gat cgc ttc act ggc agt gga       293
Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
         75                  80                  85

tct ggg acg gat ttc act ttc acc atc agc agg gtg cag gct gaa gac       341
Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Arg Val Gln Ala Glu Asp
     90                  95                  100

ctg gca gtt tat tac tgt cag caa cat tat att act ccg ctc acg ttc       389
Leu Ala Val Tyr Tyr Cys Gln Gln His Tyr Ile Thr Pro Leu Thr Phe
105                 110                 115                 120

ggt gct ggg acc aag ctg gag ctg aaa cgt acg gtg gct                   428
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg Thr Val Ala
                125                 130
```

<210> 5
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 5
ctgcccaaac taactccatg gtgaccctgg 30

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 6
gatctgctgc ccaaactaac tccatggtga 30

<210> 7
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 7
ttgtcaagag cttcaacagg aatgagtgtt ag 32

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 8
acatctggag gtgcctcagt cgtgtgcttc 30

<210> 9
<211> 405
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(405)

<400> 9
atg gaa tgg agc ggg gtc ttt atc ttt ctc ttg tca gta act gca gat 48
Met Glu Trp Ser Gly Val Phe Ile Phe Leu Leu Ser Val Thr Ala Asp
1 5 10 15

gtc cac tcc cag gtc cag ttg cag cag tct gga cct gag ctg gta agg 96
Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Arg
20 25 30

```
cct ggg act tca gtg agg ata tcc tgc aag gct tct ggc tac acc ttc       144
Pro Gly Thr Ser Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
         35                  40                  45

act att tac tgg cta ggt tgg gta aag cag aga cct gga cat gga ctt       192
Thr Ile Tyr Trp Leu Gly Trp Val Lys Gln Arg Pro Gly His Gly Leu
     50                  55                  60

gaa tgg att gga aat att ttc cct gga agt gct tac att aac tac aat       240
Glu Trp Ile Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn
 65                  70                  75                  80

gag aag ttc aag ggc aag gcc aca ctg act gca gac aca tcc tcc agc       288
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ser Ser
                 85                  90                  95

act gcc tat atg cag ctc agt agc ctg aca tct gag gac tct gct gtc       336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

tat ttc tgt gca aga gag ggt agt aat tcc ggc tac tgg ggt caa ggc       384
Tyr Phe Cys Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly
        115                 120                 125

acc act ctc aca gtc tcc tca                                           405
Thr Thr Leu Thr Val Ser Ser
    130                 135
```

<210> 10
<211> 135
<212> PRT
<213> Mus musculus

<400> 10

```
Met Glu Trp Ser Gly Val Phe Ile Phe Leu Leu Ser Val Thr Ala Asp
  1               5                  10                  15
Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Arg
             20                  25                  30
Pro Gly Thr Ser Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
         35                  40                  45
Thr Ile Tyr Trp Leu Gly Trp Val Lys Gln Arg Pro Gly His Gly Leu
     50                  55                  60
Glu Trp Ile Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn
 65                  70                  75                  80
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ser Ser
                 85                  90                  95
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110
Tyr Phe Cys Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly
        115                 120                 125
Thr Thr Leu Thr Val Ser Ser
    130                 135
```

<210> 11
<211> 399
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(399)

<400> 11

```
atg gaa tca cag act cag gtc ctc atc tcc ttg ctg ttc tgg gta tct        48
Met Glu Ser Gln Thr Gln Val Leu Ile Ser Leu Leu Phe Trp Val Ser
  1               5                  10                  15

ggt acc tgt ggg gac att gtg atg aca cag tct cca tcc tcc ctg agt        96
Gly Thr Cys Gly Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser
            20                  25                  30

gtg tca gca gga gag aag gtc act atg acc tgc aag tcc agt cag agt       144
Val Ser Ala Gly Glu Lys Val Thr Met Thr Cys Lys Ser Ser Gln Ser
         35                  40                  45

ctg tta aac agt gga aat caa cag aac tac ttg gcc tgg tac cag cag       192
Leu Leu Asn Ser Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln
     50                  55                  60

aaa cca ggg cag cct cct aaa ctg ttg atc tac ggg gca tcc act agg       240
Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg
 65                  70                  75                  80

gag tct ggg gtc cct gat cgc ttc aca ggc agt gga tct gga acc gat       288
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp
                85                  90                  95

ttc act ctc acc atc aac agt gtg cag gct gaa gac ctg gca gtt tat       336
Phe Thr Leu Thr Ile Asn Ser Val Gln Ala Glu Asp Leu Ala Val Tyr
            100                 105                 110

tac tgt cag agt gat cat att tat ccg tac acg ttc gga ggg ggg acc       384
Tyr Cys Gln Ser Asp His Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr
        115                 120                 125

aag ctg gaa ata aaa                                                   399
Lys Leu Glu Ile Lys
    130

<210> 12
<211> 133
<212> PRT
<213> Mus musculus

<400> 12
Met Glu Ser Gln Thr Gln Val Leu Ile Ser Leu Leu Phe Trp Val Ser
  1               5                  10                  15
Gly Thr Cys Gly Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser
            20                  25                  30
Val Ser Ala Gly Glu Lys Val Thr Met Thr Cys Lys Ser Ser Gln Ser
         35                  40                  45
Leu Leu Asn Ser Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln
     50                  55                  60
Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg
 65                  70                  75                  80
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp
                85                  90                  95
Phe Thr Leu Thr Ile Asn Ser Val Gln Ala Glu Asp Leu Ala Val Tyr
            100                 105                 110
Tyr Cys Gln Ser Asp His Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr
        115                 120                 125
Lys Leu Glu Ile Lys
    130

<210> 13
<211> 5
```

<212> PRT
<213> Mus musculus

<400> 13
Ile Tyr Trp Leu Gly
1 5

<210> 14
<211> 17
<212> PRT
<213> Mus musculus

<400> 14
Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe Lys
1 5 10 15
Gly

<210> 15
<211> 7
<212> PRT
<213> Mus musculus

<400> 15
Glu Gly Ser Asn Ser Gly Tyr
1 5

<210> 16
<211> 17
<212> PRT
<213> Mus musculus

<400> 16
Lys Ser Ser Gln Ser Leu Leu Asn Ser Gly Asn Gln Gln Asn Tyr Leu
1 5 10 15
Ala

<210> 17
<211> 7
<212> PRT
<213> Mus musculus

<400> 17
Gly Ala Ser Thr Arg Glu Ser
1 5

<210> 18
<211> 9
<212> PRT
<213> Mus musculus

<400> 18
Gln Ser Asp His Ile Tyr Pro Tyr Thr
1 5

<210> 19
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 19

aaggaaaaaa gcggccgcac catggaatgg agcggggtc 39

<210> 20
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 20
cgatgggccc ttggtggagg ctgaggagac tgtgagagtg gtg 43

<210> 21
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 21
ccggaattcc aagatggaat cacagactca ggtcc 35

<210> 22
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic DNA

<400> 22
agccaccgta cgttttattt ccagcttggt c 31

<210> 23
<211> 348
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(348)

<400> 23
cag gtc cag ttg gtg cag tct ggc gct gag gtg aag aag cct ggg gct 48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

tca gtg aag gtg tcc tgc aag gct tct ggc tac acc ttc act att tac 96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
20 25 30

tgg ctg ggt tgg gta cgg cag gcc cct ggt caa ggg ctt gaa tgg atg 144

Trp Leu Gly Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

ggc aat att ttc cct ggc agt gct tac att aac tac aat gag aag ttc 192
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
50 55 60

aag ggc cgg gtc aca atc act gca gac aca tcc acc agc act gcc tat 240

```
Lys Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

atg gaa ctc agt agc ctg cga tct gag gac act gct gtc tat tac tgt       288

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca cga gag ggt agt aat tcc ggc tac tgg ggt caa ggc acc ctg gtc       336
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
            100                 105                 110

aca gtc tcc tca                                                      348

Thr Val Ser Ser
        115

<210> 24
<211> 116
<212> PRT
<213> Mus musculus

<400> 24
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                   10                  15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
            20                  25                  30
Trp Leu Gly Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Ans Tyr Asn Glu Lys Phe
    50                  55                  60
Lys Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Vla
            100                 105                 110
Thr Val Ser Ser
            116

<210> 25
<211> 339
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(339)

<400> 25
gac att gtg atg aca cag tct cca gac tcc ctg gct gtg tca ctg ggt        48
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
1               5                   10                  15

gag cga gcc act atc aac tgc aag tcc agt cag agt ctg tta aac agt        96

Glu Arg Ala Thr Ile Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
            20                  25                  30

ggc aat caa cag aac tac ttg gcc tgg tac cag cag aaa cca ggg cag       144
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45
```

```
cct cct aaa ctg ttg atc tac ggg gca tcc act cgg gag tct ggg gtc       192
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
     50                  55                  60

cct gat cgc ttc tca ggc agt ggc tct ggt acc gat ttc act ctc acc       240
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65                  70                  75                  80

atc tca agt ttg cag gct gaa gac gtg gca gtt tat tac tgt cag agt       288
Ile Ser Ser Leu Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
                 85                  90                  95

gat cat att tat ccg tac acg ttc ggt caa ggg acc aag ctg gaa ata       336
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
            100                 105                 110

aaa                                                                   339
Lys

<210> 26
<211> 113
<212> PRT
<213> Mus musculus

<400> 26
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
1               5                  10                  15
Glu Arg Ala Thr Ile Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
            20                  25                  30
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
     50                  55                  60
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65                  70                  75                  80
Ile Ser Ser Leu Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
                 85                  90                  95
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
            100                 105                 110
Lys
113

<210> 27
<211> 348
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(348)
<223> Description of Artificial Sequence: HV3a sequence

<400> 27
cag gtc cag ttg gtg cag tct gga cct gag gtg aag aag cct ggg gct        48
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Ala
  1               5                  10                  15

tca gtg aag gta tcc tgc aag gct tct ggc tac acc ttc act att tac        96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
            20                  25                  30

tgg cta ggt tgg gta cgg cag gca cct gga cag gga ctt gaa tgg atg       144
Trp Leu Gly Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
```

```
          35                  40                  45

gga aat att ttc cct gga agt gct tac att aac tac aat gag aag ttc       192
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
     50                  55                  60

aag ggc aag gtc aca atc act gca gac aca tcc acc agc act gcc tat       240
Lys Gly Lys Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80

atg gag ctc agt agc ctg cga tct gag gac act gct gtc tat ttt tgt       288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95

gca aga gag ggt agt aat tcc ggc tac tgg ggt caa ggc acc ctt gtc       336
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
            100                 105                 110

aca gtc tcc tca                                                      348
Thr Val Ser Ser
        115

<210> 28
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(116)
<223> Description of Artificial Sequence: HV3a sequence

<400> 28
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Ala
  1               5                  10                  15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
            20                  25                  30
Trp Leu Gly Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
    50                  55                  60
Lys Gly Lys Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                85                  90                  95
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
            100                 105                 110
Thr Val Ser Ser
        115

<210> 29
<211> 348
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(348)
<223> Description of Artificial Sequence: HV4 sequence

<400> 29
cag gtc cag ttg gtg cag tct gga cct gag gtg aag aag cct ggg gct        48
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Ala
```

```
  1               5                  10                  15

tca gtg aag gta tcc tgc aag gct tct ggc tac acc ttc act att tac        96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
             20                  25                  30

tgg cta ggt tgg gta aag cag gca cct gga cag gga ctt gaa tgg att       144
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45

gga aat att ttc cct gga agt gct tac att aac tac aat gag aag ttc       192
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
     50                  55                  60

aag ggc cgg gtc aca atc act gca gac aca tcc acc agc act gcc tat       240
Lys Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80

atg gag ctc agt agc ctg cga tct gag gac act gct gtc tat ttt tgt       288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95

gca aga gag ggt agt aat tcc ggc tac tgg ggt caa ggc acc ctt gtc       336
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
            100                 105                 110

aca gtc tcc tca                                                      348
Thr Val Ser Ser
        115

<210> 30
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(116)
<223> Description of Artificial Sequence: HV4 sequence

<400> 30
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Ala
  1               5                  10                  15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
             20                  25                  30
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
     50                  55                  60
Lys Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
            100                 105                 110
Thr Val Ser Ser
        115

<210> 31
<211> 348
<212> DNA
<213> Artificial Sequence
```

<220>
<221> CDS
<220> (1)..(348)
<223> Description of Artificial Sequence: HV5 sequence

<400> 31
cag gtc cag ttg gtg cag tct gga gct gag gtg aag aag cct ggg gct 48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

tca gtg aag gta tcc tgc aag gct tct ggc tac acc ttc act att tac 96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
20 25 30

tgg cta ggt tgg gta aag cag gca cct gga cag gga ctt gaa tgg att 144
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

gga aat att ttc cct gga agt gct tac att aac tac aat gag aag ttc 192
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
50 55 60

aag ggc aag gcc aca atc act gca gac aca tcc acc agc act gcc tat 240
Lys Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

atg gag ctc agt agc ctg cga tct gag gac act gct gtc tat ttt tgt 288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
85 90 95

gca aga gag ggt agt aat tcc ggc tac tgg ggt caa ggc acc ctt gtc 336
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
100 105 110

aca gtc tcc tca 348
Thr Val Ser Ser
115

<210> 32
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(116)
<223> Description of Artificial Sequence: HV5 sequence

<400> 32
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
20 25 30
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
50 55 60
Lys Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
85 90 95
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Val
100 105 110

```
Thr Val Ser Ser
        115

<210> 33
<211> 348
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(348)
<223> Description of Artificial Sequence: HV10 sequence

<400> 33
cag gtc cag ttg gtg cag tct gga cct gag gtg gta aag cct ggg gct        48
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Val Lys Pro Gly Ala
  1               5                  10                  15

tca gtg aag ata tcc tgc aag gct tct ggc tac acc ttc act att tac        96
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
             20                  25                  30

tgg cta ggt tgg gta aag cag gca cct gga cag gga ctt gaa tgg att       144
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45

gga aat att ttc cct gga agt gct tac att aac tac aat gag aag ttc       192
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
     50                  55                  60

aag ggc aag gcc aca ctg act gca gac aca tcc acc agc act gcc tat       240
Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80

atg gag ctc agt agc ctg cga tct gag gac act gct gtc tat ttt tgt       288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95

gca aga gag ggt agt aat tcc ggc tac tgg ggt caa ggc acc ctt ctc       336
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Leu
            100                 105                 110

aca gtc tcc tca                                                      348
Thr Val Ser Ser
        115

<210> 34
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(116)
<223> Description of Artificial Sequence: HV10 sequence

<400> 34
Gln Val Gln Leu Val Gln Ser Gly Pro Glu Val Val Lys Pro Gly Ala
  1               5                  10                  15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
             20                  25                  30
Trp Leu Gly Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45
```

```
Gly Asn Ile Phe Pro Gly Ser Ala Tyr Ile Asn Tyr Asn Glu Lys Phe
     50                  55                  60
Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95
Ala Arg Glu Gly Ser Asn Ser Gly Tyr Trp Gly Gln Gly Thr Leu Leu
            100                 105                 110
Thr Val Ser Ser
        115
```

<210> 35
<211> 339
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(339)
<223> Description of Artificial Sequence: LV2 sequence

<400> 35

```
gac att gtg atg aca cag tct cca gac tcc ctg gct gtg tca tta gga        48
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
  1               5                  10                  15

gag cgg gtc act atc aac tgc aag tcc agt cag agt ctg tta aac agt        96
Glu Arg Val Thr Ile Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
             20                  25                  30

gga aat caa cag aac tac ttg gcc tgg tac cag cag aaa cca ggg cag       144
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
         35                  40                  45

cct cct aaa ctg ttg atc tac ggg gca tcc act agg gag tct ggg gtc       192
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
     50                  55                  60

cct gat cgc ttc tca ggc agt gga tct gga acc gat ttc act ctc acc       240
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
 65                  70                  75                  80

atc agc agt gtg cag gct gaa gac gtg gca gtt tat tac tgt cag agt       288
Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
                 85                  90                  95

gat cat att tat ccg tac acg ttc gga cag ggg acc aag ctg gaa ata       336
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
            100                 105                 110

aaa                                                                   339
Lys
```

<210> 36
<211> 113
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(113)
<223> Description of Artificial Sequence: LV2 sequence

<400> 36
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
1 5 10 15
Glu Arg Val Thr Ile Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
20 25 30
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
50 55 60
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65 70 75 80
Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
85 90 95
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
100 105 110
Lys

<210> 37
<211> 339
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(339)
<223> Description of Artificial Sequence: LV4 sequence

<400> 37
gac att gtg atg aca cag tct cca gac tcc ctg gct gtg tca gca gga 48
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Ala Gly
1 5 10 15

gag cgg gtc act atg aac tgc aag tcc agt cag agt ctg tta aac agt 96
Glu Arg Val Thr Met Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
20 25 30

gga aat caa cag aac tac ttg gcc tgg tac cag cag aaa cca ggg cag 144
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45

cct cct aaa ctg ttg atc tac ggg gca tcc act agg gag tct ggg gtc 192
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
50 55 60

cct gat cgc ttc tca ggc agt gga tct gga acc gat ttc act ctc acc 240
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65 70 75 80

atc agc agt gtg cag gct gaa gac gtg gca gtt tat tac tgt cag agt 288
Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
85 90 95

gat cat att tat ccg tac acg ttc gga cag ggg acc aag ctg gaa ata 336
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
100 105 110

aaa 339
Lys

<210> 38
<211> 113
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(113)
<223> Description of Artificial Sequence: LV4 sequence

<400> 38

```
Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Ala Gly
  1               5                  10                  15
Glu Arg Val Thr Met Asn Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser
             20                  25                  30
Gly Asn Gln Gln Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
         35                  40                  45
Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg Glu Ser Gly Val
     50                  55                  60
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
 65                  70                  75                  80
Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Ser
                 85                  90                  95
Asp His Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
            100                 105                 110
Lys
```

<210> 39
<211> 720
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(720)
<223> Description of Artificial Sequence: FLAG/Fc sequence

<400> 39

```
tct aga gca gac tac aag gac gac gat gac aag act agt gac aaa act        48
Ser Arg Ala Asp Tyr Lys Asp Asp Asp Asp Lys Thr Ser Asp Lys Thr
  1               5                  10                  15

cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca        96
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser
             20                  25                  30

gtc ttc ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg       144
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
         35                  40                  45

acc cct gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct       192
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro
     50                  55                  60

gag gtc aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc       240
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
 65                  70                  75                  80

aag aca aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc       288
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val
                 85                  90                  95

agc gtc ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac       336
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
            100                 105                 110

aag tgc aag gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc       384
```

```
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
        115                 120                 125

atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg       432
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
    130                 135                 140

ccc cca tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc       480
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
145                 150                 155                 160

ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc       528
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
                165                 170                 175

aat ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac       576
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
            180                 185                 190

tcc gac ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc       624
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
        195                 200                 205

agg tgg cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct       672
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
    210                 215                 220

ctg cac aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa       720
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
225                 230                 235                 240

<210> 40
<211> 798
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(798)
<223> Description of Artificial Sequence: Trop-2-signal sequence

<400> 40
atg aga gtg ctg att ctt ttg tgg ctg ttc aca gcc ttt cct ggt atc        48
Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
  1               5                  10                  15

ctg tct cac acg gcc gcg cag gac aac tgc acg tgt ccc acc aac aag        96
Leu Ser His Thr Ala Ala Gln Asp Asn Cys Thr Cys Pro Thr Asn Lys
             20                  25                  30

atg acc gtg tgc agc ccc gac ggc ccc ggc ggc cgc tgc cag tgc cgc       144
Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg
         35                  40                  45

gcg ctg ggc tcg ggc atg gcg gtc gac tgc tcc acg ctg acc tcc aag       192
Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys
     50                  55                  60

tgt ctg ctg ctc aag gcg cgc atg agc gcc ccc aag aac gcc cgc acg       240
Cys Leu Leu Leu Lys Ala Arg Met Ser Ala Pro Lys Asn Ala Arg Thr
 65                  70                  75                  80

ctg gtg cgg ccg agt gag cac gcg ctc gtg gac aac gat ggc ctc tac       288
```

```
Leu Val Arg Pro Ser Glu His Ala Leu Val Asp Asn Asp Gly Leu Tyr
                 85                  90                  95

gac ccc gac tgc gac ccc gag ggc cgc ttc aag gcg cgc cag tgc aac      336
Asp Pro Asp Cys Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Cys Asn
            100                 105                 110

cag acg tcg gtg tgc tgg tgc gtg aac tcg gtg ggc gtg cgc cgc acg      384
Gln Thr Ser Val Cys Trp Cys Val Asn Ser Val Gly Val Arg Arg Thr
        115                 120                 125

gac aag ggc gac ctg agc cta cgc tgc gat gag ctg gtg cgc acc cac      432
Asp Lys Gly Asp Leu Ser Leu Arg Cys Asp Glu Leu Val Arg Thr His
    130                 135                 140

cac atc ctc att gac ctg cgc cac cgc ccc acc gcc ggc gcc ttc aac      480
His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn
145                 150                 155                 160

cac tca gac ctg gac gcc gag ctg agg cgg ctc ttc cgc gag cgc tat      528
His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr
                165                 170                 175

cgg ctg cac ccc aag ttc gtg gcg gcc gtg cac tac gag cag ccc acc      576
Arg Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr
            180                 185                 190

atc cag atc gag ctg cgg cag aac acg tct cag aag gcc gcc ggt gac      624
Ile Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp
        195                 200                 205

gtg gat atc ggc gat gcc gcc tac tac ttc gag agg gac atc aag ggc      672
Val Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly
    210                 215                 220

gag tct cta ttc cag ggc cgc ggc ggc ctg gac ttg cgc gtg cgc gga      720
Glu Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly
225                 230                 235                 240

gaa ccc ctg cag gtg gag cgc acg ctc atc tat tac ctg gac gag att      768
Glu Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile
                245                 250                 255

ccc ccg aag ttc tcc atg aag cgc ctc acc                              798
Pro Pro Lys Phe Ser Met Lys Arg Leu Thr
260     265

<210> 41
<211> 681
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(681)
<223> Description of Artificial Sequence

<400> 41
atg aga gtg ctg att ctt ttg tgg ctg ttc aca gcc ttt cct ggt atc       48
Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
  1               5                  10                  15

ctg tct cac acg gcc gcg cag gac aac tgt ctg ctg ctc aag gcg cgc       96
```

```
Leu Ser His Thr Ala Ala Gln Asp Asn Cys Leu Leu Leu Lys Ala Arg
             20                  25                  30

atg agc gcc ccc aag aac gcc cgc acg ctg gtg cgg ccg agt gag cac       144
Met Ser Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His
         35                  40                  45

gcg ctc gtg gac aac gat ggc ctc tac gac ccc gac tgc gac ccc gag       192
Ala Leu Val Asp Asn Asp Gly Leu Tyr Asp Pro Asp Cys Asp Pro Glu
     50                  55                  60

ggc cgc ttc aag gcg cgc cag tgc aac cag acg tcg gtg tgc tgg tgc       240
Gly Arg Phe Lys Ala Arg Gln Cys Asn Gln Thr Ser Val Cys Trp Cys
 65                  70                  75                  80

gtg aac tcg gtg ggc gtg cgc cgc acg gac aag ggc gac ctg agc cta       288
Val Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu
                 85                  90                  95

cgc tgc gat gag ctg gtg cgc acc cac cac atc ctc att gac ctg cgc       336
Arg Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg
            100                 105                 110

cac cgc ccc acc gcc ggc gcc ttc aac cac tca gac ctg gac gcc gag       384
His Arg Pro Thr Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu
        115                 120                 125

ctg agg cgg ctc ttc cgc gag cgc tat cgg ctg cac ccc aag ttc gtg       432
Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val
    130                 135                 140

gcg gcc gtg cac tac gag cag ccc acc atc cag atc gag ctg cgg cag       480
Ala Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln
145                 150                 155                 160

aac acg tct cag aag gcc gcc ggt gac gtg gat atc ggc gat gcc gcc       528
Asn Thr Ser Gln Lys Ala Ala Gly Asp Val Asp Ile Gly Asp Ala Ala
                165                 170                 175

tac tac ttc gag agg gac atc aag ggc gag tct cta ttc cag ggc cgc       576
Tyr Tyr Phe Glu Arg Asp Ile Lys Gly Glu Ser Leu Phe Gln Gly Arg
            180                 185                 190

ggc ggc ctg gac ttg cgc gtg cgc gga gaa ccc ctg cag gtg gag cgc       624
Gly Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg
        195                 200                 205

acg ctc atc tat tac ctg gac gag att ccc ccg aag ttc tcc atg aag       672
Thr Leu Ile Tyr Tyr Leu Asp Glu Ile Pro Pro Lys Phe Ser Met Lys
    210                 215                 220

cgc ctc acc                                                          681
Arg Leu Thr
225
```

<210> 42
<211> 462
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(462)

<223> Description of Artificial Sequence

<400> 42
atg aga gtg ctg att ctt ttg tgg ctg ttc aca gcc ttt cct ggt atc 48
Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
1 5 10 15

ctg tct cac acg gcc gcg cag gac aac gat gag ctg gtg cgc acc cac 96
Leu Ser His Thr Ala Ala Gln Asp Asn Asp Glu Leu Val Arg Thr His
20 25 30

cac atc ctc att gac ctg cgc cac cgc ccc acc gcc ggc gcc ttc aac 144
His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn
35 40 45

cac tca gac ctg gac gcc gag ctg agg cgg ctc ttc cgc gag cgc tat 192
His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr
50 55 60

cgg ctg cac ccc aag ttc gtg gcg gcc gtg cac tac gag cag ccc acc 240
Arg Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr
65 70 75 80

atc cag atc gag ctg cgg cag aac acg tct cag aag gcc gcc ggt gac 288
Ile Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp
85 90 95

gtg gat atc ggc gat gcc gcc tac tac ttc gag agg gac atc aag ggc 336
Val Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly
100 105 110

gag tct cta ttc cag ggc cgc ggc ggc ctg gac ttg cgc gtg cgc gga 384
Glu Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly
115 120 125

gaa ccc ctg cag gtg gag cgc acg ctc atc tat tac ctg gac gag att 432
Glu Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile
130 135 140

ccc ccg aag ttc tcc atg aag cgc ctc acc 462
Pro Pro Lys Phe Ser Met Lys Arg Leu Thr
145 150

<210> 43
<211> 363
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(363)
<223> Description of Artificial Sequence

<400> 43
atg aga gtg ctg att ctt ttg tgg ctg ttc aca gcc ttt cct ggt atc 48
Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
1 5 10 15

ctg tct cac acg gcc gcg cag gac aac ctc ttc cgc gag cgc tat cgg 96
Leu Ser His Thr Ala Ala Gln Asp Asn Leu Phe Arg Glu Arg Tyr Arg
20 25 30

cta cac ccc aag ttc gtg gcg gcc gtg cac tac gag cag ccc acc atc 144

```
Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr Ile
         35                  40                  45

cag atc gag ctg cgg cag aac acg tct cag aag gcc gcc ggt gac gtg       192
Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp Val
     50                  55                  60

gat atc ggc gat gcc gcc tac tac ttc gag agg gac atc aag ggc gag       240
Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly Glu
 65                  70                  75                  80

tct cta ttc cag ggc cgc ggc ggc ctg gac ttg cgc gtg cgc gga gaa       288
Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly Glu
                 85                  90                  95

ccc ctg cag gtg gag cgc acg ctc atc tat tac ctg gac gag att ccc       336
Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile Pro
            100                 105                 110

ccg aag ttc tcc atg aag cgc ctc acc                                  363
Pro Lys Phe Ser Met Lys Arg Leu Thr
        115                 120

<210> 44
<211> 506
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(506)
<223> Description of Artificial Sequence: Trop-2/FLAG/Fc sequence

<400> 44
Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
  1               5                  10                  15
Leu Ser His Thr Ala Ala Gln Asp Asn Cys Thr Cys Pro Thr Asn Lys
             20                  25                  30
Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg
         35                  40                  45
Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys
     50                  55                  60
Cys Leu Leu Leu Lys Ala Arg Met Ser Ala Pro Lys Asn Ala Arg Thr
 65                  70                  75                  80
Leu Val Arg Pro Ser Glu His Ala Leu Val Asp Asn Asp Gly Leu Tyr
                 85                  90                  95
Asp Pro Asp Cys Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Cys Asn
            100                 105                 110
Gln Thr Ser Val Cys Trp Cys Val Asn Ser Val Gly Val Arg Arg Thr
        115                 120                 125
Asp Lys Gly Asp Leu Ser Leu Arg Cys Asp Glu Leu Val Arg Thr His
    130                 135                 140
His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn
145                 150                 155                 160
His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr
                165                 170                 175
Arg Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr
            180                 185                 190
Ile Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp
        195                 200                 205
Val Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly
    210                 215                 220
Glu Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly
```

```
225                 230                 235                 240
Glu Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile
                245                 250                 255
Pro Pro Lys Phe Ser Met Lys Arg Leu Thr Ser Arg Ala Asp Tyr Lys
            260                 265                 270
Asp Asp Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro Cys
        275                 280                 285
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
    290                 295                 300
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
305                 310                 315                 320
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
                325                 330                 335
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
            340                 345                 350
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
        355                 360                 365
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
    370                 375                 380
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
385                 390                 395                 400
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
                405                 410                 415
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
            420                 425                 430
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
        435                 440                 445
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
    450                 455                 460
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
465                 470                 475                 480
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
                485                 490                 495
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
            500                 505
```

<210> 45
<211> 467
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(467)
<223> Description of Artificial Sequence: mutant A sequence

<400> 45

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  1               5                  10                  15
Leu Ser His Thr Ala Ala Gln Asp Asn Cys Leu Leu Leu Lys Ala Arg
             20                  25                  30
Met Ser Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His
         35                  40                  45
Ala Leu Val Asp Asn Asp Gly Leu Tyr Asp Pro Asp Cys Asp Pro Glu
     50                  55                  60
Gly Arg Phe Lys Ala Arg Gln Cys Asn Gln Thr Ser Val Cys Trp Cys
 65                  70                  75                  80
Val Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu
                 85                  90                  95
Arg Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg
            100                 105                 110
His Arg Pro Thr Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu
        115                 120                 125
```

```
Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val
    130                 135                 140
Ala Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln
145                 150                 155                 160
Asn Thr Ser Gln Lys Ala Ala Gly Asp Val Asp Ile Gly Asp Ala Ala
                165                 170                 175
Tyr Tyr Phe Glu Arg Asp Ile Lys Gly Glu Ser Leu Phe Gln Gly Arg
            180                 185                 190
Gly Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg
        195                 200                 205
Thr Leu Ile Tyr Tyr Leu Asp Glu Ile Pro Pro Lys Phe Ser Met Lys
    210                 215                 220
Arg Leu Thr Ser Arg Ala Asp Tyr Lys Asp Asp Asp Asp Lys Thr Ser
225                 230                 235                 240
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
                245                 250                 255
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
            260                 265                 270
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
        275                 280                 285
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
    290                 295                 300
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
305                 310                 315                 320
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
                325                 330                 335
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
            340                 345                 350
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
        355                 360                 365
Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser
    370                 375                 380
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
385                 390                 395                 400
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
                405                 410                 415
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
            420                 425                 430
Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
        435                 440                 445
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
    450                 455                 460
Pro Gly Lys
465
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<210> 46
<211> 394
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(394)
<223> Description of Artificial Sequence: mutant B sequence

<400> 46

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  1               5                  10                  15
Leu Ser His Thr Ala Ala Gln Asp Asn Asp Glu Leu Val Arg Thr His
             20                  25                  30
His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn
         35                  40                  45
His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr
```

```
     50                  55                  60
Arg Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr
 65                  70                  75                  80
Ile Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp
                 85                  90                  95
Val Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly
            100                 105                 110
Glu Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly
        115                 120                 125
Glu Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile
    130                 135                 140
Pro Pro Lys Phe Ser Met Lys Arg Leu Thr Ser Arg Ala Asp Tyr Lys
145                 150                 155                 160
Asp Asp Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro Cys
                165                 170                 175
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
            180                 185                 190
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
        195                 200                 205
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
    210                 215                 220
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
225                 230                 235                 240
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
                245                 250                 255
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
            260                 265                 270
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
        275                 280                 285
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
    290                 295                 300
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
305                 310                 315                 320
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
                325                 330                 335
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
            340                 345                 350
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
        355                 360                 365
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
    370                 375                 380
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
385                 390
```

<210> 47
<211> 361
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(361)
<223> Description of Artificial Sequence: mutant C sequence

<400> 47

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  1               5                  10                  15
Leu Ser His Thr Ala Ala Gln Asp Asn Leu Phe Arg Glu Arg Tyr Arg
             20                  25                  30
Leu His Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr Ile
         35                  40                  45
Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala Gly Asp Val
     50                  55                  60
```

```
Asp Ile Gly Asp Ala Ala Tyr Tyr Phe Glu Arg Asp Ile Lys Gly Glu
 65                  70                  75                  80
Ser Leu Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly Glu
                 85                  90                  95
Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu Asp Glu Ile Pro
            100                 105                 110
Pro Lys Phe Ser Met Lys Arg Leu Thr Ser Arg Ala Asp Tyr Lys Asp
        115                 120                 125
Asp Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro
    130                 135                 140
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
145                 150                 155                 160
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
                165                 170                 175
Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
            180                 185                 190
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
        195                 200                 205
Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
    210                 215                 220
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
225                 230                 235                 240
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
                245                 250                 255
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu
            260                 265                 270
Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
        275                 280                 285
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
    290                 295                 300
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
305                 310                 315                 320
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val
                325                 330                 335
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln
            340                 345                 350
Lys Ser Leu Ser Leu Ser Pro Gly Lys
        355                 360
```

<210> 48
<211> 344
<212> PRT
<213> Artificial Sequence

<220>
<221> PEPTIDE
<220> (1)..(344)
<223> Description of Artificial Sequence: myc/His-tagged Trop-2 sequence

<400> 48

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Met Ala Arg Gly Pro Gly Leu Ala Pro Pro Pro Leu Arg Leu Pro Leu
  1               5                  10                  15
Leu Leu Leu Val Leu Ala Ala Val Thr Gly His Thr Ala Ala Gln Asp
             20                  25                  30
Asn Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly
         35                  40                  45
Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly Met Ala Val
     50                  55                  60
Asp Cys Ser Thr Leu Thr Ser Lys Cys Leu Leu Leu Lys Ala Arg Met
 65                  70                  75                  80
Ser Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala
                 85                  90                  95
Leu Val Asp Asn Asp Gly Leu Tyr Asp Pro Asp Cys Asp Pro Glu Gly
```

```
                100                 105                 110
Arg Phe Lys Ala Arg Gln Cys Asn Gln Thr Ser Val Cys Trp Cys Val
        115                 120                 125
Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg
    130                 135                 140
Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
145                 150                 155                 160
Arg Pro Thr Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu
                165                 170                 175
Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val Ala
            180                 185                 190
Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
        195                 200                 205
Thr Ser Gln Lys Ala Ala Gly Asp Val Asp Ile Gly Asp Ala Ala Tyr
    210                 215                 220
Tyr Phe Glu Arg Asp Ile Lys Gly Glu Ser Leu Phe Gln Gly Arg Gly
225                 230                 235                 240
Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg Thr
                245                 250                 255
Leu Ile Tyr Tyr Leu Asp Glu Ile Pro Pro Lys Phe Ser Met Lys Arg
            260                 265                 270
Leu Thr Ala Gly Leu Ile Ala Val Ile Val Val Val Val Val Ala Leu
        275                 280                 285
Val Ala Gly Met Ala Val Leu Val Ile Thr Asn Arg Arg Lys Ser Gly
    290                 295                 300
Lys Tyr Lys Lys Val Glu Ile Lys Glu Leu Gly Glu Leu Arg Lys Glu
305                 310                 315                 320
Pro Ser Leu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His
                325                 330                 335
Thr Gly His His His His His His
            340
```

<210> 49
<211> 1032
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<220> (1)..(1032)
<223> Description of Artificial Sequence: myc/His-tagged Trop-2 sequence

<400> 49

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atg gct cgg ggc ccc ggc ctc gcg ccg cca ccg ctg cgg ctg ccg ctg        48
Met Ala Arg Gly Pro Gly Leu Ala Pro Pro Pro Leu Arg Leu Pro Leu
  1               5                  10                  15

ctg ctg ctg gtg ctg gcg gcg gtg acc ggc cac acg gcc gcg cag gac        96
Leu Leu Leu Val Leu Ala Ala Val Thr Gly His Thr Ala Ala Gln Asp
             20                  25                  30

aac tgc acg tgt ccc acc aac aag atg acc gtg tgc agc ccc gac ggc       144
Asn Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly
         35                  40                  45

ccc ggc ggc cgc tgc cag tgc cgc gcg ctg ggc tcg ggc atg gcg gtc       192
Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly Met Ala Val
     50                  55                  60

gac tgc tcc acg ctg acc tcc aag tgt ctg ctg ctc aag gcg cgc atg       240
Asp Cys Ser Thr Leu Thr Ser Lys Cys Leu Leu Leu Lys Ala Arg Met
 65                  70                  75                  80
```

```
agc gcc ccc aag aac gcc cgc acg ctg gtg cgg ccg agt gag cac gcg       288
Ser Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala
                 85                  90                  95

ctc gtg gac aac gat ggc ctc tac gac ccc gac tgc gac ccc gag ggc       336
Leu Val Asp Asn Asp Gly Leu Tyr Asp Pro Asp Cys Asp Pro Glu Gly
            100                 105                 110

cgc ttc aag gcg cgc cag tgc aac cag acg tcg gtg tgc tgg tgc gtg       384
Arg Phe Lys Ala Arg Gln Cys Asn Gln Thr Ser Val Cys Trp Cys Val
        115                 120                 125

aac tcg gtg ggc gtg cgc cgc acg gac aag ggc gac ctg agc cta cgc       432
Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg
    130                 135                 140

tgc gat gag ctg gtg cgc acc cac cac atc ctc att gac ctg cgc cac       480
Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
145                 150                 155                 160

cgc ccc acc gcc ggc gcc ttc aac cac tca gac ctg gac gcc gag ctg       528
Arg Pro Thr Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu
                165                 170                 175

agg cgg ctc ttc cgc gag cgc tat cgg ctg cac ccc aag ttc gtg gcg       576
Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val Ala
            180                 185                 190

gcc gtg cac tac gag cag ccc acc atc cag atc gag ctg cgg cag aac       624
Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
        195                 200                 205

acg tct cag aag gcc gcc ggt gac gtg gat atc ggc gat gcc gcc tac       672
Thr Ser Gln Lys Ala Ala Gly Asp Val Asp Ile Gly Asp Ala Ala Tyr
    210                 215                 220

tac ttc gag agg gac atc aag ggc gag tct cta ttc cag ggc cgc ggc       720
Tyr Phe Glu Arg Asp Ile Lys Gly Glu Ser Leu Phe Gln Gly Arg Gly
225                 230                 235                 240

ggc ctg gac ttg cgc gtg cgc gga gaa ccc ctg cag gtg gag cgc acg       768
Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg Thr
                245                 250                 255

ctc atc tat tac ctg gac gag att ccc ccg aag ttc tcc atg aag cgc       816
Leu Ile Tyr Tyr Leu Asp Glu Ile Pro Pro Lys Phe Ser Met Lys Arg
            260                 265                 270

ctc acc gcc ggc ctc atc gct gtc atc gtg gtg gtc gtg gtg gcc ctc       864
Leu Thr Ala Gly Leu Ile Ala Val Ile Val Val Val Val Ala Leu
        275                 280                 285

gtc gcc ggc atg gcc gtc ctg gtg atc acc aac cgg aga aag tcg ggg       912
Val Ala Gly Met Ala Val Leu Val Ile Thr Asn Arg Arg Lys Ser Gly
    290                 295                 300

aag tac aag aag gtg gag atc aag gaa ctg ggg gag ttg aga aag gaa       960
Lys Tyr Lys Lys Val Glu Ile Lys Glu Leu Gly Glu Leu Arg Lys Glu
305                 310                 315                 320

ccg agc ttg gaa caa aaa ctc atc tca gaa gag gat ctg aat atg cat      1008
Pro Ser Leu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His
                325                 330                 335
```

```
acc ggt cat cat cac cat cac cat                                    1032
Thr Gly His His His His His His
            340
```

Claims

1. I A monoclonal antibody or an antibody fragment thereof against human Trop-2, which binds to at least domain I in an extracellular region of human Trop-2.

2. The monoclonal antibody or the antibody fragment thereof according to claim 1, which binds to at least one amino acid in amino acids at positions 34 to 72 in the amino acid sequence represented by SEQ ID NO:1.

3. The monoclonal antibody or the antibody fragment thereof according to claim 1 or 2, which has a dissociation constant (K_D) of 5×10^{-10}M or less against an antigen of an antibody.

4. The monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 3, which has high ADCC activity and antitumor activity.

5. The monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 4, wherein CDR1 to 3 of an H chain of the antibody comprise the amino acid sequences represented by SEQ ID NOs:13 to 15, respectively; and CDR1 to 3 of an L chain of the antibody comprise the amino acid sequences represented by SEQ ID NOs:16 to 18, respectively.

6. A monoclonal antibody or an antibody fragment thereof, which binds to an epitope which is the same as an epitope in an extracellular region of Trop-2 to which a monoclonal antibody comprising VH comprising the amino acid sequence represented by SEQ ID NO:10 and VL comprising the amino acid sequence represented by SEQ ID NO: 12 binds.

7. A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of Trop-2 while competing with the antibody according to claim 5 or 6.

8. The monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 7, which is a recombinant antibody.

9. The monoclonal antibody or the antibody fragment thereof according to claim 8, which is a recombinant antibody selected from a human chimeric antibody, a humanized antibody and a human antibody.

10. The monoclonal antibody or the antibody fragment thereof according to claim 9, which is a humanized antibody and comprises at least one of the following (a) VH and (b) VL:

    (a) VH which comprises the amino acid sequence represented by SEQ ID NO:24 or an amino acid sequence in which at least one modification in amino acid modifications for substituting Ala at position 9 with Pro, Lys at position 12 with Val, Val at position 20 with Ile, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Tyr at position 95 with Phe and Val at position 112 with Leu is introduced in the amino acid sequence represented by SEQ ID NO:24,
    (b) VL which comprises the amino acid sequence represented by SEQ ID NO:26 or an amino acid sequence in which at least one modification in amino acid modifications for substituting Leu at position 15 with Ala, Ala at position 19 with Val, Ile at position 21 with Met and Leu at position 84 with Val is introduced in the amino acid sequence represented by SEQ ID NO:26.

11. The monoclonal antibody or the antibody fragment thereof according to claim 10, which is selected from the following (1) to (5):

    (1) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:28 and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:26,
    (2) a humanized antibody which comprises at least one ofVH of the antibody comprising the amino acid sequence

represented by SEQ ID NO:30 and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:26,
(3) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:32 and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:26,
(4) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:34 and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:36,
(5) a humanized antibody which comprises at least one of VH of the antibody comprising the amino acid sequence represented by SEQ ID NO:34 and VL of the antibody comprising the amino acid sequence represented by SEQ ID NO:38.

12. The antibody fragment according to any one of claims 1 to 11, which is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv) and a peptide comprising CDR.

13. A DNA which encodes the monoclonal antibody or the antibody fragment according to any one of claims 1 to 12.

14. A recombinant vector comprising the DNA according to claim 13.

15. A transformant obtainable by introducing the recombinant vector according to claim 14 into a host cell.

16. A process for producing the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12, which comprises culturing the transformant according to claim 14 in a medium to thereby form and accumulate the antibody or the antibody fragment thereof according to any one of claims 1 to 12 in culture, and recovering the antibody or the antibody fragment thereof from the culture.

17. A reagent for detecting or measuring human Trop-2, which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12.

18. A diagnostic agent for a disease relating to human Trop-2-positive cells, which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 as an active ingredient.

19. The diagnostic agent according to claim 18, wherein the disease relating to human Trop-2-positive cells is a cancer.

20. A therapeutic agent for a disease relating to human Trop-2-positive cells, which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 as an active ingredient.

21. The therapeutic agent according to claim 20, wherein the disease relating to human Trop-2-positive cells is a cancer.

22. A method for diagnosing a disease relating to human Trop-2-positive cells, which comprises using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 to detect or measure human Trop-2-positive cells.

23. A method for diagnosing a disease relating to human Trop-2-positive cells, which comprises using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 to detect or measure human Trop-2.

24. The diagnosing method according to claim 22 or 23, wherein the disease relating to human Trop-2-positive cells is a cancer.

25. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 for manufacture of a diagnostic agent for a disease relating to human Trop-2-positive cells.

26. The use of the monoclonal antibody or the antibody fragment thereof according to claim 25, wherein the disease relating to human Trop-2-positive cells is a cancer.

27. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 12 for manufacture of a therapeutic agent for a disease relating to human Trop-2-positive cells.

28. The use of the monoclonal antibody or the antibody fragment thereof according to claim 27, wherein the disease relating to human Trop-2-positive cells is a cancer.

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Fig. 1

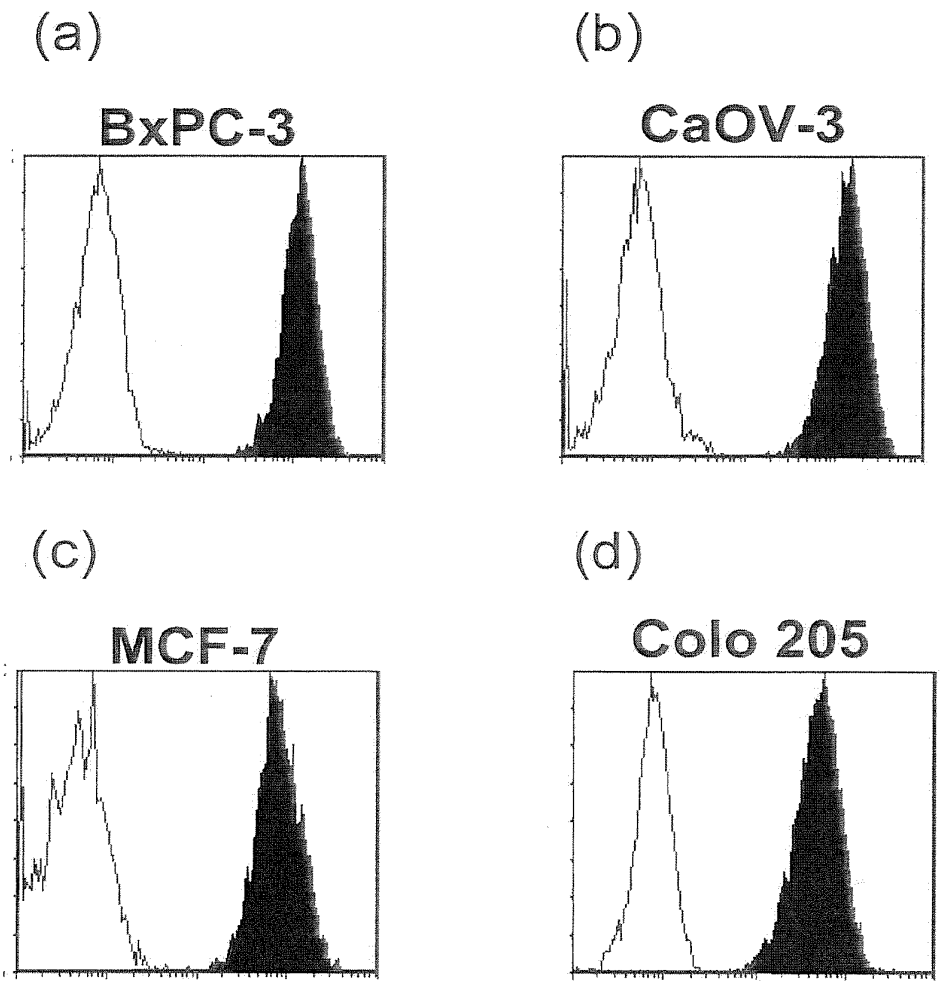

Fig. 2

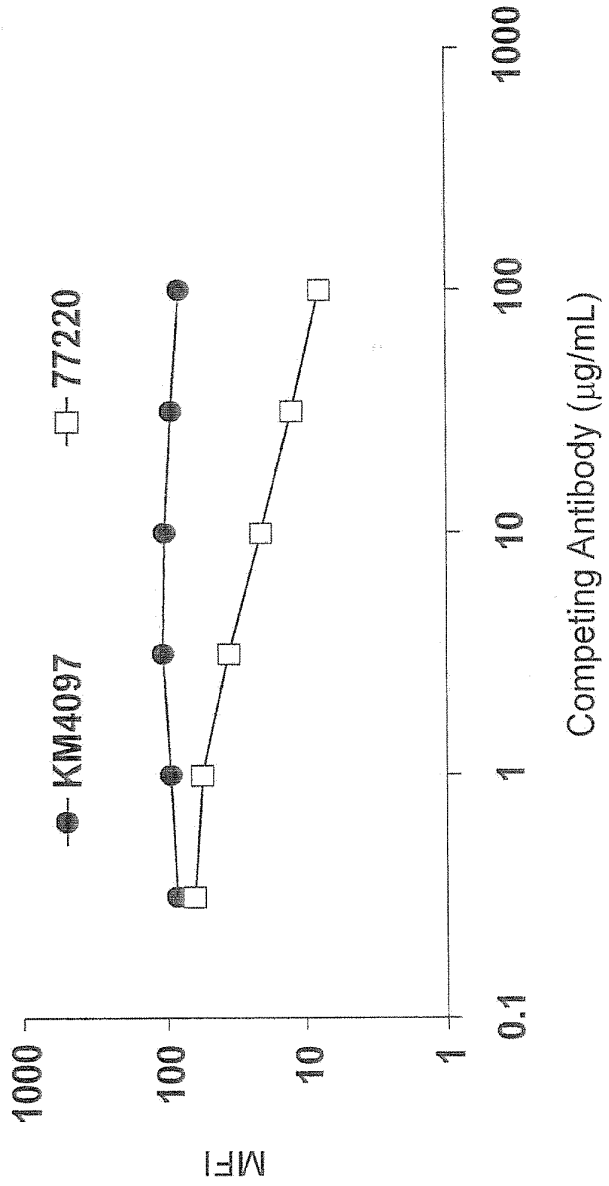
KM4097
77220
MFI
1000
100
10
1
0.1
1
10
100
1000
Competing Antibody (µg/mL)

Fig. 3

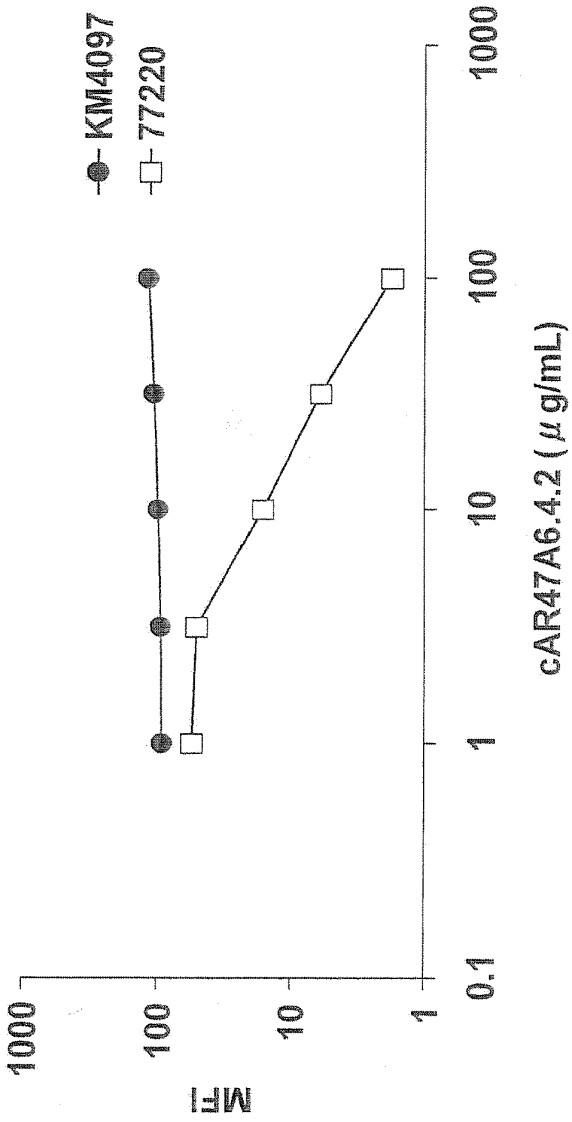

Fig. 4

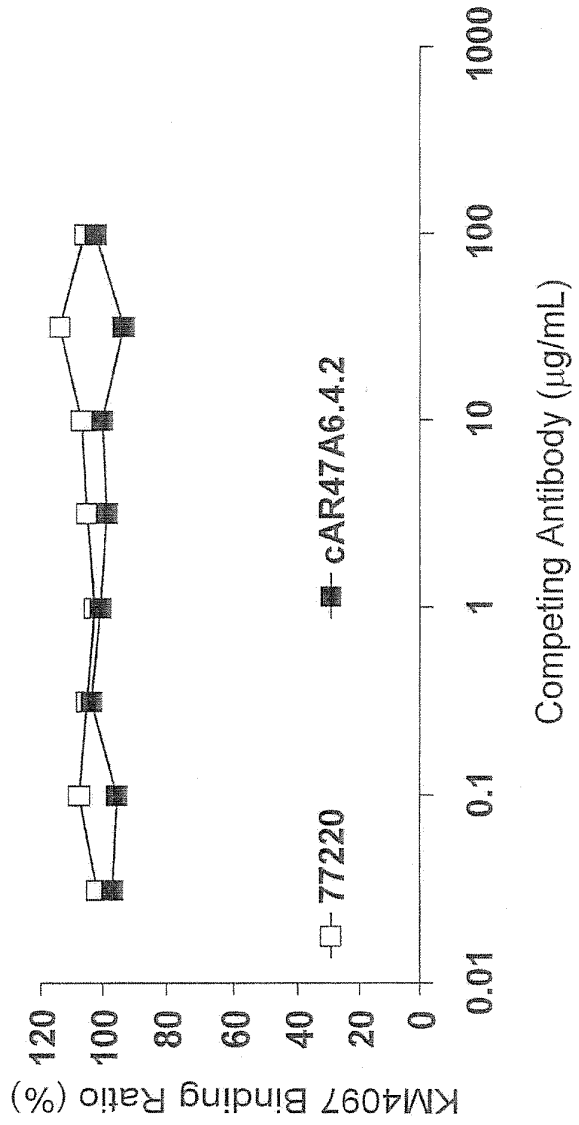
KM4097 Binding Ratio (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Competing Antibody (μg/mL)
77220
cAR47A6.4.2

Fig. 5

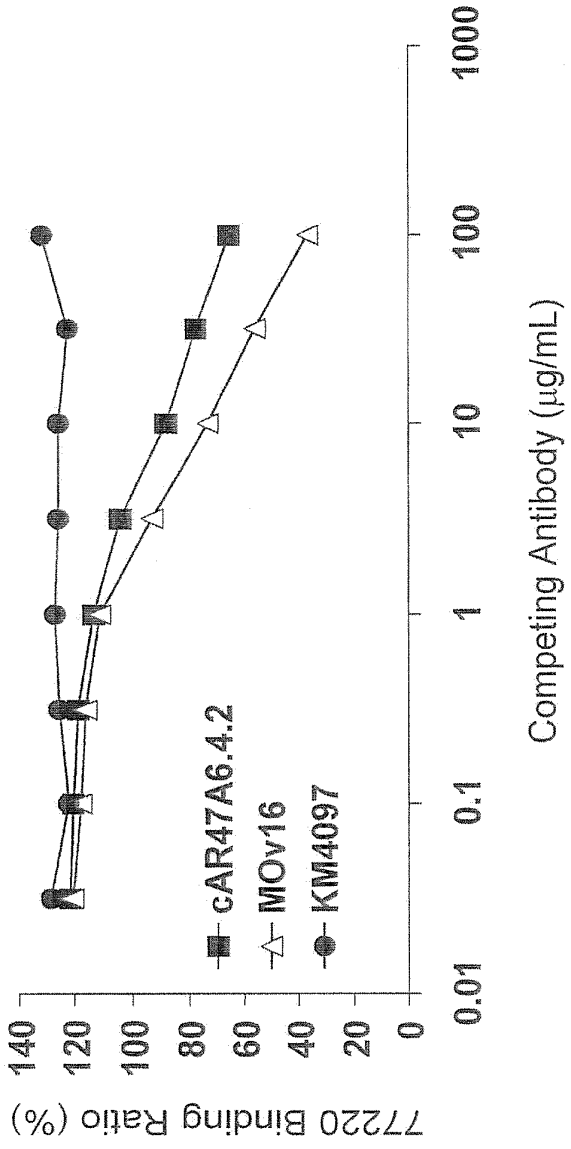
77220 Binding Ratio (%)
140
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Competing Antibody (μg/mL)
cAR47A6.4.2
MOv16
KM4097

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Fig. 6

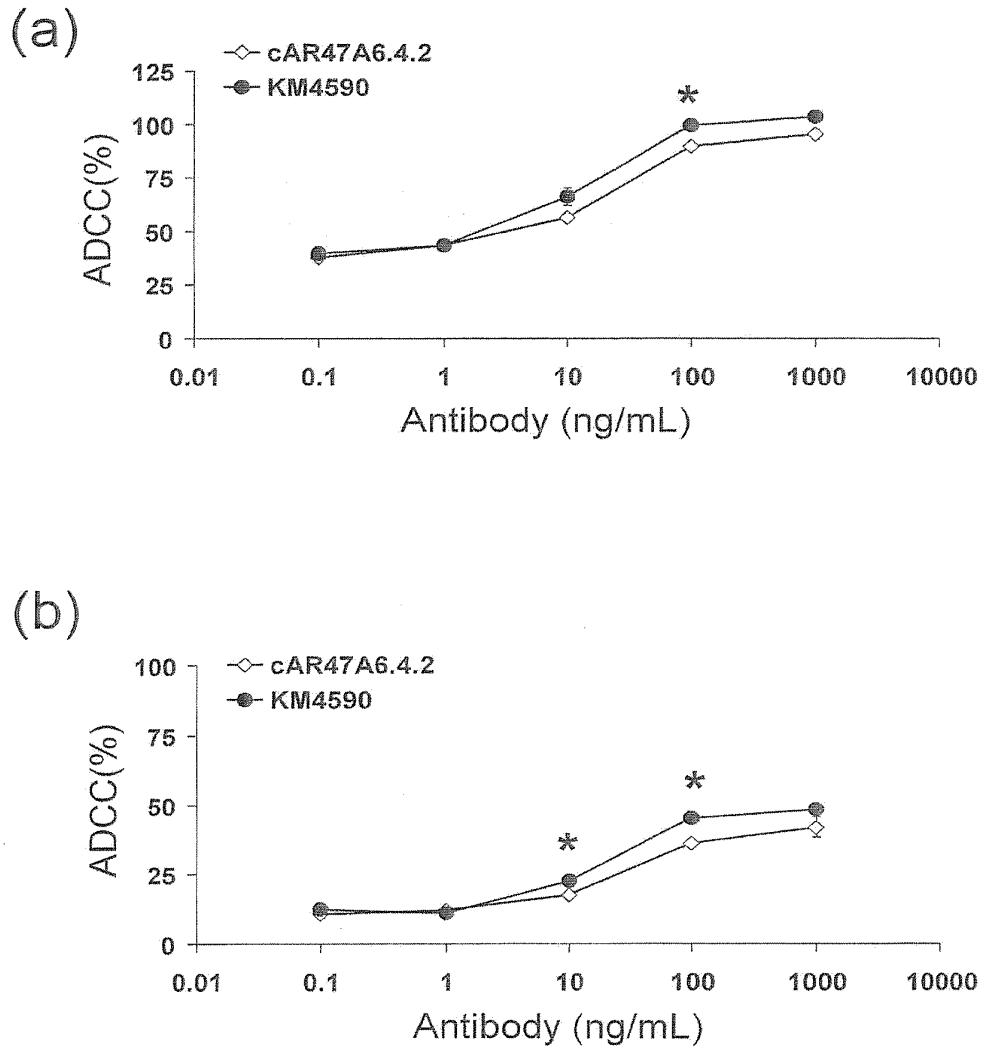

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Fig. 7

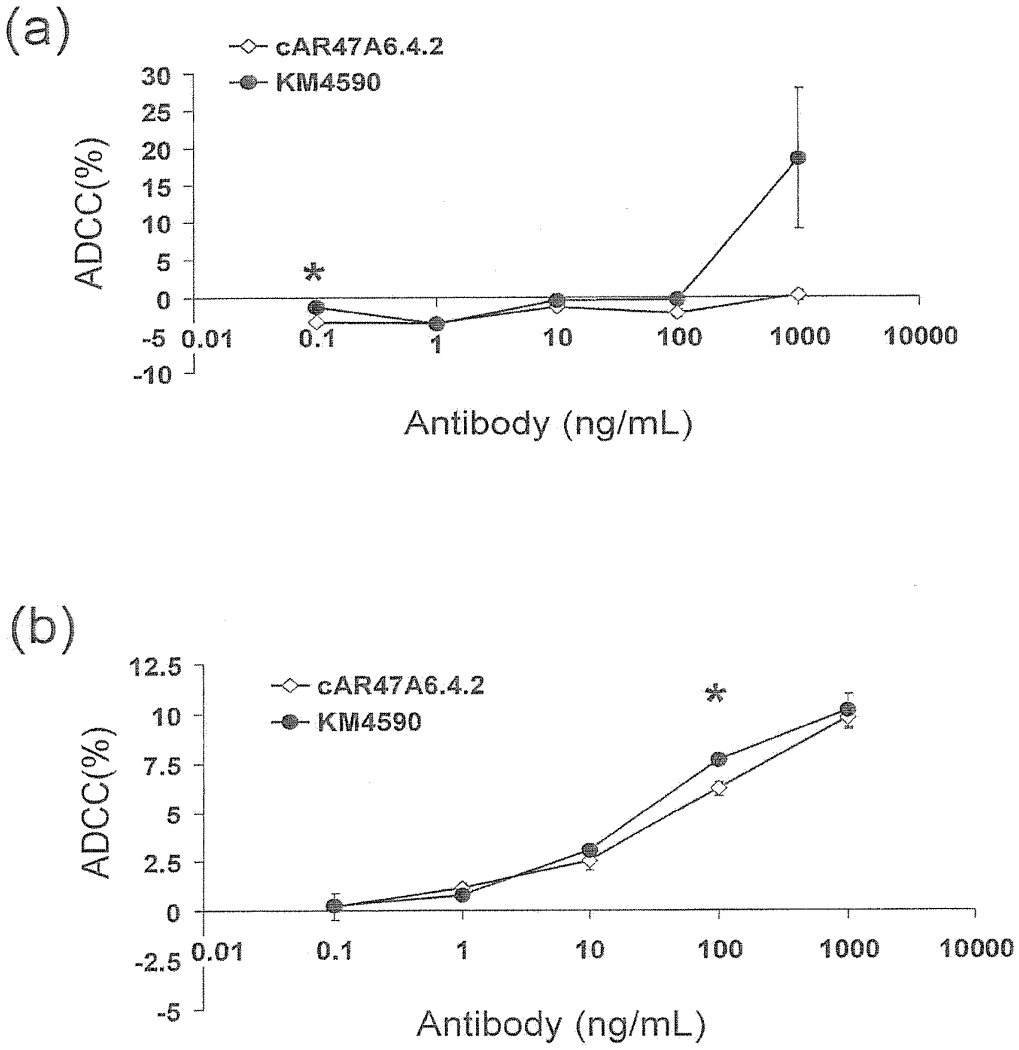

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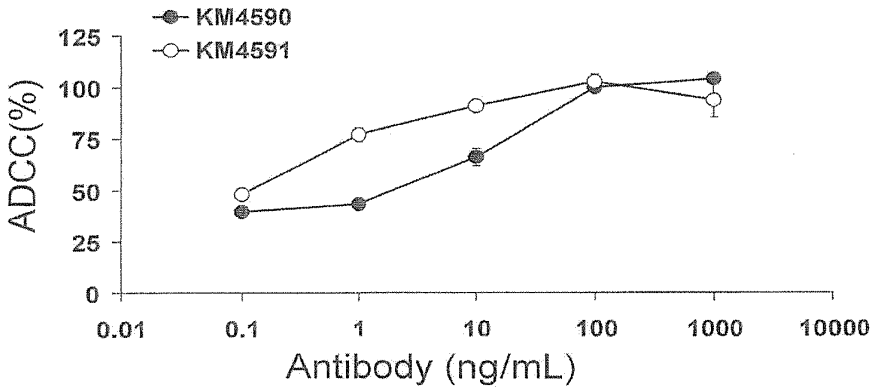
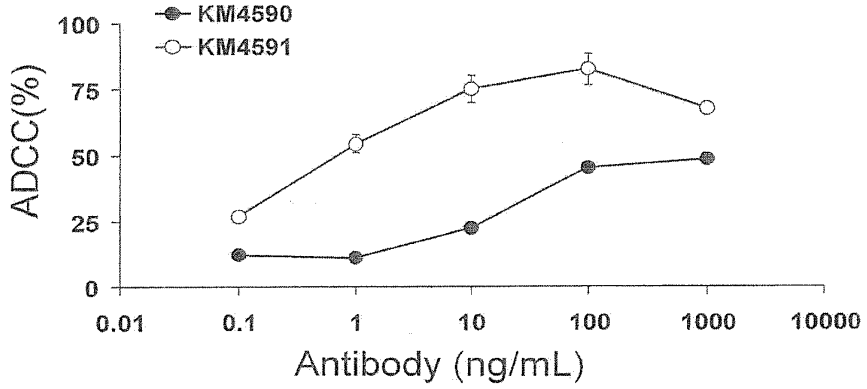
Fig. 8

(a)



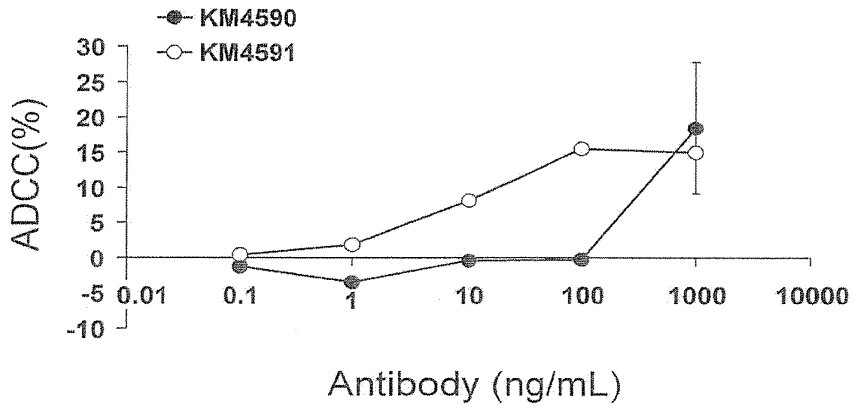
(b)



EP 2 594 589 A1

Fig. 9

(a)



(b)

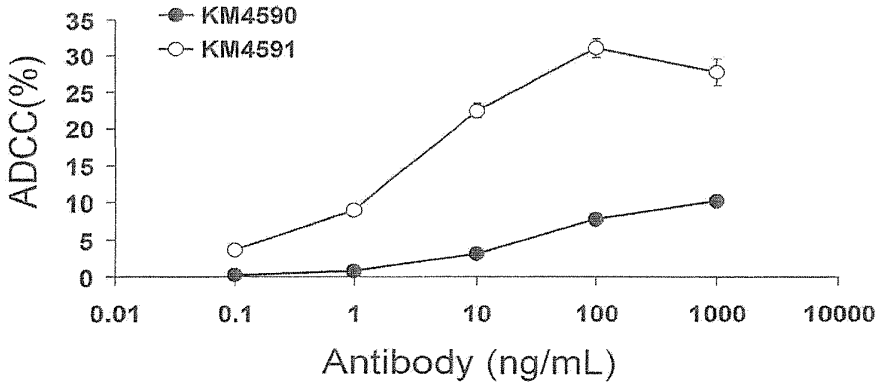


Fig. 10

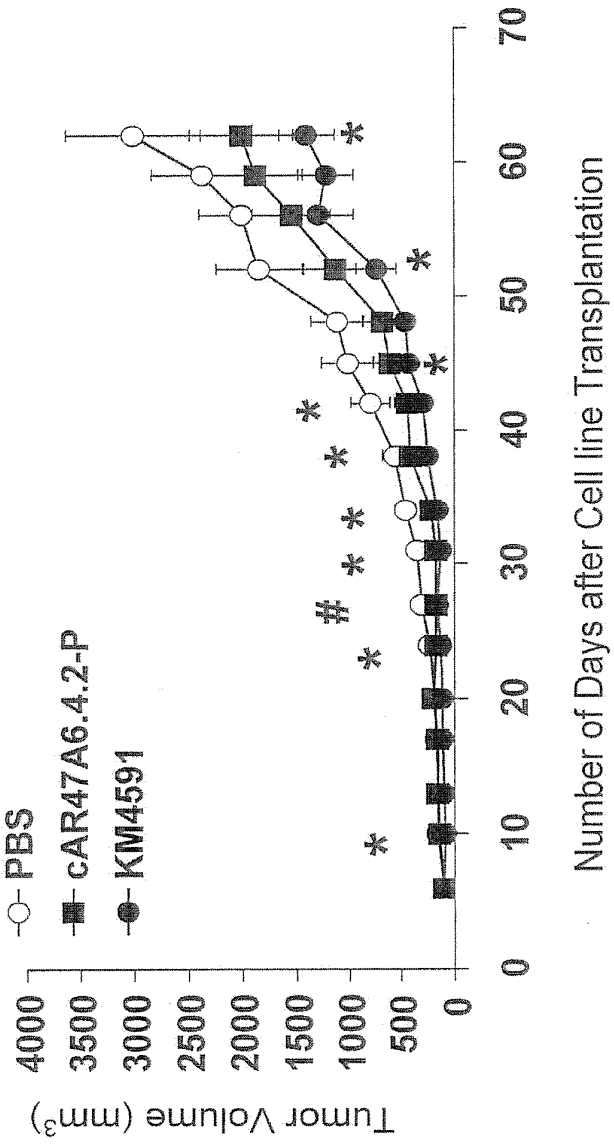

Tumor Volume (mm3)
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
Number of Days after Cell line Transplantation
PBS
cAR47A6.4.2-P
KM4591
*
#

Fig. 11

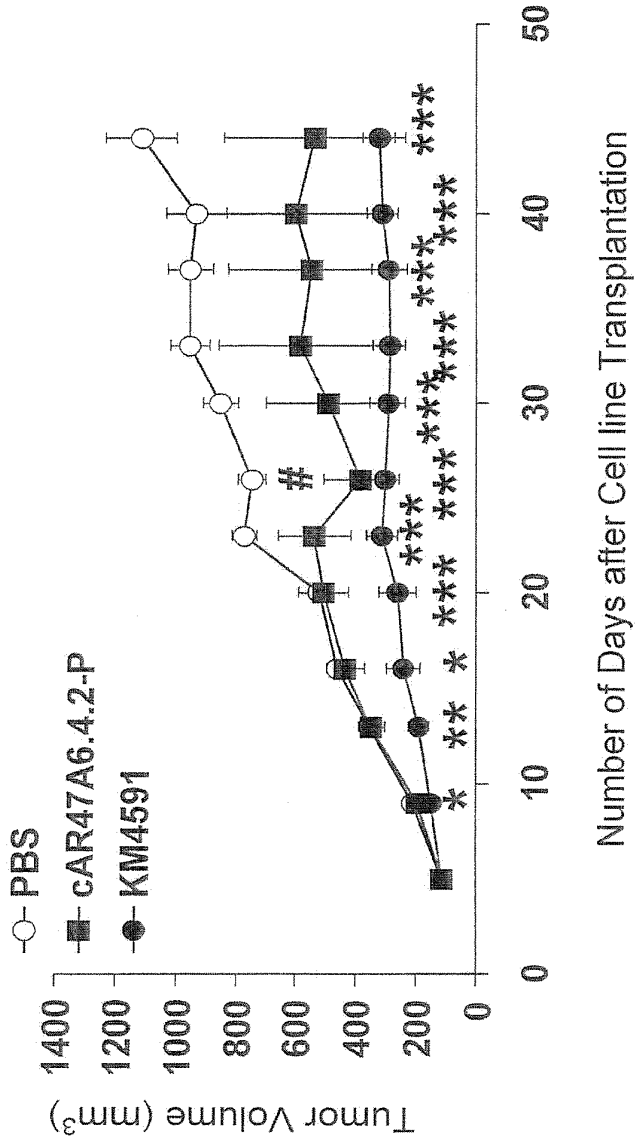

PBS
cAR47A6.4.2-P
KM4591
Tumor Volume (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Number of Days after Cell line Transplantation

Fig. 12

	1	2	3	4	5	6
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
KM4097HV0	QVQLVQSGAE	VKKPGASVKV	SCKASGYTFT	IYWLGWVRQA	PGQGLEWMGN	IFPGSAYINY
KM4097HV2				K	I	
KM4097HV3a	P					
KM4097HV3b				K	I	
KM4097HV4	P			K	I	
KM4097HV5a	P					
KM4097HV5b	P			K	I	
KM4097HV5c				K	I	
KM4097HV6	P			K	I	
KM4097HV8	P	I		K	I	
KM4097HV9	P V	I		K	I	
KM4097HV10	P V	I		K	I	

	7	8	9	10	11
	1234567890	1234567890	1234567890	1234567890	123456
KM4097HV0	NEKFKGRVTI	TADTSTSTAY	MELSSLRSED	TAVYYCAREG	SNSGYWGQGT	LVTVSS
KM4097HV2						
KM4097HV3a	K			F		
KM4097HV3b				F		
KM4097HV4				F		
KM4097HV5a	KA L			F		
KM4097HV5b	K			F		
KM4097HV5c	KA			F		
KM4097HV6	KA			F		
KM4097HV8	KA L			F		
KM4097HV9	KA L			F		
KM4097HV10	KA L			F	L	

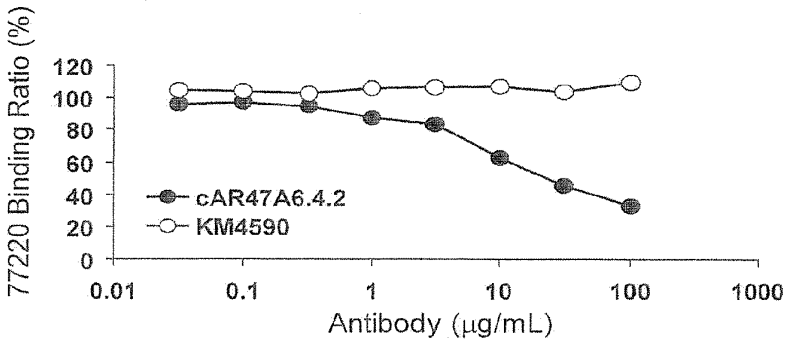
Fig. 13

	1	2	3	4	5	6
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
KM4097LV0	DIVMTQSPDS	LAVSLGERAT	INCKSSQSLL	NSGNQQNYLA	WYQQKPGQPP	KLLIYGASTR
KM4097LV2		V				
KM4097LV3a		A V				
KM4097LV3b		V M				
KM4097LV4		A V M				

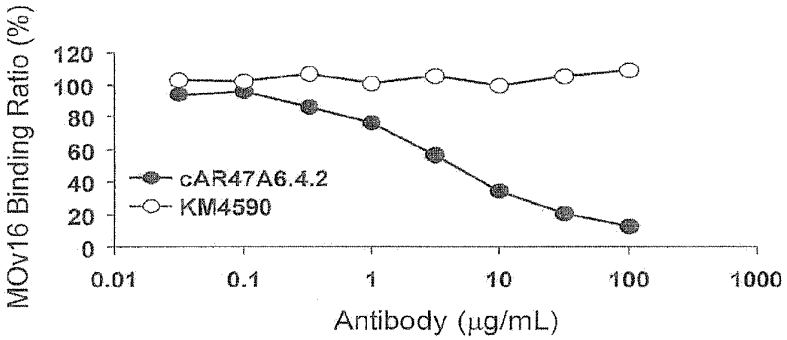
	7	8	9	10	11
	1234567890	1234567890	1234567890	1234567890	1234567890 123
KM4097LV0	ESGVPDRFSG	SGSGTDFTLT	ISSLQAEDVA	VYYCQSDHIY	PYTFGQGTKL EIK
KM4097LV2			V		
KM4097LV3a			V		
KM4097LV3b			V		
KM4097LV4			V		

Fig. 14

(a)


77220 Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

(b)


MOv16 Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

(c)

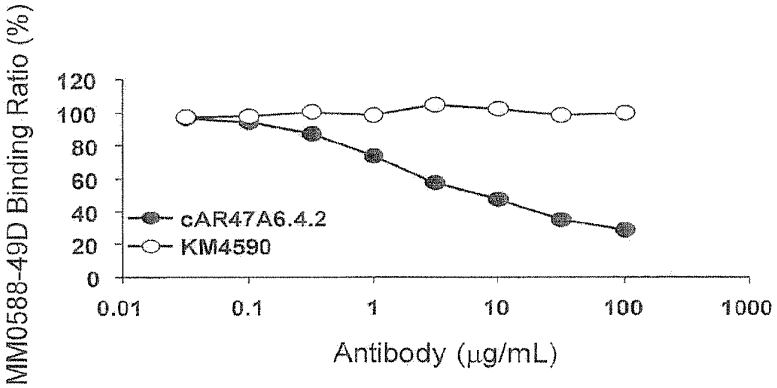
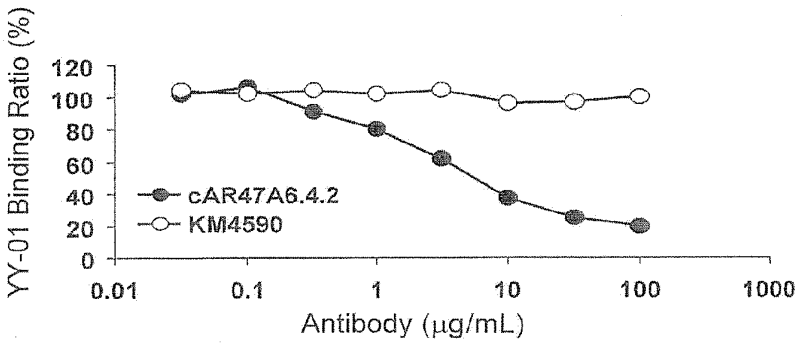
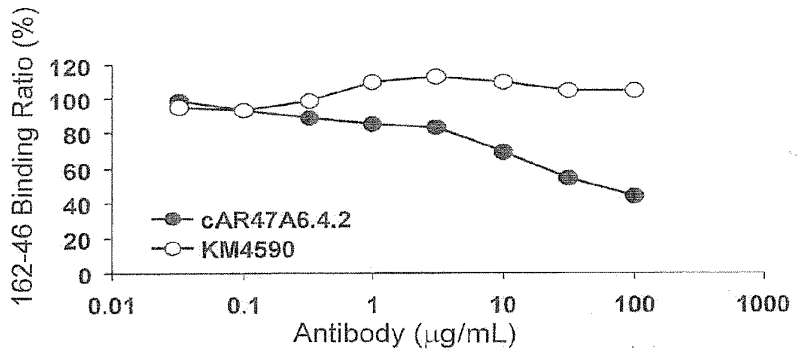

MM0588-49D Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

Fig. 14

(d)


YY-01 Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

(e)


162-46 Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

(f)

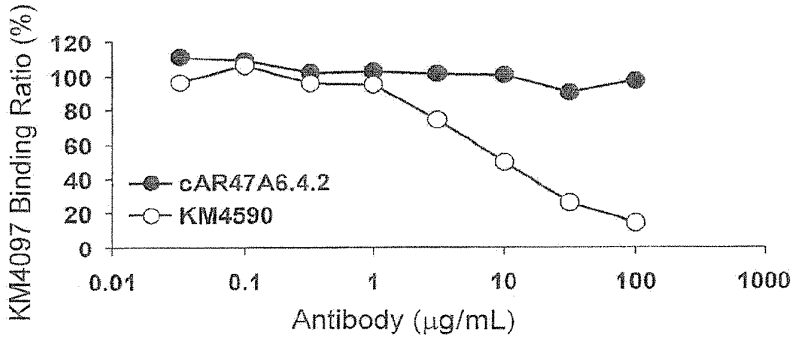

KM4097 Binding Ratio (%)
120
100
80
60
40
20
0
cAR47A6.4.2
KM4590
0.01
0.1
1
10
100
1000
Antibody (μg/mL)

Fig. 15

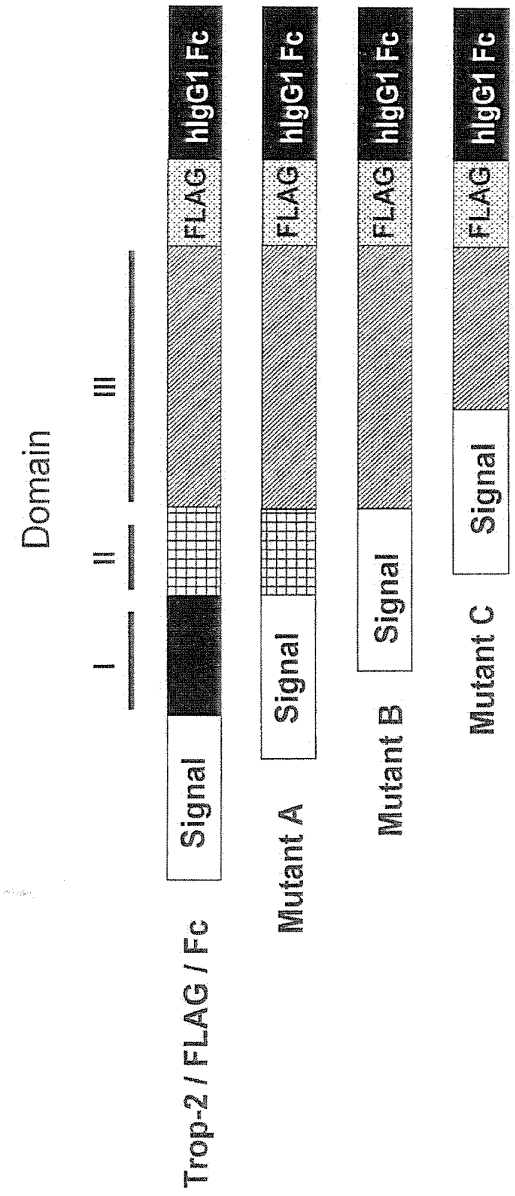

Domain
I
II
III
Trop-2 / FLAG / Fc
Signal
FLAG
hIgG1 Fc
Mutant A
Signal
FLAG
hIgG1 Fc
Mutant B
Signal
FLAG
hIgG1 Fc
Mutant C
Signal
FLAG
hIgG1 Fc

Fig. 16

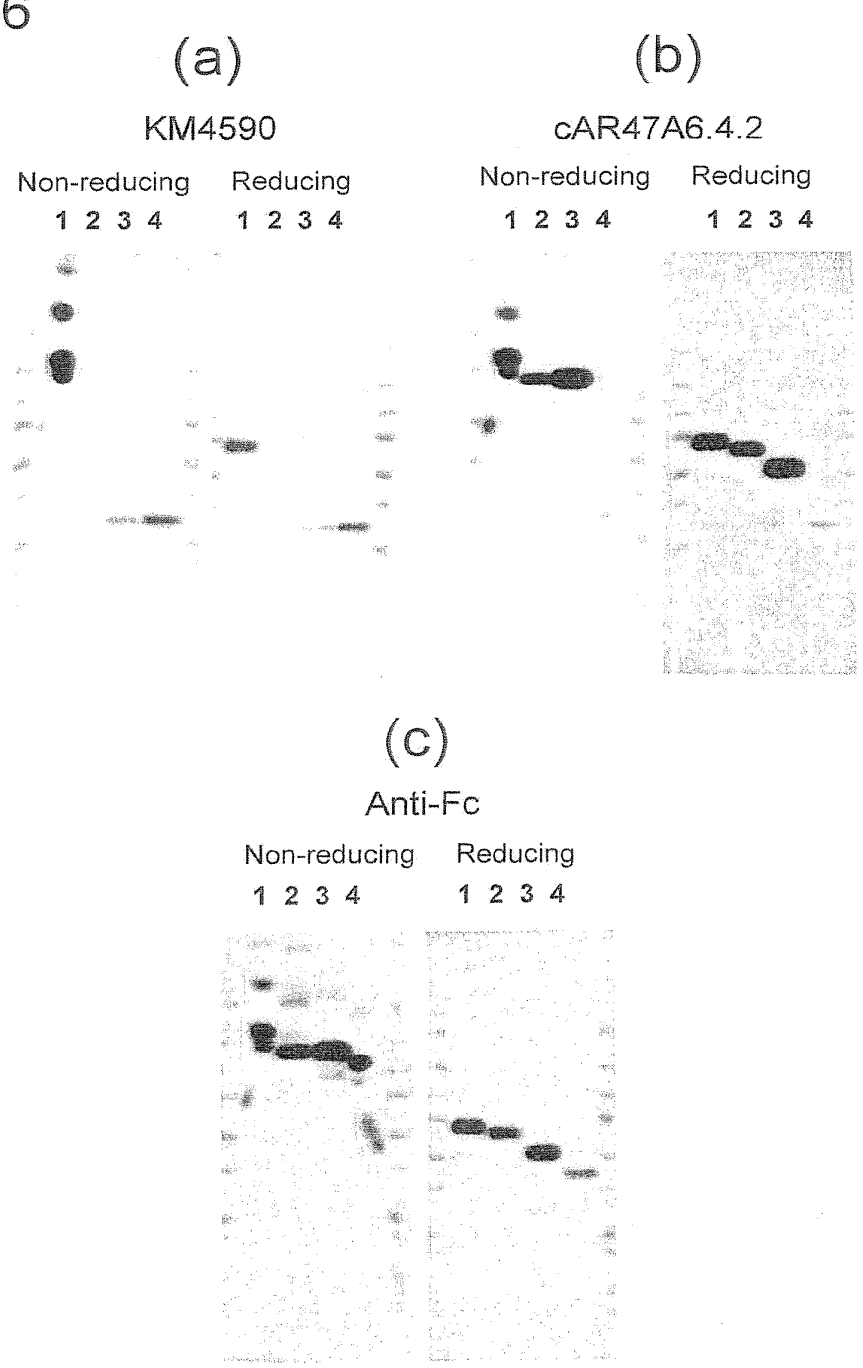

(a)
KM4590
Non-reducing
1 2 3 4
Reducing
1 2 3 4
(b)
cAR47A6.4.2
Non-reducing
1 2 3 4
Reducing
1 2 3 4
(c)
Anti-Fc
Non-reducing
1 2 3 4
Reducing
1 2 3 4

Fig. 17

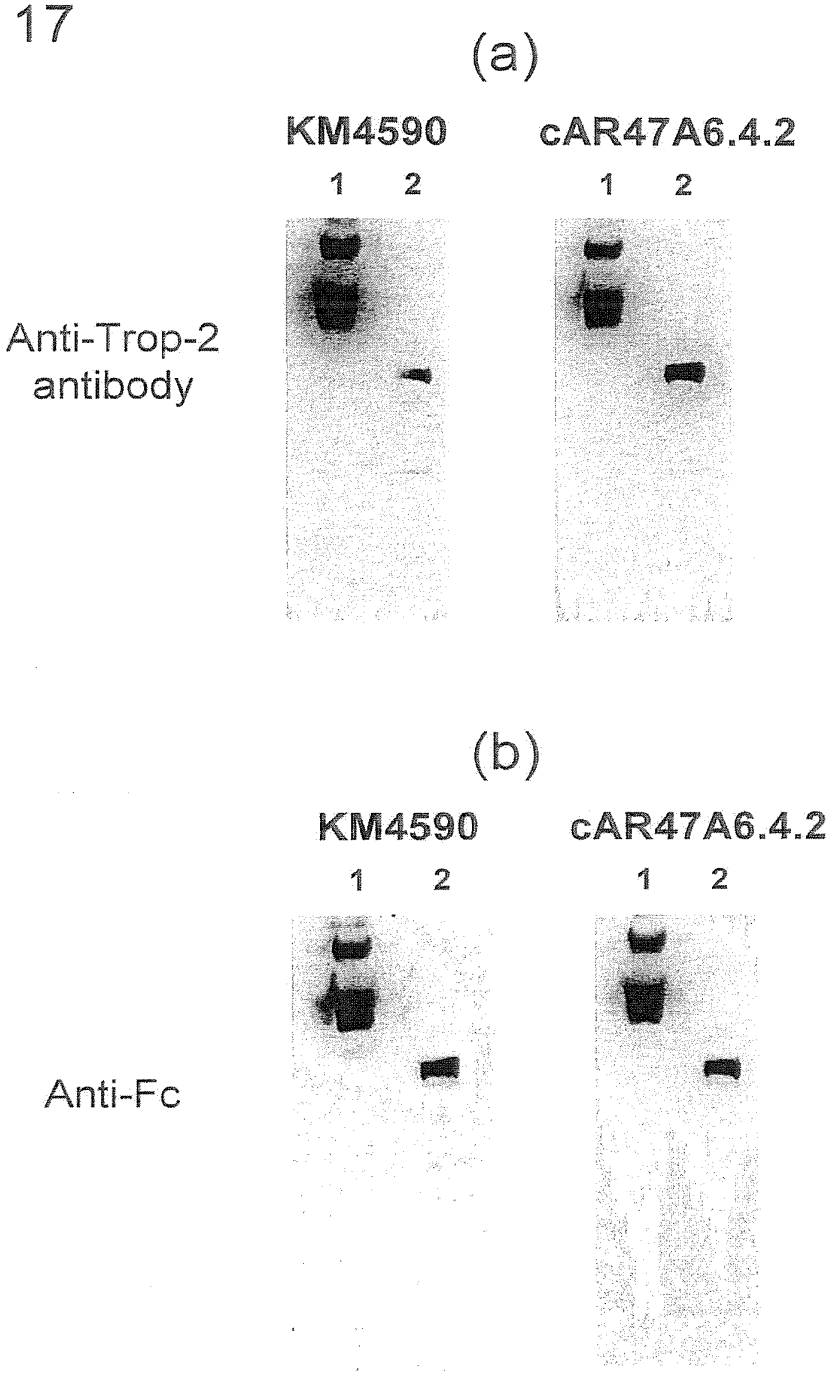

(a)
KM4590
cAR47A6.4.2
1
2
1
2
Anti-Trop-2 antibody
(b)
KM4590
cAR47A6.4.2
1
2
1
2
Anti-Fc

Fig. 18

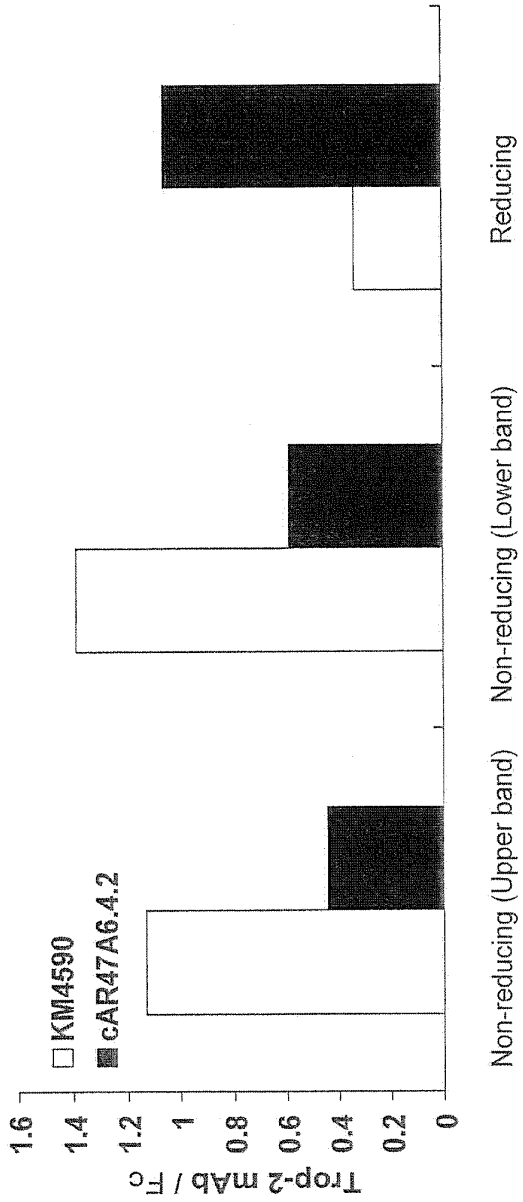
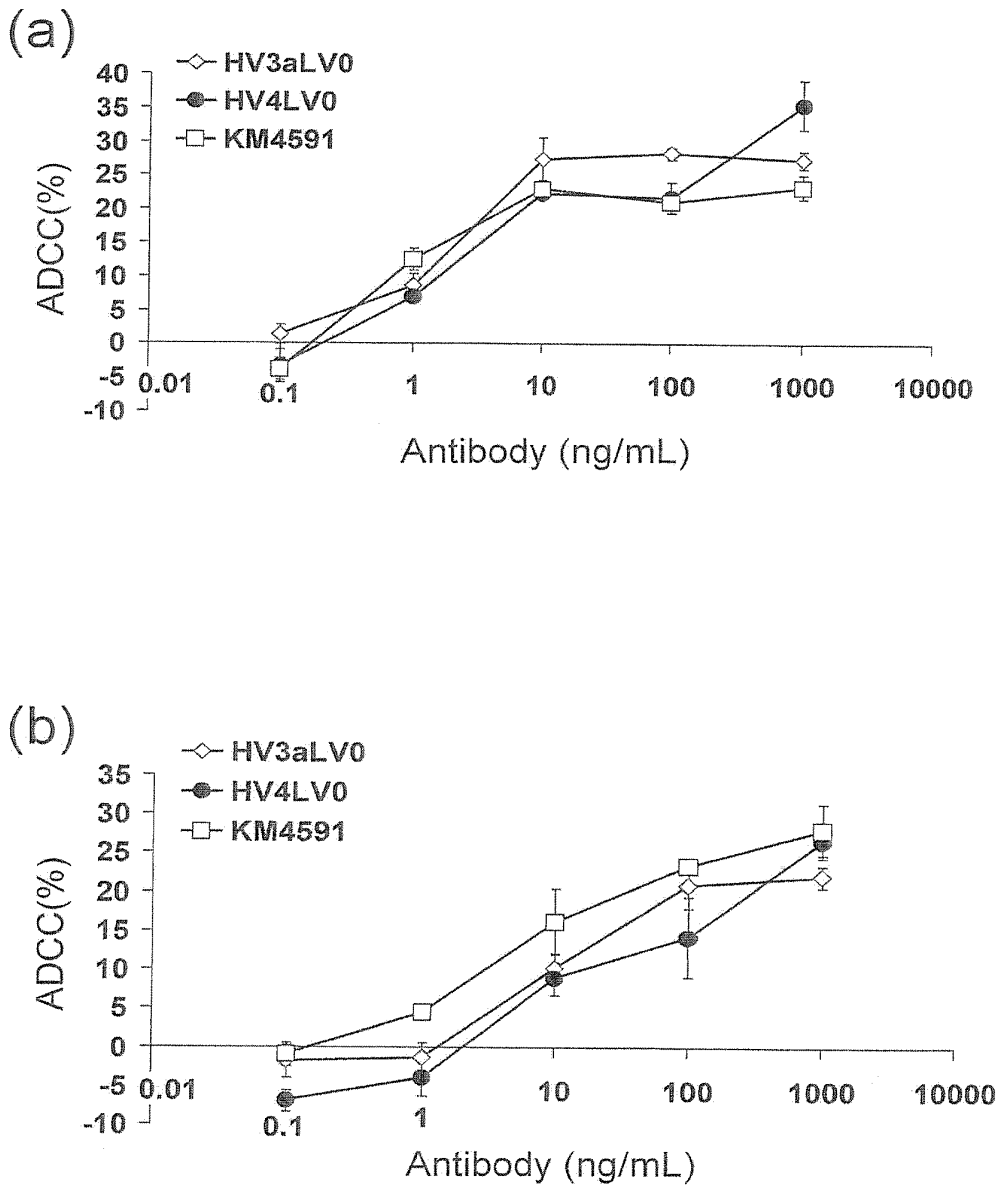

Trop-2 mAb / Fc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KM4590
cAR47A6.4.2
Non-reducing (Upper band)
Non-reducing (Lower band)
Reducing

Fig. 19


(a)
HV3aLV0
HV4LV0
KM4591
ADCC(%)
40
35
30
25
20
15
10
5
0
-5
-10
0.01
0.1
1
10
100
1000
10000
Antibody (ng/mL)
(b)
HV3aLV0
HV4LV0
KM4591
ADCC(%)
35
30
25
20
15
10
5
0
-5
-10
0.01
0.1
1
10
100
1000
10000
Antibody (ng/mL)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063294

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/28(2006.01)i, *A61K39/395*(2006.01)i, *A61K45/00*(2006.01)i, *C12N5/10*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/08*(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)
C07K16/28, A61K39/395, A61K45/00, C12N5/10, C12N15/09, C12P21/08

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 1996-2011
Kokai Jitsuyo Shinan Koho 1971-2011 Toroku Jitsuyo Shinan Koho 1994-2011

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, SwissProt/PIR/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y/A	FORNARO, M. et al, Cloning of the gene encoding TROP-2, a cell-surface glycoprotein expressed by human carcinomas., International Journal of Cancer, 1995, Vol. 62, No. 5, p. 610-618	1-4,6-9, 12-21,25-28/ 5,10,11
Y/A	WO 97/14796 A1 (BRISTOL-MYERS SQIBB CO.), 24 April 1997 (24.04.1997), a whole article & JP 2001-501801 A & US 5840854 A & EP 856054 A	1-4,6-9, 12-21,25-28/ 5,10,11

☒ 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
30 August, 2011 (30.08.11)	06 September, 2011 (06.09.11)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063294

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y/A	WO 2003/74566 A2 (IMMUNOMEDICS, INC.), 12 September 2003 (12.09.2003), a whole article & US 2004/0001825 A1 & US 2007/0212350 A1 & US 2010/0221177 A1 & US 2008/0131363 A1 & US 2011/0070156 A & EP 1483295 A & EP 2287201 A2 & WO 2003/074566 A2	1-4,6-9, 12-21,25-28/ 5,10,11
Y/A	WO 2007/95748 A1 (ARIUS RESEARCH, INC.), 30 August 2007 (30.08.2007), a whole article & JP 2009-528995 A & US 2007/0202043 A1 & EP 1996700 A	1-4,6-9, 12-21,25-28/ 5,10,11
Y/A	WO 2007/95749 A1 (ARIUS RESEARCH, INC.), 30 August 2007 (30.08.2007), a whole article & JP 2009-527230 A & US 2007/0202043 A1 & EP 1996701 A	1-4,6-9, 12-21,25-28/ 5,10,11
Y/A	WO 2008/144891 A1 (ARIUS RESEARCH, INC.), 04 December 2008 (04.12.2008), a whole article & JP 2010-528056 A & US 2008/0131428 A1 & EP 2155791 A	1-4,6-9, 12-21,25-28/ 5,10,11

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063294

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.: 22-24
because they relate to subject matter not required to be searched by this Authority, namely:
The inventions set forth in claims 22 to 24 pertain to diagnostic methods to be practiced on the human body and thus relate to a subject matter on which the International Searching Authority is not required to carry out a 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 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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

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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(54) ANTI-HUMAN TROP-2 ANTIBODY EXHIBITING ANTITUMOR ACTIVITY IN VIVO

(57) The present invention provides: an antibody which specifically reacts with hTROP-2 and has anti-tumor activity *in vivo* (particularly, a humanized antibody); a hybridoma which produces the aforementioned antibody; a conjugate of the aforementioned antibody and a drug; a pharmaceutical composition for diagnosing or treating a tumor; a method for detecting a tumor; and a kit for detecting or diagnosing a tumor.

EP 2 799 452 A1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to an anti-human TROP-2 antibody having anti-tumor activity, and particularly, to an anti-human TROP-2 antibody having anti-tumor *activity in vivo.* In addition, the present invention relates to a hybridoma, which produces the aforementioned antibody, and a use of the aforementioned antibody.

BACKGROUND OF THE INVENTION

[0002] Human TROP-2 (Tacstd2, GA733-1 and EGP-1) (hereinafter also referred to as "hTROP-2") is a single transmembrane, type 1 cell membrane protein consisting of 323 amino acid residues (see SEQ ID NO: 2), and this protein has been known to be overexpressed in various types of epidermal cell carcinomas. The presence of a cell membrane protein associated with immunological resistance, which is commonly expressed in both human trophoblasts and cancer cells, had been long suggested (Non-Patent Document 1). An antigen molecule recognized by mouse monoclonal antibodies (162-25.3, 162-46.2) reacting with the cell membrane protein of a human choriocarcinoma cell line BeWo was identified. This antigen molecule was considered as one of the molecules expressed in human trophoblasts, and was named as Trop-2 (Non-Patent Document 2). Thereafter, the same molecule was discovered by other researchers. That is to say, a tumor antigen recognized by a mouse monoclonal antibody GA733 which is obtained by immunization with stomach cancer cells SW948 was named as GA733-1 (Non-Patent Document 3), and an epithelial glycoprotein recognized by a mouse monoclonal antibody RS7-3G11 which is obtained by immunization with non-small cell lung cancer cells was named as an epithelial/carcinoma antigen, EGP-1 (Non-Patent Document 4). In 1995, the Trop-2 gene was cloned, and as a result, it was confirmed that these are the same molecules (Non-Patent Document 5). Moreover, it was clarified that the molecule has a function to amplify intracellular calcium signals in cancer cells (Non-Patent Document 6), and therefore, it is also referred to as a tumor-associated calcium signal transducer 2 (TACSTD2).

[0003] The hTROP-2 gene is mapped on chromosome 1p32, and it constitutes a TACSTD gene family together with GA733-2 having a homology of approximately 50% therewith (which has been known as "TACSTD1," "epithelial glycoprotein EGP-2," "EpCAM" or "Trop-1") (Non-Patent Document 7). The hTROP-2 protein (323 amino acid residues; SEQ ID NO: 2) has a molecular weight of approximately 36K Dalton, and this protein consists of a hydrophilic signal peptide (1st to 26th amino acids), an extracellular domain (27th to 274th amino acids), a transmembrane domain (275th to 297th amino acids) and an intracellular domain (298th to 323rd amino acids). The extracellular domain has four heterogeneous N-linked glycosylation sites, and its apparent molecular weight is increased by 11 to 13K Dalton due to addition of sugar chains (Non-Patent Document 5). It is considered that TACSTD gene family has a characteristic thyroglobulin (TY) sequence in the extracellular domain and is associated with the proliferation, invasion and metastasis of cancer cells.

[0004] To date, a physiological ligand of hTROP-2 has not been identified, and the molecular function thereof has not been clarified. However, it has been described that hTROP-2 transmits a calcium signal in tumor cells (Non-Patent Document 6). In addition, from the facts that intracellular serine 303 is phosphorylated by protein kinase C (PKC) that is Ca^{2+}-dependent kinase (Non-Patent Document 4) and that hTROP-2 has a PIP2-binding sequence in its intracellular domain, it has been suggested that hTROP-2 has a signaling function in tumor cells (Non-Patent Document 8).

[0005] As a result of analyses such as immunohistochemistry (IHC) and flow cytometry, overexpression of hTROP-2 in many types of epithelium-derived carcinomas such as stomach cancer, lung cancer, colorectal cancer, ovarian cancer, breast cancer, prostate cancer, pancreatic cancer, liver cancer and esophagus cancer has been reported. In contrast, the expression of hTROP-2 in normal tissues is limited to cells in the epithelial region, and the expression level of hTROP-2 in normal cells is lower than that in cancer cells. Thus, the association of TROP-2 with tumor formation is suggested (Patent Documents 1-3 and 9).

[0006] Moreover, it has been demonstrated that the expression of hTROP-2 used as a biomarker in clinical samples correlates with the malignancy of colorectal cancer (Non-Patent Documents 10 and 11), pancreatic cancer (Non-Patent Document 12) or oral cancer (Non-Patent Document 13), and that when hTROP-2 is overexpressed, the possibility of metastasis or recurrence of such cancer is significantly high. Furthermore, in a large-scale gene expression analysis using a cDNA microarray technique, hTROP-2 has been identified as a gene cluster, which is overexpressed at the highest level in severe papillary adenocarcinoma of the ovary, in comparison with in normal ovary epithelium (Non-Patent Document 14).

[0007] Still further, in recent years, an important role of hTROP-2 in tumor formation has been demonstrated in the models by using colon cancer cells (Non-Patent Document 15). Since the expression of hTROP-2 promotes the anchorage-independent cell proliferation of tumor cells and is required for the tumor formation and proliferation of cancer cells subcutaneously transplanted in immunodeficient mice, it raised the possibility that hTROP-2 would act as a functional tumor antigen and would be used as a new therapeutic target.

[0008] To date, studies regarding the anti-tumor effects of several anti-hTROP-2 antibodies have been reported. An

RS7 antibody (Patent Document 1) has been examined by *employing in vivo* models, in which radioactive substance-labeled antibodies were used, and anti-tumor activity was demonstrated in nude mouse xenograft models. However, the anti-tumor effects by antibody alone (a naked antibody) have not been reported.

[0009] In addition, the cytotoxicity of a cytotoxin-attached anti-hTROP-2 monoclonal antibody BR110 (Patent Document 2) on human cancer cell lines H3619, H2987, MCF-7, H3396 and H2981 in *in vitro* experiments has been reported. However, the cytotoxicity of a naked antibody or an immunoconjugate of BR110 *in vivo* has not been disclosed.

[0010] In recent years, it has been reported that an isolated monoclonal antibody, which was produced from a hybridoma cell line AR47A6.4.2 or AR52A301.5 obtained by immunizing mice with human ovarian cancer tissues, bound to hTROP-2, and that, for the first time, it exhibited, as a naked antibody, anti-tumor activity on nude mouse xenograft models, as well as cytotoxicity *in vitro* (Patent Documents 3 and 4). In these patent documents, the aforementioned antibody exhibited anti-tumor effects by treatment with antibody alone in mouse xenograft models, into which pancreatic cancer cell lines BxPC-3 and PL45, a prostate cancer cell line PC-3, a breast cancer cell line MCF-7 and a colon cancer cell line Colo205 had been transplanted. The therapeutic effects of the antibody have appeared in the models, into which BxPC-3 cells had been transplanted. Other than this, tumor formation and proliferation were only partially (approximately 40% to 60%) suppressed by the preventive administration of the antibody, and an extremely large amount (approximately 20 mg/kg) of the antibody was necessary for such suppression of tumor formation and proliferation.

[0011] Based on the above-mentioned previous findings, the potential use of the anti-hTROP-2 antibody as an anti-tumor antibody has been suggested. However, not all of the anti-hTROP-2 antibodies exhibit anti-tumor effects by treatment with antibody alone as naked antibodies *in vivo*. The antibodies exhibit different actions on hTROP-2, depending on a binding site, affinity and the properties of a monoclonal antibody.

Patent Document 1: U.S. Patent No. 6653104
Patent Document 2: U.S. Patent No. 5840854
Patent Document 3: U.S. Patent No. 7420040
Patent Document 4: U.S. Patent No. 7420041
Non-Patent Document 1: Faulk WP, et al., Proc. Natl. Acad. Sci. U.S.A., 75(4), pp. 1947-1951 (1978)
Non-Patent Document 2: Lipinski M, et al., Proc. Natl. Acad. Sci. U.S.A., 78(8), pp. 5147-5150 (1981)
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Non-Patent Document 13: Fong D, et al., Mod. Pathol., 21(2), pp. 186-191 (2008)
Non-Patent Document 14: Santin AD, et al:, Int. J. Cancer, 112(1), pp. 14-25 (2004)
Non-Patent Document 15: Wang J, et al., Mol. Cancer Ther., 7(2), pp. 280-285 (2008)

SUMMARY OF THE INVENTION

[0012] Under the aforementioned circumstances, it has been desired to develop an anti-hTROP-2 antibody (an anti-hTROP-2 monoclonal antibody) having high anti-tumor *activity in vivo,* and specifically, an anti-hTROP-2 antibody or the like, which has an anti-tumor effect as a naked antibody alone *in vivo* and further, which has the anti-tumor effect at a low dose, and particularly, such an anti-hTROP-2 antibody, which is a humanized antibody.

[0013] The present invention has been completed, while taking into consideration the aforementioned circumstances. The present invention provides an anti-hTROP-2 antibody (an anti-hTROP-2 monoclonal antibody), a hybridoma, which produces the antibody, a fragment of the antibody, a conjugate (an immunoconjugate) of the antibody or the like and a drug, a pharmaceutical composition for diagnosing or treating a tumor, a method for detecting a tumor, a kit for detecting or diagnosing a tumor, and the like, which will be described below.

(1) An antibody against human TROP-2 in which an H chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 92 or 98, and an L chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 93.

In the antibody according to (1) above, the amino acid sequences of CDR 1 to 3 of the H chain V region of the antibody are shown in SEQ ID NOS: 36 to 38, respectively, and/or the amino acid sequences of CDR 1 to 3 of the

L chain V region of the antibody are shown in SEQ ID NOS: 41 to 43, respectively.

(2) An antibody against human TROP-2 in which an H chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 94 or 95, and an L chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 96.

[0014] In the antibody according to (2) above, the amino acid sequences of CDR 1 to 3 of the H chain V region of the antibody are shown in SEQ ID NOS: 66 to 68, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region of the antibody are shown in SEQ ID NOS: 71 to 73, respectively.

[0015] An example of the antibody according to (1) and (2) above is a humanized antibody.

[0016] An example of the antibody according to (1) and (2) above is an antibody having anti-tumor activity *in vivo.*

[0017] An example of the antibody according to (1) and (2) above is an antibody exhibiting 50% or more of tumor growth inhibitory activity at a dosage of 5 to 20 mg/kg body weight. Herein, the frequency of administration for exhibiting the tumor growth inhibitory activity is, for example, at most once a week.

[0018] An example of the antibody according to (1) and (2) above is an antibody exhibiting 50% or more of the tumor growth inhibitory activity by a single administration of the antibody at a dosage of 10 mg/kg body weight.

[0019] An example of the antibody according to (1) and (2) above is an antibody having anti-tumor activity on two or more types of human tumor cell lines.

[0020] An example of the antibody according to (1) and (2) above is an antibody having a dissociation constant (Kd value) of 1.0×10^{-10} M or less.

[0021] An example of the antibody according to (1) and (2) above is a monoclonal antibody.

[0022] Herein, the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. The tumor is preferably at least one type selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer.

[0023] In addition, the tumor is, for example, a recurrent cancer or a metastatic cancer.

[0024] Moreover, the tumor cell lines are, for example, at least two types selected from the group consisting of a human pancreatic cancer cell line PK-59, a human pancreatic cancer cell line BxPC-3, a human pancreatic cancer cell line KP-3L, a human pancreatic cancer cell line KP-2, a human pancreatic cancer cell line PK-1, a human pancreatic cancer cell line PK-45H, a human pancreatic cancer cell line PK-45P, a human pancreatic cancer cell line TCC-PAN2, a human pancreatic cancer cell line SUIT-2, a human colon cancer cell line CACO-2, a human colon cancer cell line SW480, a human colon cancer cell line DLD-1, a human colon cancer cell line HCT 116, a human breast cancer cell line JIMT-1, a human breast cancer cell line HCC1143, a human breast cancer cell line MCF-7, a human breast cancer cell line MBA-MB-468, a human prostate cancer cell line DU145, a human prostate cancer cell line PC-3, a human lung cancer cell line Calu-3, a human ovarian cancer cell line SK-OV-3 and a human bile duct cancer cell line TFK-1. Among others, the tumor cell lines are preferably at least two types selected from the group consisting of a human pancreatic cancer cell line PK-59, a human pancreatic cancer cell line BxPC-3, a human colon cancer cell line SW480, a human lung cancer cell line Calu-3, a human breast cancer cell line MBA-MD-468 and a human ovarian cancer cell line SK-OV-3. (3) An antibody fragment derived from the antibody according to (1) and (2) above.

[0025] Examples of the antibody fragment according to (3) above include an antibody fragment comprising the amino acid sequence shown in SEQ ID NO: 92 or 98 and/or the amino acid sequence shown in SEQ ID NO: 93, or an antibody fragment comprising the amino acid sequence shown in SEQ ID NO: 94 or 95 and/or the amino acid sequence shown in SEQ ID NO: 96.

(4) An antibody-drug conjugate, which comprises the antibody according to (1) and (2) above and a substance having anti-tumor activity and/or cell-killing activity.

(5) An antibody fragment-drug conjugate, which comprises the antibody fragment according to (3) above and a substance having anti-tumor activity and/or cell-killing activity.

In the conjugate according to (4) and (5) above, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one type selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer. Moreover, the tumor is, for example, a recurrent cancer or a metastatic cancer.

(6) A pharmaceutical composition, which comprises at least one type selected from the group consisting of the antibody according to (1) and (2) above, the antibody fragment according to (3) above and the conjugate according to (4) and (5) above.

Examples of the composition according to (6) above include a composition which is used in the treatment of tumor, a composition which does not cause weight reduction as a side effect and a composition which is used in the

diagnosis of tumor. Herein, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer. Moreover, the tumor is, for example, a recurrent cancer or a metastatic cancer

(7) A tumor therapeutic agent, which comprises at least one type selected from the group consisting of the antibody according to (1) and (2) above, the antibody fragment according to (3) above and the conjugate according to (4) and (5) above.

An example of the tumor therapeutic agent according to (7) above is a tumor therapeutic agent which does not cause weight reduction as a side effect. Herein, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer.

(8) A tumor diagnostic agent, which comprises at least one type selected from the group consisting of the antibody according to (1) and (2) above, the antibody fragment according to (3) above and the conjugate according to (4) and (5) above.

In the tumor diagnostic agent according to (8) above, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer.

(9) A method for detecting a tumor, which comprises: allowing at least one type selected from the group consisting of the antibody according to (1) and (2) above, the antibody fragment according to (3) above and the conjugate according to (4) and (5) above, to react with a sample collected from a living body; and then detecting a signal(s) of the reacted antibody and/or antibody fragment.

In the method for detecting a tumor according to (9) above, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer.

(10) A kit for treating, diagnosing or detecting a tumor, which comprises at least one type selected from the group consisting of the antibody according to (1) and (2) above, the antibody fragment according to (3) above and the conjugate according to (4) and (5) above.

In the kit for treating, diagnosing or detecting a tumor according to (10) above, the tumor is, for example, at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer. Among others, the tumor is preferably at least one selected from the group consisting of human pancreatic cancer, human colorectal cancer, human breast cancer, human lung cancer and human ovarian cancer.

(11) A polynucleotide encoding the antibody according to (1) and (2) above.

(12) A polynucleotide encoding the antibody fragment according to (3) above.

(13) A recombinant vector comprising the polynucleotide according to (11) or (12) above.

(14) A transformant comprising the recombinant vector according to (13) above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026]

Figure 1 shows the measurement of the antigen binding affinity (Kd: dissociation constant) of an anti-hTROP-2 monoclonal antibody (K5-70). Abt: Antibody (total); Agf: Antigen (free).

Figure 2 shows the reactivity of a culture supernatant of hybridoma producing an anti-hTROP-2 monoclonal antibody, with HuH-7 cells (hTROP-2-negative) and HuH-7-hTROP-2 cells. The filled histogram indicates HuH-7 cells, and the open histogram indicates HuH-7-hTROP-2 cells.

Figure 3 shows the reactivity of an anti-hTROP-2 monoclonal antibody with a human pancreatic cancer cell line (PK-59 cells), which endogenously expresses hTROP-2 on the cell surface. The filled histogram indicates the reaction of the cell line only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cell line with each anti-hTROP-2 monoclonal antibody.

Figure 4 shows the reactivity of an anti-hTROP-2 monoclonal antibody with a human pancreatic cancer cell line

(BxPC-3 cells), which endogenously expresses hTROP-2 on the cell surface. The filled histogram indicates the reaction of the cell line only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cell line with each anti-hTROP-2 monoclonal antibody.

Figure 5 shows the reactivity of an anti-hTROP-2 monoclonal antibody (K5-70) with human pancreatic cancer cell lines. The filled histogram indicates the reaction of the cell line only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cell line with each anti-hTROP-2 monoclonal antibody.

Figure 6 shows the reactivity of an anti-hTROP-2 monoclonal antibody (K5-70) with human colon cancer cell lines (Colo320, CACO2, SW480, DLD1, CW2 and HCT 116), human breast cancer cell lines (JIMT-1 and HCC1143) and human prostate cancer cell lines (PC-3 and DU145). The filled histogram indicates the reaction of the cell line only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cell line with the anti-hTROP-2 monoclonal antibody.

Figure 7 shows the cross-reactivity of anti-hTROP-2 monoclonal antibodies with mouse TROP-2. Cells prepared by allowing a mouse TROP-2 gene to be transiently expressed in CHO-K1 cells were used, and a T2-102 antibody (mouse IgG1) exhibiting cross-reactivity with mouse TROP-2 was used as a positive control antibody. The filled histogram indicates the reaction of the cells only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cells with each anti-hTROP-2 monoclonal antibody.

Figure 8 shows the cross-reactivity of anti-hTROP-2 monoclonal antibodies with human EpCAM/TROP-1. Cells prepared by allowing a human EpCAM/TROP-1 gene to be transiently expressed in CHO-K1 cells were used, and a PE-labeled anti-human EpCAM monoclonal antibody (Becton, Dickinson and Company) was used as a positive control antibody. The filled histogram indicates the reaction of the cells only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cells with each anti-hTROP-2 monoclonal antibody.

Figure 9 shows the cell growth inhibitory activity of anti-hTROP-2 antibodies (T6-16, T5-86, K5-70 and K5-107) on a human pancreatic cancer cell line (PK-59 cells). mIgG indicates a control antibody (mouse IgG), and YY01 indicates a commercially available anti-hTROP-2 antibody (Santa Cruz). White column: 0 μg/mL; gray column: 0.1 μg/mL; black column: 1 μg/mL. The activity level was expressed as a ratio of the actual value to the value obtained when an antibody had not been added (0 μg/mL). The error bar indicates a standard deviation. *P < 0.05, **P < 0.01 (by Student's t-test).

Figure 10 shows a scratch assay of a human pancreatic cancer cell line (PK-59 cells) in the presence of anti-hTROP-2 antibodies (T6-16 and K5-70).

Figure 10A shows representative examples of photographs of the scratch regions of PK-59 cells. Day 0 shows a representative example immediately after scratching. mIgG (Day 1) shows a photograph taken 1 day (24 hours) after scratching and then adding a control antibody (mouse IgG, 1 μg/mL) to the medium. K5-70 (Day 1) shows a photograph taken 1 day (24 hours) after scratching and then adding a K5-70 antibody (1 μg/mL) to the medium. T6-16 (Day 1) shows a photograph taken 1 day (24 hours) after scratching and then adding a T6-16 antibody (1 μg/mL) to the medium. Each arrow in each photograph indicates the width of a scratch region.

Figure 10B. The area of a scratch region was analyzed using image analysis software (Scion Image), and based on the obtained value, the value of each test antibody was calculated using the value obtained on Day 0 of the control antibody (mIgG) addition group as a standard value of 1.

*P <0.05, **P < 0.01 (by Student's t-test).

Figure 11 is a view illustrating FACS showing the expression of a stem cell marker in a human pancreatic cancer cell line PK-59. Figure 11A is a view illustrating FACS showing the expression of EpCAM in the PK-59 cells. The filled histogram indicates the reaction of the cells only with a secondary antibody (PE-labeled anti-mouse IgG), and the open histogram indicates the reaction of the cells with an anti-human EpCAM antibody (Becton, Dickinson and Company). Figures 11B and C are views illustrating FACS showing the expression of P-glycoprotein/MCR1 in the PK-59 cells (Figure 11B), and the expression of ABCG2 in the PK-59 cells (Figure 11C). The blue histogram indicates the reaction of the cells only with a secondary antibody, and the red histogram indicates the reaction of the cells with an anti-human P-glycoprotein/MDR1 antibody (BD Biosciences Pharmingen) (Figure 11B), or with an anti-human ABCG2 antibody (BD Biosciences Pharmingen) (Figure 11C). Figure 11D shows FACS analysis, in which the PK-59 cells were double stained with pancreatic cancer stem cell markers, an FITC-labeled anti-human CD24 antibody (BD Biosciences Pharmingen) and a PE-labeled anti-human CD44 antibody (BD Biosciences Pharmingen). Each number in Figure 11D indicates the existing ratio of the cells in each fraction.

Figure 12 shows the evaluation of the anti-tumor activity of a novel anti-hTROP-2 monoclonal antibody clone K5-70 (mouse IgG2a) on xenograft treatment models using PK-59 cells.

Figure 12A shows the time course of tumor growth of a control group (●: mouse IgG) and a K5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05, ** P < 0.01 (by Student's t-test).

Figure 12B shows the plotted tumor weight of each mouse at the time of the 21st day (Day 21) (the final day of experiment) in the test of Figure 12A. The numerical value on each plot indicates a mean value ± standard deviation. ** P < 0.01 (by Student's t-test).

Figure 13 shows the evaluation of the anti-tumor activity of a clone K5-107 (A), a clone T6-16 (B) and a clone K5-116-2-1 (C) on xenograft treatment models using PK-59 cells. The symbol "●" indicates a control group (mouse IgG), and the symbol " ○" indicates an anti-hTROP-2 antibody (10 mg/kg body weight) administration group. The arrow in the graph indicates an antibody administration period, and the numerical value on each plot indicates a mean value ± standard deviation. * P < 0.05 (by Student's t-test).

Figure 14 shows the evaluation of the anti-tumor activity of a clone K5-70 (Figure 14A), a clone T6-16 (Figure 14B) and a clone K5-116-2-1 (Figure 14C) in xenograft prevention models using PK-59 cells. The symbol "●" indicates a control group (mouse IgG), and the symbol "○" indicates an anti-hTROP-2 antibody (10 mg/kg body weight) administration group. The arrow in the graph indicates an antibody administration period, and the numerical value on each plot indicates a mean value ± standard deviation. ** P < 0.01 (by Student's t-test).

Figure 15 shows the evaluation of the anti-tumor activity of a clone K5-70 on xenograft prevention and treatment models using BxPC-3 cells. Figure 15A shows the time course of tumor growth of a control group (●: mouse IgG) and a K5-70 antibody (10 mg/kg body weight) administration group (○) in prevention models (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** P < 0.01 (by Student's t-test). Figure 15B shows the time course of tumor growth of a control group (●: mouse IgG) and a K5-70 antibody (10 mg/kg body weight) administration group (○) in treatment models (a mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05 (by Student's t-test).

Figure 16 shows the dose-dependent anti-tumor activity of a clone K5-70 on xenograft prevention models using PK-59 cells. The volume of a tumor is expressed as a mean value ± standard deviation.

Figure 16A shows the time course of tumor growth of a control group (●: mouse IgG) and K5-70 antibody administration groups (□: 1 mg/kg body weight, Δ: 5 mg/kg body weight) at different doses (a mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05 (by Student's t-test), ** P < 0.01 (by Student's t-test).

Figure 16B shows the plotted tumor weight of each mouse at the time of the 17th day (Day 17) (the final day of experiment) in the test of Figure 16A. The numerical number on each plot indicates a mean value ± standard deviation. ** P < 0.01 (by Student's t-test).

Figure 17 is a schematic view of a human/mouse chimeric TROP-2 protein used in the experiment. SP: signal sequence; TY domain: thyroglobulin type 1 region; TM: transmembrane region; C: intracellular region, wherein the filled region is a polypeptide derived from hTROP-2, whereas the open region is a polypeptide derived from mouse TROP-2. The number in the upper position of the schematic view of the chimeric protein indicates the amino acid number of a mouse TROP-2 protein, and the number in the lower position thereof indicates the amino acid number of an hTROP-2 protein.

Figure 18 shows the results obtained by identifying an anti-hTROP-2 monoclonal antibody-binding region, using human/mouse chimeric TROP-2. Using HEK293 cells, which constantly express either human/mouse chimeric TROP-2-C (hmTROP-2-C) or mouse/human chimeric TROP-2-D (mhTROP-2-D) proteins, the reactivity with the anti-hTROP-2 monoclonal antibodies shown in the figure was studied. As a negative control, mouse IgG2b was used.

Figure 19 shows the results obtained by identifying the antibody-binding region of an anti-hTROP-2 monoclonal antibody.

An hTROP-2 gene and each human/mouse chimeric TROP-2 gene were introduced into HEK293 cells, and FACS analysis was then carried out using the cells, in which the genes were transiently expressed. In Figure 19(A), the reactivity of K5-70, K5-107, T5-86 and K5-116-2-1 antibodies with hTROP-2 (upper case), with hmTROP-2-A (middle case) and with hmTROP-2-B (lower case) was studied. As a negative control, mouse IgG2b was used. In Figure 19(B), the reactivity of T6-4 and T6-16 antibodies with hTROP-2 (upper case), with mhTROP-2-E (middle case) and with mhTROP-2-F (lower case) was studied. As a negative control, mouse IgG2b was used.

Figure 20 shows the expression of hTROP-2 in human normal tissues. Human normal tissue arrays were immunostained with an anti-hTROP-2 monoclonal antibody clone K5-63-17. (A) skin, (B) esophagus, (C) kidney (cortex), (D) kidney (medulla), (E) pancreas, (F) prostate, (G) bladder, (H) tonsil, (I) heart, (J) liver (magnification: × 200)

Figure 21 shows the expression of hTROP-2 in cancer tissues. Human cancer tissue arrays were immunostained with an anti-hTROP-2 monoclonal antibody clone K5-63-17. (A) breast cancer , (B) lung cancer, (C) esophagus cancer, (D) stomach cancer, (E) pancreatic cancer, (F) colorectal cancer , (G) bladder cancer, (H) prostate cancer, (I) ovarian cancer (magnification: × 100)

Figure 22 shows the anti-tumor activity of a clone K5-70 by a single administration on xenograft prevention models using PK-59 cells.

Figure 22A shows the time course of tumor formation in a control group (●: mouse IgG) and in a K5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates antibody

administration. * P < 0.05 (by Student's t-test), ** P < 0.01 (by Student's t-test).

Figure 22B shows the plotted tumor weight of each mouse at the time of the 28th day (Day 28) (the final day of experiment) in the test of Figure 22A. ** P < 0.01 (by Student's t-test).

Figure 22C shows the time course of tumor formation in each mouse in a control group (●: mouse IgG) and in a K5-70 antibody (10 mg/kg body weight) administration group (○). The arrow indicates antibody administration.

Figure 23 shows the anti-tumor activity of a clone K5-70 on xenograft treatment models using human colon cancer SW480 cells.

Figure 23A shows the time course of tumor formation in a control group (●: mouse IgG) and in a K5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** P < 0.01 (by Student's t-test).

Figure 23B shows the plotted tumor weight of each mouse at the time of the 44th day (Day 44) (the final day of experiment) in the test of Figure 23A. ** P < 0.01 (by Student's t-test).

Figure 24 shows the anti-tumor activity of a clone K5-116-2-1 on xenograft treatment models using SW480 cells.

Figure 24A shows the time course of tumor formation in a control group (●: mouse IgG) and in a K5-116-2-1 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. **P < 0.01 (by Student's t-test).

Figure 24B shows the plotted tumor weight of each mouse at the time of the 42nd day (Day 42) (the final day of experiment) in the test of Figure 24A. ** P < 0.01 (by Student's t-test).

Figure 25 shows the anti-tumor activity of a clone T6-16 on xenograft treatment models using SW480 cells.

Figure 25A shows the time course of tumor formation in a control group (●: mouse IgG) and in a T6-16 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05 (by Student's t-test).

Figure 25B shows the plotted tumor weight of each mouse at the time of the 42nd day (Day 42) (the final day of experiment) in the test of Figure 25A. * P < 0.05 (by Student's t-test).

Figure 26 shows the dose-dependent anti-tumor activity of a clone K5-70 on xenograft treatment models using SW480 cells.

Figure 26A shows the time course of tumor formation in a control group (●: mouse IgG) and in a K5-70 antibody administration group (○: 1 mg/kg body weight, Δ: 5 mg/kg body weight, □: 10 mg/kg body weight) (a mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05 (by Student's t-test).

Figure 26B shows the plotted tumor weight of each mouse at the time of the 42nd day (Day 42) (the final day of experiment) in the test of Figure 26A. * P < 0.05 (by Student's t-test).

Figure 27 shows the anti-tumor activity of a clone K5-70 on xenograft treatment models using SW480 cells.

Figure 27A shows the anti-tumor activity of a K5-70 antibody at administration intervals of once a week. Time course of tumor formation in a control group (● mouse IgG) and in a K5-70 antibody (10 mg/kg body weight) administration group (○: 10 mg/kg) is shown (a mean value ± standard deviation). The arrow heads (Days 10, 17, 24, 31, and 38) indicate administration of a K5-70 antibody. * P < 0.05 by Student's t-test.

Figure 27B is a view showing the anti-tumor activity of a K5-70 antibody at administration intervals of once every ten days (q10d) or once every two weeks (q14d). The figure shows the time course of tumor formation in a control group (● mouse IgG, 10 mg/kg) and in a K5-70 antibody administration group (○: q10d, 10 mg/kg, Δ: q14d, 10 mg/kg) (a mean value ± standard deviation). The filled arrowheads (▼: Days 9, 19, and 29) and the open arrowheads (▽: Days 9, 23, and 37) indicate administration of a K5-70 antibody. * P < 0.05, ** P < 0.01 by Student's t-test.

Figure 28 shows the dose-dependent anti-tumor activity of a clone T6-16 on xenograft treatment models using SW480 cells.

Figure 28A shows the time course of tumor formation in a control group (● mouse IgG) and in a T6-16 antibody administration group (○: 1 mg/kg body weight, Δ : 5 mg/kg body weight, □ : 10 mg/kg body weight) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** P < 0.01 (by Student's t-test).

Figure 28B shows the plotted tumor weight of each mouse at the time of the 43rd day (Day 43) (the final day of experiment) in the test of Figure 28A. ** P < 0.01 (by Student's t-test).

Figure 29 shows the anti-tumor activity of a clone T6-16 on xenograft treatment models using SW480 cells. Time course of tumor formation in a control group (● mouse IgG, 10 mg/kg body weight) and in a T6-16 antibody (10 mg/kg body weight) administration group (○: q7d, Δ: q10d) is shown (a mean value ± standard deviation). The arrowheads (Days 10, 17, 24, 31, and 38) and the arrows (Days 10, 20, 30, and 40) indicate administration of a T6-16 antibody. Administration was carried out once every three days to the control group. * P < 0.05, ** P < 0.01 by Student's t-test.

Figure 30 shows the anti-tumor activity of a clone K5-70 on xenograft prevention models using human prostate DU-145 cells.

Figure 30A shows the time course of tumor formation in a control group (●: mouse IgG) and in a K5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an

antibody administration period. * P < 0.05 (by Student's t-test). Figure 30B shows the plotted tumor weight of each mouse at the time of the 40th day (Day 40) (the final day of experiment) in the test of Figure 30A. * P < 0.05 (by Student's t-test).

Figure 31 shows the metastasis-inhibitory activity of a clone K5-70 on liver metastatic models using PK-59 cells. Figures 31A and 31B show the excised liver image of a control group (● mouse IgG) (A) and a K5-70 antibody (10 mg/kg body weight) administration group (B), which were taken 6 weeks after the cell transplantation. The arrows indicate liver metastatic foci.

Figure 32 shows the anti-tumor activity of K5-70 on xenograft models using SW480 cells, which are recurrent cancer models after administration of irinotecan hydrochloride. This figure shows the time course of tumor formation in a non-treat group (◆), in an irinotecan hydrochloride (40 mg/kg body weight) + K5-70 antibody (○: 10 mg/kg body weight) administration group, and in an irinotecan hydrochloride (40 mg/kg body weight) + mouse IgG (●: 10 mg/kg body weight) administration group (a mean value ± standard deviation). The arrowheads (Days 11, 14, and 17) indicate administration of irinotecan hydrochloride. The K-70 antibody or the mouse IgG was administered once every three days from Day 20. The arrow indicates an antibody administration period. *P < 0.05, **P < 0.01 by Student's t-test.

Figure 33 shows the cDNA nucleotide sequence of a clone K5-70 H chain variable region (VH) (SEQ ID NO: 34) and the deduced amino acid sequence (SEQ ID NO: 35). A signal peptide is shown in italics. The double-underlined glutamine (Q) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *IYWIN, NIYPSDSYTNYNQKFKD,* and *TSMADY)* were determined in accordance with the definitions of Kabat et al. (Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-70 VH are shown in SEQ ID NOS: 36 to 38, respectively.

Figure 34 shows the cDNA nucleotide sequence of a clone K5-70 L chain variable region (VL) (SEQ ID NO: 39) and the deduced amino acid sequence (SEQ ID NO: 40). A signal peptide is shown in italics. The double-underlined aspartic acid (D) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *RASQSIGTSIH, YASESIS,* and *QQSNSWPFT)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-70 VL are shown in SEQ ID NOS: 41 to 43, respectively.

Figure 35 shows the cDNA nucleotide sequence of a clone K5-107 H chain variable region (VH) (SEQ ID NO: 44) and the deuced amino acid sequence (SEQ ID NO: 45). A signal peptide is shown in italics. The double-underlined glutamine (Q) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *SYWMH, NIYPGGGYTNYDEKFKS,* and *SSVFDY)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-107 VH are shown in SEQ ID NOS: 46 to 48, respectively.

Figure 36 shows the cDNA nucleotide sequence of a clone K5-107 L chain variable region (VL) (SEQ ID NO: 49) and the deduced amino acid sequence (SEQ ID NO: 50). A signal peptide is shown in italics. The double-underlined aspartic acid (D) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *RASQNIGTSIH, YASESIS,* and *QQSNSWPFT)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-107 VL are shown in SEQ ID NOS: 51 to 53, respectively.

Figure 37 shows the cDNA nucleotide sequence of a clone K5-116-2-1 H chain variable region (VH) (SEQ ID NO: 54) and the deduced amino acid sequence (SEQ ID NO: 55). A signal peptide is shown in italics. The double-underlined glutamine (Q) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *SYWIT, NIYPSDSYTNYNQKFRD,* and *LFDY)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-116-2-1 VH are shown in SEQ ID NOS: 56 to 58, respectively.

Figure 38 shows the cDNA nucleotide sequence of a clone K5-116-2-1 L chain variable region (VL) (SEQ ID NO: 59) and the deduced amino acid sequence (SEQ ID NO: 60). A signal peptide is shown in italics. The double-underlined aspartic acid (D) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *RASQSIGTSIH, YASESIS,* and *QQSNSWPFT)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-116-2-1 VL are shown in SEQ ID NOS: 61 to 63, respectively.

Figure 39 shows the cDNA nucleotide sequence of a clone T6-16 H chain variable region (VH) (SEQ ID NO: 64) and the deduced amino acid sequence (SEQ ID NO: 65). A signal peptide is shown in italics. The double-underlined glutamic acid (E) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *DYNMH, YIYPYNGGTGYNQRFKS,* and *EDYGSSPSYAMDY)* were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone T6-16 VH are shown in SEQ ID NOS: 66 to 68, respectively.

Figure 40 shows the cDNA nucleotide sequence of a clone T6-16 L chain variable region (VL) (SEQ ID NO: 69) and the deduced amino acid sequence (SEQ ID NO: 70). A signal peptide is shown in italics. The double-underlined aspartic acid (D) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *RSSQSLVHGNGNTYLH, KVSNRFS,* and *SQTTHVPT*) were determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone T6-16 VL are shown in SEQ ID NOS: 71 to 73, respectively.

Figure 41 shows an alignment of the amino acid sequence (SEQ ID NO: 35) of the H chain variable region of a clone K5-70 (K5-70 VH), the amino acid sequence (SEQ ID NO: 75) of the H chain variable region of a humanized K5-70 (HuK5-70 VH), and the amino acid sequence (SEQ ID NO: 85) of the H chain variable region of an acceptor (Genbank accession No. DA980102; SEQ ID NO: 84) used for the production of the humanized antibody (DA980102 VH). (It is to be noted that each of the amino acid sequences shown in the figure indicates a portion of the amino acid sequence of each H chain variable region (specifically, the amino acid sequence of a mature protein portion from which a signal peptide portion is removed)).

The amino acid sequence underlined in the K5-70 VH indicates a CDR sequence determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). In addition, the number in the upper position of the amino acid sequence indicates the position number of an amino acid determined in accordance with the aforementioned definitions of Kabat et al. Each CDR sequence in the DA980102 VH is expressed with the symbol "---," and thus the description is omitted. Since the amino acid underlined in the HuK5-70 VH was assumed to be important for the maintenance of the structure of CDR, the sequence of the K5-70 VH was maintained. In addition, with regard to the amino acid double-underlined in the HuK5-70 VH, the amino acid of the corresponding DA980102 VH (methionine (M)) is rarely found at this position. Hence, for the purpose of decreasing antigenicity, the amino acid double-underlined in the HuK5-70 VH was substituted with leucine (L) as a representative amino acid belonging to the same subgroup.

Figure 42 shows an alignment of the amino acid sequence (SEQ ID NO: 40) of the L chain variable region of a clone K5-70 (K5-70 VL), the amino acid sequence (SEQ ID NO: 77) of the L chain variable region of a humanized K5-70 (HuK5-70 VL), and the amino acid sequence (SEQ ID NO: 87; Genbank accession No. AAA64877) of the L chain variable region of an acceptor (Genbank accession No. L41174; SEQ ID NO: 86) used for the production of the humanized antibody (L41174 VL). (It is to be noted that each of the amino acid sequences shown in the figure indicates a portion of the amino acid sequence of each L chain variable region (specifically, the amino acid sequence of a mature protein portion from which a signal peptide portion is removed)).

The amino acid sequence underlined in the K5-70 VL indicates a CDR sequence determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). In addition, the number in the upper position of the amino acid sequence indicates the position number of an amino acid determined in accordance with the aforementioned definitions of Kabat et al. Each CDR sequence in the L41174 VL is expressed with the symbol "---," and thus the description is omitted. Since the amino acid underlined in the HuK5-70 VL was assumed to be important for the maintenance of the structure of CDR, the sequence of the K5-70 VL was maintained.

Figure 43 shows an alignment of the amino acid sequence (SEQ ID NO: 65) of the H chain variable region of a clone T6-16 (T6-16 VH), the amino acid sequences (SEQ ID NOS: 79 and 81, respectively) of the H chain variable regions of two types of humanized T6-16 (HuT6-16 VH1 and HuT6-16 VH2), and the amino acid sequence (SEQ ID NO: 89) of the H chain variable region of an acceptor (Genbank accession No. DA935238; SEQ ID NO: 88) used for the production of the humanized antibody (DA935238 VH). (It is to be noted that each of the amino acid sequences shown in the figure indicates a portion of the amino acid sequence of each H chain variable region (specifically, the amino acid sequence of a mature protein portion from which a signal peptide portion is removed)).

The amino acid sequence underlined in the HuT6-16 VH indicates a CDR sequence determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). In addition, the number in the upper position of the amino acid sequence indicates the position number of an amino acid determined in accordance with the aforementioned definitions of Kabat et al. Each CDR sequence in the DA935238 VH is expressed with the symbol "---," and thus the description is omitted. Since the amino acids underlined in the HuT6-16 VH1 and HuT6-16 VH2 were assumed to be important for the maintenance of the structure of CDR, the sequence of the T6-16 VH was maintained. In addition, the lysine (K) at position 73 in the HuT6-16 VH1 was substituted with a threonine (T) derived from DA935238 as an acceptor sequence in the HuT6-16 VH2.

Figure 44 shows an alignment of the amino acid sequence (SEQ ID NO: 70) of the L chain variable region of a clone T6-16 (T6-16 VL), the amino acid sequence (SEQ ID NO: 83) of the L chain variable region of a humanized T6-16 (HuT6-16 VL), and the amino acid sequence (SEQ ID NO: 91; Genbank accession No. AAA60341) of the L chain variable region of an acceptor (Genbank accession No. M99608; SEQ ID NO: 90) used for the production of the humanized antibody (M99608 VL). (It is to be noted that each of the amino acid sequences shown in the figure indicates a portion of the amino acid sequence of each L chain variable region (specifically, the amino acid sequence

of a mature protein portion from which a signal peptide portion is removed)).

The amino acid sequence underlined in the T6-16 VL indicates a CDR sequence determined in accordance with the definitions of Kabat et al. (as described above; U.S. Department of Health and Human Services, 1991). In addition, the number in the upper position of the amino acid sequence indicates the position number of an amino acid determined in accordance with the aforementioned definitions of Kabat et al. Each CDR sequence in the M99608 VL is expressed with the symbol "---," and thus the description is omitted.

Figure 45 shows the gene sequence (SEQ ID NO: 74) and amino acid sequence (SEQ ID NO: 75) of HuK5-70 VH. The upper position of each line indicates the gene sequence (cDNA sequence) and the lower position thereof indicates the amino acid sequence. In the amino acid sequence, a signal peptide portion is underlined with a dashed line and each CDR sequence (CDR 1 to 3) is underlined with a solid line (the amino acid sequence of only the mature protein portion, from which the signal peptide portion is removed, is shown in SEQ ID NO: 92). An EcoRI site (GAA TTC) and a Kozak sequence (ACC ACC) were added to the 5' end of the HuK5-70 VH gene, and an NheI site (GCT AGC) was added to the 3' end thereof.

Figure 46 shows the gene sequence (SEQ ID NO: 76) and amino acid sequence (SEQ ID NO: 77) of HuK5-70 VL. The upper position of each line indicates the gene sequence (cDNA sequence) and the lower position thereof indicates the amino acid sequence. In the amino acid sequence, a signal peptide portion is underlined with a dashed line and each CDR sequence (CDR 1 to 3) is underlined with a solid line (the amino acid sequence of only the mature protein portion, from which the signal peptide portion is removed, is shown in SEQ ID NO: 93). An AgeI site (ACC GGT) and a Kozak sequence (ACC ACC) were added to the 5' end of the HuK5-70 VL gene, and a BsiWI site (CGT ACG) was added to the 3' end thereof.

Figure 47 shows the gene sequence (SEQ ID NO: 78) and amino acid sequence (SEQ ID NO: 79) of HuT6-16 VH1. The upper position of each line indicates the gene sequence (cDNA sequence) and the lower position thereof indicates the amino acid sequence. In the amino acid sequence, a signal peptide portion is underlined with a dashed line and each CDR sequence (CDR 1 to 3) is underlined with a solid line (the amino acid sequence of only the mature protein portion, from which the signal peptide portion is removed, is shown in SEQ ID NO: 94). An EcoRI site (GAA TTC) and a Kozak sequence (ACC ACC) were added to the 5' end of the HuT6-16 VH1 gene, and an NheI site (GCT AGC) was added to the 3' end thereof.

Figure 48 shows the gene sequence (SEQ ID NO: 80) and amino acid sequence (SEQ ID NO: 81) of HuT6-16 VH2. The upper position of each line indicates the gene sequence (cDNA sequence) and the lower position thereof indicates the amino acid sequence. In the amino acid sequence, a signal peptide portion is underlined with a dashed line and each CDR sequence (CDR 1 to 3) is underlined with a solid line (the amino acid sequence of only the mature protein portion, from which the signal peptide portion is removed, is shown in SEQ ID NO: 95). An EcoRI site (GAA TTC) and a Kozak sequence (ACC ACC) were added to the 5' end of the HuT6-16 VH2 gene, and an NheI site (GCT AGC) was added to the 3' end thereof.

Figure 49 shows the gene sequence (SEQ ID NO: 82) and amino acid sequence (SEQ ID NO: 83) of HuT6-16 VL. The upper position of each line indicates the gene sequence (cDNA sequence) and the lower position thereof indicates the amino acid sequence. In the amino acid sequence, a signal peptide portion is underlined with a dashed line and each CDR sequence (CDR 1 to 3) is underlined with a solid line (the amino acid sequence of only the mature protein portion, from which the signal peptide portion is removed, is shown in SEQ ID NO: 96). An AgeI site (ACC GGT) and a Kozak sequence (ACC ACC) were added to the 5' end of the HuT6-16 VL gene, and a BsiWI site (CGT ACG) was added to the 3' end thereof.

Figure 50 shows the results obtained by confirming the expression of the HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody.

Figure 50(A) The expression vectors pFUSE-CHIg-HuK5-70 and pFUSE2-CLIg-HuK5-70 were introduced into 293F cells, and the expression of the HuK5-70 antibody in the culture supernatant was analyzed by Western blotting. Lane 1 indicates the culture supernatant of 293F cells into which no genes were introduced (negative control), and lane 2 indicates the culture supernatant of 293F cells into which the aforementioned expression vectors were introduced. The heavy chain and light chain proteins of the HuK5-70 antibody were detected with a biotin-labeled anti-human IgG $F(ab')^2$ antibody.

Figure 50(B) The expression vectors pFUSE-CHIg-HuT6-16-1 and pFUSE2-CLIg-HuT6-16 (lane 3), and the expression vectors pFUSE-CHIg-HuT6-16-2 and pFUSE2-CLIg-HuT6-16 (lane 4), were introduced into 293F cells in these combinations. Then, the expression of the HuT6-16-1 antibody and HuT6-16-2 antibody was analyzed by Western blotting. The heavy chain proteins of the HuT6-16-1 antibody and HuT6-16-2 antibody were detected with a biotin-labeled anti-human IgG Fc antibody, and the light chain proteins of the HuT6-16-1 antibody and HuT6-16-2 antibody were detected with a biotin-labeled anti-human IgG $F(ab')^2$ antibody.

Figure 51 shows the results obtained by staining the purified HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody with Coomassie.

The purified HuK5-70 antibody (lane 1), HuT6-16-1 antibody (lane 2) and HuT6-16-2 antibody (lane 3) were loaded

in amounts of 1 μg each on SDS-PAGE, and were then stained with Coomassie.

Figure 52 shows the results obtained by analyzing the antigen-binding ability of the HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody, using a flow cytometer.

The reactivity of each antibody shown in the figure with HEK293-hTROP-2 cells (Figure 52A) and PK-59 cells (Figure 52B) was analyzed by FACS. Secondary antibody alone was used as a negative control (filled), and the reactivity of each antibody was indicated with a gray line.

Figure 53 shows the results obtained by measuring the antigen-binding ability of the HuK5-70 antibody according to an ELISA method.

The antigen-binding ability of the K5-70 antibody and HuK5-70 antibody was analyzed according to an antigen-coated ELISA method. The symbol ▲ indicates the measurement results of the K5-70 antibody, and the symbol ● indicates the measurement results of the HuK5-70 antibody.

Figure 54 shows the results obtained by measuring the antigen-binding ability of the HuT6-16-1 antibody and HuT6-16-2 antibody according to an ELISA method.

The antigen-binding ability of the T6-16 antibody, HuT6-16-1 antibody and HuT6-16-2 was analyzed according to an antigen-coated ELISA method. The symbol ▲ indicates the measurement results of the T6-16 antibody, the symbol ● indicates the measurement results of the HuT6-16-1 antibody, and the symbol ■ indicates the measurement results of the HuT6-16-2 antibody.

Figure 55 shows the anti-tumor activity of a humanized anti-hTROP-2 antibody (HuK5-70 antibody) on xenograft treatment models using human colon cancer SW480 cells.

Figure 55A shows the time course of tumor formation in a control group (●: PBS) and in a HuK5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. * $P < 0.05$, ** $P < 0.01$ (by Student's t-test).

Figure 55B shows the plotted tumor weight of each mouse at the time of the 39th day (Day 39) (the final day of experiment) in the test of Figure 55A. * $P < 0.05$ (by Student's t-test).

Figure 56 shows the dose-dependent anti-tumor activity of a humanized anti-hTROP-2 antibody (HuK5-70) on xenograft treatment models using human colon cancer SW480 cells.

Figure 56A shows the time course of tumor formation in a control group (●: PBS) and in a HuK5-70 antibody administration groups (○: 1 mg/kg body weight, △: 5 mg/kg body weight, □: 10 mg/kg body weight) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** $P < 0.01$ (by Student's t-test).

Figure 56B shows the plotted tumor weight of each mouse at the time of the 48th day (Day 48) (the final day of experiment) after cancer cell transplantation in the test of Figure 56A. ** $P < 0.01$ (by Student's t-test).

Figure 57 shows the dose-dependent anti-tumor activity of a humanized anti-hTROP-2 antibody (HuT6-16-2) on xenograft treatment models using human colon cancer SW480 cells.

Figure 57A shows the time course of tumor formation in a control group (●: PBS) and in a HuT6-16-2 antibody administration groups (○: 1 mg/kg body weight, △: 5 mg/kg body weight, □: 10 mg/kg body weight) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** $P < 0.01$ (by Student's t-test).

Figure 57B shows the plotted tumor weight of each mouse at the time of the 48th day (Day 48) (the final day of experiment) after cancer cell transplantation in the test of Figure 57A. * $P < 0.05$ (by Student's t-test).

Figure 58 shows the anti-tumor activity of mouse anti-hTROP-2 antibodies (K5-70 and T6-16) on xenograft treatment models using human ovarian cancer SK-OV-3 cells.

Figure 58A shows the time course of tumor formation in a control group (●: PBS), in a K5-70 antibody (10 mg/kg body weight) administration group (○), and in a T6-16 antibody (10mg/kg body weight) administration group (△) (a mean value ± standard deviation). The arrow indicates an antibody administration period. * $P < 0.01$ (by Student's t-test).

Figure 58B shows the plotted tumor weight of each mouse at the time of the 56th day (Day 56) (the final day of experiment) after cancer cell transplantation in the test of Figure 58A. * $P < 0.05$ (by Student's t-test).

Figure 59 shows the anti-tumor activity of mouse anti-hTROP-2 antibodies (K5-70 and T6-16) on xenograft treatment models using human breast cancer MDA-MB-468 cells.

Figure 59A shows the time course of tumor formation in a control group (●: PBS), in a K5-70 antibody (10 mg/kg body weight) administration group (○), and in a T6-16 antibody (10mg/kg body weight) administration group (△) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** $P < 0.01$ (by Student's t-test).

Figure 59B shows the plotted tumor weight of each mouse at the time of the 54th day (Day 54) (the final day of experiment) after cancer cell transplantation in the test of Figure 59A. * $P < 0.05$, ** $P < 0.01$ (by Student's t-test).

Figure 60 shows the anti-tumor activity of mouse anti-hTROP-2 antibodies (K5-70 and T6-16) on xenograft treatment models using human lung cancer Calu-3 cells.

Figure 60A shows the time course of tumor formation in a control group (●: PBS), in a K5-70 antibody (10 mg/kg body weight) administration group (○), and in a T6-16 antibody (10mg/kg body weight) administration group (△) (a

mean value ± standard deviation). The arrow indicates an antibody administration period. * P < 0.05, ** P < 0.01 (by Student's t-test).

Figure 60B shows the plotted tumor weight of each mouse at the time of the 41st day (Day 41) (the final day of experiment) after cancer cell transplantation in the test of Figure 60A. * P < 0.05, ** P < 0.01 (by Student's t-test).

Figure 61 shows the anti-tumor activity of a mouse anti-hTROP-2 antibody K5-70 on xenograft prevention models using human bile duct cancer TFK-1 cells.

Figure 61A shows the time course of tumor formation in a control group (●: PBS) and in a K5-70 antibody (10 mg/kg body weight) administration group (○) (a mean value ± standard deviation). The arrow indicates an antibody administration period. ** P < 0.01 (by Student's t-test).

Figure 61B shows the plotted tumor weight of each mouse at the time of the 31st day (Day 31) (the final day of experiment) after cancer cell transplantation in the test of Figure 61A. ** P < 0.01 (by Student's t-test).

Figure 62 shows the results obtained by analyzing the binding activity of the HuK5-70 and HuT6-16-2 antibodies according to low-density antigen-coated ELISA. A 96-well plate was coated with a 0.1 μg/mL recombinant hTACSTD2-Fc-His protein, and thereafter, test antibodies (K5-70, HuK5-70, T6-16 and HuT6-16-2 antibodies), which had been prepared in concentrations from 20 μg/mL to a series of two-fold dilutions, were allowed to react with the protein. In Figure 62(A), K5-70 (▲) and HuK5-70 (●) antibodies were used as test antibodies, and in Figure 62(B), T6-16 (▲) and HuT6-16-2 antibody (●) were used as test antibodies.

Figure 63 shows the results obtained by analyzing the binding activity of hTROP-2 to K5-70 and HuK5-70 antibodies by ELISA. A 96-well plate was coated with the K5-70 and HuK5-70 antibodies via anti-mouse IgG (γ chain specific) and anti-human IgG1 (Fcγ specific), and thereafter, these antibodies were allowed to react with hTROP-2-EC-His proteins, which had been prepared in concentrations from 5 μg/mL to a series of two-fold dilutions. The binding of such a hTROP-2-EC-His protein was detected using an anti-His tag antibody.
(▲) K5-70 antibody and (●) HuK5-70 antibody.

Figure 64 shows the nucleotide sequence (upper case; SEQ ID NO: 99) and amino acid sequence (lower case; SEQ ID NO: 35) of a K5-70 VH gene that was prepared by gene synthesis. With regard to this nucleotide sequence, an EcoRI site (GAA TTC) and a Kozak sequence (ACC ACC) were added to the 5' end, and an NheI site (GCT AGC) was added to the 3' end. The amino acid sequence is shown by single letter code. A signal peptide on the N-terminal side is shown in italics. The double-underlined glutamine (Q) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *IYWIN, NIYPSDSYTNYNQKFKD,* and *TSMADY)* were determined in accordance with the definitions of Kabat et al. (Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-70 VH are shown in SEQ ID NOS: 36 to 38, respectively.

Figure 65 shows the nucleotide sequence (upper case; SEQ ID NO: 100) and amino acid sequence (lower case; SEQ ID NO: 40) of a K5-70 VL gene that was prepared by gene synthesis. With regard to this nucleotide sequence, an AgeI site (ACC GGT) and a Kozak sequence (ACC ACC) were added to the 5' end, and a BsiWI site (CGT ACG) was added to the 3' end. The amino acid sequence is shown by single letter code. A signal peptide on the N-terminal side is shown in italics. The double-underlined aspartic acid (D) indicates the N-terminal amino acid residue of a mature peptide. The CDR sequences (underlined; *RASQSIGTSIH, YASESIS,* and *QQSNSWPFT)* were determined in accordance with the definitions of Kabat et al. (as described above, U.S. Department of Health and Human Services, 1991). The amino acid sequences of CDR 1 to 3 of the clone K5-70 VL are shown in SEQ ID NOS: 41 to 43, respectively.

Figure 66 shows the binding activity of ChK5-70, HuK5-70, HuVH/MuVL (in which the VL of the HuK5-70 antibody is substituted with the VL of the K5-70 antibody) and MuVH/HuVL (in which the VH of the HuK5-70 antibody is substituted with the VH of the K5-70 antibody) antibodies to hTROP-2. A 96-well plate was coated with a 0.1 μg/mL recombinant hTACSTD2-Fc-His protein. A culture supernatant of cells, in which test antibodies (ChK5-70, HuK5-70, HuVH/MuVL and MuVH/HuVL antibodies) had been transiently expressed, was diluted to result in antibody concentrations of 1, 0.1, 0.01 and 0.001 μg/mL. Thus, the diluted test antibodies were allowed to react with the antigen.
(▲) ChK5-70 antibody, (Δ) MuVH/HuVL antibody, (○) HuVH/MuVL antibody and (●) HuK5-70 antibody.

Figure 67 shows the amino acid sequences of HuK5-70 VH and its amino acid substitution mutants. Amino acids are shown by single letter code. The amino acid of each amino acid substitution mutant, which is the same as that of the HuK5-70 VH, is indicated with the symbol "-," and only the substituted amino acids are shown by single letter code. The number in the upper position of the sequence indicates an amino acid number (Kabat et al., 1991).

Figure 68 shows the binding activity of ChK5-70, HuK5-70, HuK5-70 VH A40R (a mutant in which the alanine at position 40 of the VH of the HuK5-70 antibody is substituted with an arginine) and HuK5-70 VH R44G (a mutant in which the arginine at position 44 of the VH of the HuK5-70 antibody is substituted with a glycine) antibodies to hTROP-2. A 96-well plate was coated with a 0.1 μg/mL recombinant hTACSTD2-Fc-His protein. A culture supernatant of cells, in which test antibodies (ChK5-70, HuK5-70, HuK5-70 VH A40R and HuK5-70 VH R44G antibodies) had been transiently expressed, was diluted to result in concentrations from 0.5 μg/mL to a series of two-fold dilutions

(six samples). Thus, the diluted test antibodies were allowed to react with the antigen. (▲) ChK5-70 antibody, (Δ) HuK5-70 VH R44G antibody, (○) HuK5-70 VH A40R antibody and (●) HuK5-70 antibody.

Figure 69 shows the nucleotide sequence (upper case) and amino acid sequence (lower case) of a HuK5-70 VH R44G gene that was prepared by gene synthesis. With regard to this nucleotide sequence, an EcoRI site (GAA TTC) and a Kozak sequence (ACC ACC) were added to the 5' end, and an NheI site (GCT AGC) was added to the 3' end. The amino acid sequence is shown by single letter code. A signal peptide on the N-terminal side is shown in italics. The amino acid (Q: glutamine) on the N-terminal side of mature VH is double-underlined, and the CDR sequence (Kabat et al., 1991) is underlined.

Figure 70 shows SDS-PAGE performed on a purified HuK5-70-2 antibody. The HuK5-70-2 antibody (1 μg) was loaded on a 11% SDS-PAGE gel under reducing conditions. Lane 1: a molecular weight marker (Precision Plus Dual Standard (BIO-RAD)), lane 2: a HuK5-70-2 antibody. The numerical value on the left side of the figure indicates a molecular weight.

Figure 71 shows the binding activity of K5-70, HuK5-70 and HuK5-70-2 antibodies to hTROP-2. A 96-well plate was coated with a 0.1 μg/mL recombinant hTACSTD2-Fc-His protein. The purified test antibodies (K5-70, HuK5-70 and HuK5-70-2 antibodies) were diluted to result in concentrations from 1 μg/mL to a series of two-fold dilutions (ten samples). Thus, the diluted test antibodies were allowed to react with the antigen. (■) K5-70 antibody, (Δ) HuK5-70-2 antibody, and (●) HuK5-70 antibody.

Figure 72A shows the ADCC activity of humanized anti-hTROP-2 antibodies (open column: HuK5-70, and filled column: HuT6-16-2). More specifically, a HuK5-70 antibody and a HuT6-16-2 antibody (all of which were in concentrations of 0, 0.1, 0.3, 1, 3, and 10 μg/mL) and healthy human peripheral blood monocytes were added to a human colon cancer cell line SW480, and they were then cultured for 6 hours. Thereafter, the activity of LDH released into the culture supernatant was measured, so that the ADCC activity could be measured (a mean value ± standard deviation (N = 3), effector/target (E/T) = 40). The antibody concentration that is 0 indicates non-addition of the antibody. *P< 0.05, **P < 0.01 (by Student's t-test).

Figure 72B shows the ADCC activity of humanized anti-hTROP-2 antibodies (open column: HuK5-70, gray column: HuK5-70-2, and filled column: HuT6-16-2). More specifically, a HuK5-70 antibody, a HuK5-70-2 antibody and a HuT6-16-2 antibody (all of which were in concentrations of 0, 0.3, 1, 3, 10 and 30 μg/mL) and healthy human peripheral blood monocytes were added to a human pancreatic cancer cell line (PK-59), and they were then cultured for 6 hours. Thereafter, the activity of LDH released into the culture supernatant was measured, so that the ADCC activity could be measured (a mean value ± standard deviation (N = 3), effector/target (E/T) = 40). The antibody concentration that is 0 indicates non-addition of the antibody. **P < 0.01 (by Student's t-test).

Figure 72C shows the ADCC activity of humanized anti-hTROP-2 antibodies (open column: HuK5-70, gray column: HuK5-70-2, and filled column: HuT6-16-2). More specifically, a HuK5-70 antibody, a HuK5-70-2 antibody and a HuT6-16-2 antibody (all of which were in concentrations of 0, 0.3, 1, 3, 10 and 30 μg/mL) and healthy human peripheral blood monocytes were added to a human prostate cancer cell line (PC-3), and they were then cultured for 6 hours. Thereafter, the activity of LDH released into the culture supernatant was measured, so that the ADCC activity could be measured (a mean value ± standard deviation (N = 3), effector/target (E/T) = 40). The antibody concentration that is 0 indicates non-addition of the antibody. **P < 0.01 (by Student's t-test).

DETAILED DESCRIPTION OF THE INVENTION

[0027] Hereinafter, the present invention will be described in detail. The following descriptions are not intended to limit the scope of the present invention. Other than the following examples, the present invention may be modified and may be carried out, as appropriate, within a range that does not impair the intention of the present invention

[0028] The present specification includes all of the contents as disclosed in the specification of U.S. Provisional Patent Application No. 61/562,672 (filed on November 22, 2011), which is a priority document of the present application.

[0029] In addition, all publications cited in the present specification, which include prior art documents and patent documents such as laid-open application publications and patent publications, are incorporated herein by reference in their entirety.

1. Summary of the present invention

[0030] As mentioned above, human TROP-2 (hTROP-2) is a single transmembrane, type 1 membrane protein having a full length of 323 amino acid residues. It has been known that an hTROP-2 gene and a gene product thereof are expressed in various types of cancer cells.

[0031] As mentioned above, it has been desired to develop an anti- hTROP-2 antibody (an anti-hTROP-2 monoclonal antibody) or the like having high anti-tumor activity *in vivo.* Under such circumstances, the present inventor performed a screening through an extremely large number of clones, and as a result, the inventor succeeded in obtaining a clone

having high anti-tumor activity *in vivo.* Specifically, the present invention provides a monoclonal antibody, which specifically recognizes the extracellular region of hTROP-2 *in vivo,* and particularly, a monoclonal antibody exhibiting high affinity at a picomole (pM) order. The antibody of the present invention is extremely useful in that it is an anti-hTROP-2 monoclonal antibody (particularly, a humanized antibody), which exhibits significant tumor growth inhibitory activity at a lower dose than that of the existing anti-hTROP-2 antibody (for example, at a dosage of 1/20) when it is administered singly as a naked antibody, and which also exhibits significant tumor growth inhibitory activity on tumor-bearing mouse treatment models, in which multiple types of human cancer cells are used.

2. Production of anti-hTROP-2 antibody

(1) Preparation of antigen

[0032] Information regarding the amino acid sequence (SEQ ID NO: 2) of hTROP-2 is disclosed under "Accession number NP_002344" in the website of, for example, NCBI (GenBank) (http://www.ncbi.nlm.nih.gov/). Information regarding a nucleotide sequence (SEQ ID NO: 1) encoding the amino acid sequence of hTROP-2 is disclosed under "Accession number NM_002353" in the same website as described above.

[0033] As an antigen, a polypeptide or peptide (which is also simply referred to as a peptide) comprising at least a portion (the entire or a part) of the amino acid sequence of hTROP-2 can be used, and preferably, a peptide comprising at least a portion (the entire or a part) of the amino acid sequence of the extracellular region of hTROP-2 can be used. The extracellular region (including a signal peptide) of hTROP-2 indicates a region comprising the amino acids at positions 1 to 274 from the amino acid sequence shown in SEQ ID NO: 2 (the signal peptide: the amino acids at positions 1 to 26). Herein, with regard to a peptide used as an antigen, the above description "at least a portion of the amino acid sequence" is not particularly limited in terms of length. For example, a region comprising the amino acids at positions 1 to 145 from the amino acid sequence shown in SEQ ID NO: 2, a region comprising the amino acids at positions 146 to 274 from the same amino acid sequence as described above, and the like are preferable.

[0034] A peptide used as an antigen may be produced either by chemical synthesis, or by synthesis according to a genetic engineering method using *Escherichia coli* or the like. A method well known to persons skilled in the art may be applied.

[0035] When a peptide is produced by chemical synthesis, it can be synthesized by a well known peptide synthesis method. In addition, either a solid-phase synthesis method or a liquid-phase synthesis method can be applied to the peptide synthesis. A commercially available peptide synthesizer (for example, PSSM-8 manufactured by Shimadzu Corporation, etc.) may also be used.

[0036] When a peptide is synthesized by a genetic engineering method, first, DNA encoding the peptide is designed and synthesized. The design and synthesis of such DNA can be carried out according to a PCR method, using a vector comprising an entire-length hTROP-2 gene or the like as a template, and also using primers designed to be able to synthesize a desired DNA region. Thereafter, the DNA is ligated to a suitable vector to obtain a recombinant vector used for protein expression, and this recombinant vector is then introduced into a host so that a gene of interest can be expressed therein, thereby obtaining a transformant (Sambrook J. et al., Molecular Cloning, A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001).

[0037] As a vector, a phage or a plasmid capable of autonomously replicating in a host microorganism is used. Further, an animal virus or an insect virus vector can also be used. To produce a recombinant vector, a purified DNA may be cleaved with suitable restriction enzymes, and the thus cleaved DNA portion may be then inserted into the restriction enzyme site or the like of a suitable vector DNA, so as to ligate it to the vector. The type of a host used in transformation is not particularly limited, as long as it is able to express a gene of interest. Examples of such a host include bacteria *(Escherichia coli, Bacillus subtilis,* etc.), yeasts, animal cells (COS cells, CHO cells, etc.), insect cells, and insects. A mammal such as a goat may also be used as such a host. A method of introducing a recombinant vector into a host is publicly known.

[0038] The above-described transformant is cultured, and a peptide used as an antigen is then collected from the culture. The term "culture" is used herein to mean both (a) a culture supernatant, and (b) cultured cells, a cultured cell mass or a disintegrated product thereof.

[0039] After completion of the culture, when a peptide of interest is produced in a cell mass or in cells, the peptide is extracted by disintegrating the cell mass or the cells. On the other hand, when a peptide of interest is produced outside a cell mass or outside cells, a culture solution is directly used, or the cell mass or the cells are removed from the culture solution by centrifugation or the like. Thereafter, the peptide of interest can be isolated and purified by a single use of biochemical methods commonly used in the isolation and purification of peptides, such as ammonium sulfate precipitation, gel filtration, ion exchange chromatography and affinity chromatography, or by appropriately combining the aforementioned biochemical methods.

[0040] In the present invention, a peptide used as an antigen can also be obtained by *in vitro* translation using a cell-

free synthesis system. In this case, two methods, namely, a method using RNA as a template and a method using DNA as a template (transcription/translation) can be applied. As such a cell-free synthesis system, a commercially available system, such as Expressway™ system (Invitrogen), PURESYSTEM (registered trademark; Post Genome Institute) or TNT system (registered trademark; Promega) can be used.

[0041] The thus obtained peptide may bind to a suitable carrier protein such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), human thyroglobulin or chicken γ-globulin.

[0042] Moreover, the antigen may be a peptide consisting of an amino acid sequence comprising a deletion, substitution or addition of one or more amino acids with respect to the amino acid sequence of hTROP-2 (SEQ ID NO: 2) or a partial sequence thereof as described above. There can be used, for example, a peptide consisting of an amino acid sequence, in which one or more (preferably one or several (for example, 1 to 10, and more preferably 1 to 5)) amino acids are deleted, or one or more (preferably one or several (for example, 1 to 10, and more preferably 1 to 5)) amino acids are substituted with other amino acids, or one or more (preferably one or several (for example, 1 to 10, and more preferably 1 to 5)) other amino acids are added, with respect to the amino acid sequence of hTROP-2 or a partial sequence thereof.

[0043] In the present invention, a gene to be introduced into a cell or the like is a gene encoding an hTROP-2 protein or a partial fragment thereof, or a mutant protein thereof or a fragment thereof. As such a gene, a gene having the nucleotide sequence shown in SEQ ID NO: 1 or a partial sequence thereof can be used, for example.

[0044] Furthermore, as a gene to be introduced into a cell or the like, there may also be used a nucleotide sequence hybridizing under stringent conditions with a sequence complementary to the nucleotide sequence shown in SEQ ID NO: 1 and encoding a protein having hTROP-2 activity, or a partial sequence thereof.

[0045] The description "stringent conditions" is used herein to mean washing conditions after completion of the hybridization. As such stringent conditions, a salt (sodium) concentration in buffer is 10 to 500 mM and a temperature is 42°C to 72°C, and preferably, the aforementioned salt condition is 50 to 300 mM and a temperature is 55°C to 68°C.

[0046] Mutation can be introduced into a gene according to a known method such as a Kunkel method or a Gapped duplex method, using, for example, a mutation introduction kit which utilizes site-directed mutagenesis, such as Gene-Tailor™ Site-Directed Mutagenesis System (manufactured by Invitrogen) or TaKaRa Site-Directed Mutagenesis System (Mutan-K, Mutan-Super Express Km, etc.; manufactured by Takara Bio Inc.).

(2) Production of polyclonal antibody

[0047] The prepared antigen is administered to a mammal for immunization. The type of such a mammal is not particularly limited. For example, a rat, a mouse and a rabbit can be used, and among them, a mouse is preferable.

[0048] The dosage of the antigen per animal can be determined, as appropriate, depending on the presence or absence of an adjuvant. Examples of such an adjuvant include a Freund's complete adjuvant (FCA), a Freund's incomplete adjuvant (FIA) and an aluminum hydroxide adjuvant. Immunization can be carried out mainly by injecting the antigen into the vein, footpad, subcutis or abdominal cavity of an animal. In addition, immunization interval is not particularly limited, and immunization is carried out at intervals of several days to several weeks, preferably at intervals of 1 week, 1 to 10 times, and preferably 2 or 3 times. Three to seven days after the final immunization day, an antibody titer is measured by enzyme immunoassay (ELISA or EIA), radioimmunoassay (RIA) or other methods. On the date at which a desired antibody titer is obtained, blood can be collected to obtain antiserum. In the above-described method of collecting an antibody, if it is necessary to purify the antibody, the antibody can be purified by selecting a suitable method from known methods such as ammonium sulfate precipitation, ion exchange chromatography, gel filtration chromatography and affinity chromatography, or by combining the above-mentioned methods, as appropriate. Thereafter, the reactivity of a polyclonal antibody in the antiserum is measured by the ELISA method or the like.

(3) Production of monoclonal antibody

(3-1) Collection of antibody-producing cells

[0049] The anti-hTROP-2 antibody of the present invention is not limited, but it is preferably a monoclonal antibody.

[0050] The prepared antigen is administered to a mammal, such as a rat, a mouse or a rabbit, for immunization. The dosage of the antigen per animal can be determined, as appropriate, depending on the presence or absence of an adjuvant. The same adjuvants as described above can be used herein. Also, the same immunization methods as described above can be applied herein. One to sixty days, and preferably, one to fourteen days after the final immunization day, antibody-producing cells are collected. Examples of such antibody-producing cells include splenic cells, lymph node cells and peripheral blood cells. Of these, lymph node cells and splenic cells are preferable.

(3-2) Cell fusion

[0051] In order to obtain hybridomas (an antibody-producing cell line), the cell fusion of antibody-producing cells with myeloma cells is carried out. As myeloma cells to be fused with antibody-producing cells, commonly available established cells from animals such as mice can be used. The cell line used herein is preferably a cell line, which has drug selectivity, cannot survive in an unfused state in a HAT selective medium (containing hypoxanthine, aminopterin and thymidine), and can survive only in a state fused with antibody-producing cells.

[0052] Examples of myeloma cells include mouse myeloma cell lines such as P3-X63-Ag8.653, P3-X63-Ag8(X63), P3-X63-Ag8.U1(P3U1), P3/NS I/1-Ag4-1(NS1) and Sp2/0-Ag14(Sp2/0). Myeloma cells can be selected, while taking into consideration the compatibility with antibody-producing cells, as appropriate.

[0053] Subsequently, myeloma cells are fused with antibody-producing cells. For such cell fusion, antibody-producing cells (1×10^6 to 1×10^7 cells/mL) are mixed with myeloma cells (2×10^5 to 2×10^6 cells/mL) in a medium for animal cells, such as DMEM or an RPMI-1640 medium containing no serum. The cell ratio between the antibody-producing cells and the myeloma cells (antibody-producing cells : myeloma cells) is not limited. In general, the cell ratio is preferably 1 : 1 to 10 : 1, and more preferably 3 : 1. Thereafter, a fusion reaction is carried out in the presence of a cell fusion promoter. As such a cell fusion promoter, polyethylene glycol having a mean molecular weight of 1,000 to 6,000 Dalton (D) or the like can be used. In addition, it is also possible to fuse antibody-producing cells with myeloma cells, employing a commercially available cell fusion apparatus which utilizes electrical stimulation (for example, electroporation).

(3-3) Selection and cloning of hybridomas

[0054] Hybridomas of interest are selected from the cells after the cell fusion treatment. As a method of selecting hybridomas, the cell suspension is appropriately diluted with, for example, a fetal bovine serum-containing RPMI-1640 medium, and the diluted solution is then dispersed on a microtiter plate. Thereafter, a selective medium is added to each well. While the selective medium is appropriately exchanged with a fresh one, culture is carried out. As a result, approximately 14 days after initiation of the culture on the selective medium, cells growing from the selective medium can be obtained as hybridomas.

[0055] Subsequently, screening is carried out to examine whether or not an antibody reacting with hTROP-2 is present in a culture supernatant of the growing hybridomas. The screening of hybridomas may be carried out according to an ordinary method, and thus, the screening method is not particularly limited. For example, an aliquot is collected from the culture supernatant of the growing hybridomas contained in the well, and screening is then carried out by ELISA, EIA, RIA or the like.

[0056] The cloning of the fused cells can be carried out by a limiting dilution method or the like. An antibody showing high reactivity with hTROP-2 is determined by flow cytometry or the like, and a hybridoma producing this antibody is then selected. The selected hybridoma is established as a clone.

(3-4) Collection of monoclonal antibody

[0057] As a method of culturing the established hybridoma and then collecting a monoclonal antibody from the obtained culture, a common cell culture method, an ascites formation method or the like can be adopted. The term "culture" is used herein to mean allowing hybridomas to grow in a culture dish or a culture bottle, or allowing hybridomas to grow in the abdominal cavity of an animal, as described below.

[0058] In the case of the cell culture method, hybridomas are cultured in a medium for animal cells, such as a 10% fetal bovine serum-containing RPMI-1640 medium, an MEM medium or a serum-free medium, under common culture conditions (for example, 37°C, 5% CO_2 concentration) for 7 to 14 days, and thereafter, an antibody can be obtained from the culture supernatant.

[0059] In the case of the ascites formation method, approximately 1×10^7 hybridomas are administered into the abdominal cavity of an animal of the same species as the mammal from which myeloma cells are derived, so that large quantities of hybridomas are allowed to proliferate. Thereafter, 2 to 3 weeks later, ascites is preferably collected.

[0060] In the above-described antibody collection methods, if it is necessary to purify the antibody, the antibody can be purified by appropriately selecting a suitable method from known methods such as ammonium sulfate precipitation, ion exchange chromatography, gel filtration and affinity chromatography, or by combining the above-mentioned methods.

(3-5) Selection of clone having anti-tumor activity

[0061] The anti-hTROP-2 antibody of the present invention is an antibody having anti-tumor *activity in vivo.*

[0062] The term "anti-tumor activity" is used herein to mean activity of killing tumor cells (cancer cells) or activity of inhibiting tumor growth. Preferred examples of such anti-tumor activity include activity of inhibiting the growth of cancer

cells and activity of inhibiting tumor angiogenesis. The type of human tumor (tumor cells), on which the antibody of the present invention is able to exhibit anti-tumor activity, includes various types of known human tumors, in which the expression of hTROP-2 has been confirmed. The type of such human tumor is not particularly limited. For example, one or two or more types selected from various human tumors such as human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer are preferable; and one or two or more types selected from human pancreatic cancer, human colorectal cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer and human lung cancer are more preferable. Human pancreatic cancer and/or human colorectal cancer are further preferable.

[0063] Moreover, the above-described tumor may be a recurrent cancer or a metastatic cancer. The antibody of the present invention is also able to exhibit excellent anti-tumor activity on these types of tumors.

[0064] The presence of anti-tumor activity *in vivo* can be confirmed, for example, by employing a tumor-bearing mouse (a mouse xenograft model), into the subcutis of which desired tumor cells have been transplanted, and then by administering the antibody as obtained above to the mouse. In this case, the antibody may be administered immediately after the transplantation of tumor cells (prevention models). Alternatively, it may be administered after confirming that the transplanted tumor has reached a predetermined volume (treatment models). The administration method is not limited. For example, the antibody may be administered once every three days, every one week, every ten days, or every two weeks, or by a single administration (only one time), at a dosage of 5 to 20 mg/kg body weight, via intraperitoneal administration or the like. In the case of prevention models, the presence or absence of anti-tumor activity and the level thereof can be evaluated based on tumor formation frequency and tumor volume. In the case of treatment models, the presence or absence of anti-tumor activity and the level thereof can be evaluated based on tumor volume.

[0065] In the present invention, a preferred example of the anti-hTROP-2 antibody having anti-tumor activity *in vivo* is an antibody in which the amino acid sequences of CDR 1 to 3 of the H chain V region thereof are shown in SEQ ID NOS: 36 to 38, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region thereof are shown in SEQ ID NOS: 41 to 43, respectively. A preferred example of the H chain V region is an H chain V region consisting of the amino acid sequence shown in SEQ ID NO: 35. A preferred example of the L chain V region is an L chain V region consisting of the amino acid sequence shown in SEQ ID NO: 40.

[0066] As another embodiment of the anti-hTROP-2 antibody of the present invention, a preferred example is an antibody in which the amino acid sequences of CDR 1 to 3 of the H chain V region thereof are shown in SEQ ID NOS: 46 to 48, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region thereof are shown in SEQ ID NOS: 51 to 53, respectively. A preferred example of the H chain V region is an H chain V region consisting of the amino acid sequence shown in SEQ ID NO: 45. A preferred example of the L chain V region is an L chain V region consisting of the amino acid sequence shown in SEQ ID NO: 50.

[0067] Likewise, as a further embodiment of the anti-hTROP-2 antibody of the present invention, a preferred example is an antibody in which the amino acid sequences of CDR 1 to 3 of the H chain V region thereof are shown in SEQ ID NOS: 56 to 58, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region thereof are shown in SEQ ID NOS: 61 to 63, respectively. A preferred example of the H chain V region is an H chain V region consisting of the amino acid sequence shown in SEQ ID NO: 55. A preferred example of the L chain V region is an L chain V region consisting of the amino acid sequence shown in SEQ ID NO: 60.

[0068] Likewise, as a further embodiment of the anti-hTROP-2 antibody of the present invention, a preferred example is an antibody in which the amino acid sequences of CDR 1 to 3 of the H chain V region thereof are shown in SEQ ID NOS: 66 to 68, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region thereof are shown in SEQ ID NOS: 71 to 73, respectively. A preferred example of the H chain V region is an H chain V region consisting of the amino acid sequence shown in SEQ ID NO: 65. A preferred example of the L chain V region is an L chain V region consisting of the amino acid sequence shown in SEQ ID NO: 70.

[0069] In the present invention, more specifically, preferred examples of an anti-hTROP-2 antibody having anti-tumor activity *in vivo* include: an anti-hTROP-2 monoclonal antibody (clone name: K5-70) produced by a hybridoma with Accession number FERM BP-11251; an anti-hTROP-2 monoclonal antibody (clone name: K5-107) produced by a hybridoma with Accession number FERM BP-11252; an anti-hTROP-2 monoclonal antibody (clone name: K5-116-2-1) produced by a hybridoma with Accession number FERM BP-11253; an anti-hTROP-2 monoclonal antibody (clone name: T6-16) produced by a hybridoma with Accession number FERM BP-11346; and an anti-hTROP-2 monoclonal antibody (clone name: T5-86) produced by a hybridoma with Accession number FERM BP-11254.

[0070] Herein, the hybridoma with Accession number FERM BP-11251 was named as "Mouse-Mouse Hybridoma K5-70" and was deposited on May 12, 2010; the hybridoma with Accession number FERM BP-11252 was named as "Mouse-Mouse Hybridoma K5-107" and was deposited on May 12, 2010; the hybridoma with Accession number FERM BP-11253 was named as "Mouse-Mouse Hybridoma K5-116-2-1" and was deposited on May 12, 2010; the hybridoma with Accession number FERM BP-11346 was named as "Mouse-Mouse Hybridoma T6-16" and was deposited on March 1, 2011; and the hybridoma with Accession number FERM BP-11254 was named as "Mouse-Mouse Hybridoma T5-86" and was deposited on May 12, 2010. All of these hybridomas were deposited with the International Patent Organism

Depositary (IPOD), the National Institute of Advanced Industrial Science and Technology, an Independent Administrative Institution under the Ministry of Economy, Trade and Industry (the AIST, Tsukuba Central 6, Higashi 1-1-1, Tsukuba, Ibaraki, Japan, postal code: 305-8566).

[0071] Still further, another preferred example of the anti-hTROP-2 antibody of the present invention is an anti-hTROP-2 antibody that binds to a site (e.g. an epitope), to which a monoclonal antibody produced by the hybridoma having accession No. FERM BP-11251, FERM BP-11252, FERM BP-11253, FERM BP-11346 or FERM BP-11254 binds (recognizes).

[0072] Preferred examples of such an epitope will be given in (3-6) below.

(3-6) Epitope of anti-hTROP-2 antibody

[0073] The type of an epitope (an antigenic determinant) of the anti-hTROP-2 antibody of the present invention is not limited, as long as it is at least a portion of hTROP-2 as an antigen. For example, such an epitope is preferably at least a portion of a region formed by removing a region consisting of amino acids at positions 252 to 260 from the amino acid sequence of hTROP-2 shown in SEQ ID NO: 2, more preferably at least a portion of a region consisting of amino acids at positions 1 to 69 or at least a portion of a region consisting of amino acids at positions 100 to 274 (except for a region consisting of amino acids at position 252 to 260), and further preferably at least a portion of a region consisting of amino acids at positions 27 to 69 or a region consisting of amino acids at positions 109 to 206. Particularly preferred examples of the above-described epitope include a region consisting of amino acids at positions 43 to 65, a region consisting of amino acids at positions 152 to 165, a region consisting of amino acids at positions 171 to 183, a region consisting of amino acids at positions 109 to 120, a region consisting of amino acids at positions 193 to 206, a region consisting of amino acids at positions 43 to 56, and a portion comprising such a region, in the amino acid sequence of hTROP-2 shown in SEQ ID NO: 2. Further particularly preferred examples include a region consisting of amino acids at positions 43 to 65, a region consisting of amino acids at positions 152 to 165, a region consisting of amino acids at positions 171 to 183, a region consisting of amino acids at positions 109 to 120, and a portion comprising such a region. An anti-hTROP-2 antibody, which recognizes the above-described regions (binds to the above-described regions or portions comprising such regions), has high internalization activity into tumor cells, for example, and thus it is extremely useful as an immunoconjugate as described later.

(3-7) Characteristics of anti-hTROP-2 antibody

[0074] As described above, the anti-hTROP-2 antibody of the present invention is an antibody having high anti-tumor activity *in vivo* at a low dose. Specifically, it is preferable that the present anti-hTROP-2 antibody exhibits tumor growth inhibitory activity of 50% or more (preferably 80% or more, more preferably 90% or more, further preferably 95% or more, and particularly preferably almost 100% (for example, 98% or more, or 99% or more)) at a dose (as a naked antibody) of 20 mg/kg body weight or less (preferably 10 mg/kg body weight or less, more preferably 5 mg/kg body weight or less, and further preferably 1 mg/kg body weight or less) with respect to a tumor-bearing animal model.

[0075] Herein, the tumor growth inhibitory activity (%) can be calculated, for example, by the following formula:

$$\text{Tumor growth inhibitory activity (\%)} = 100 - [(\text{tumor volume or tumor weight of antibody administration group}) / (\text{tumor volume or tumor weight of control group})] \times 100$$

[0076] In addition, the anti-hTROP-2 antibody of the present invention preferably has anti-tumor activity on two or more types of human tumor cell lines. The type of such a human tumor cell line is not limited. For example, such human tumor cell lines are at least two types selected from the group consisting of various types of human pancreatic cancer cell lines, human prostate cancer cell lines, human colorectal cancer cell lines, human breast cancer cell lines, human ovarian cancer cell lines, human lung cancer cell lines and human bile duct cancer cell lines. Specifically, preferred examples of such human tumor cell lines include at least two types selected from the group consisting of a human pancreatic cancer cell line PK-59, a human pancreatic cancer cell line BxPC-3, a human pancreatic cancer cell line KP-3L, a human pancreatic cancer cell line KP-2, a human pancreatic cancer cell line PK-1, a human pancreatic cancer cell line PK-45H, a human pancreatic cancer cell line PK-45P, a human pancreatic cancer cell line TCC-PAN2, a human pancreatic cancer cell line SUIT-2, a human colon cancer cell line CACO-2, a human colon cancer cell line SW480, a human colon cancer cell line DLD-1, a human colon cancer cell line HCT 116, a human breast cancer cell line JIMT-1, a human breast cancer cell line HCC1143, a human breast cancer cell line MCF-7, a human breast cancer cell line MDA-MB-468, a human prostate cancer cell line DU145, a human prostate cancer cell line PC-3, a human ovarian cancer cell line SK-OV-3, a human lung cancer cell line Calu-3 and a human bile duct cancer cell line TFK-1. Of these, as the above-described two or more types of human tumor cell lines, at least two types selected from the group consisting of the human pancreatic cancer cell line PK-59, the human pancreatic cancer cell line BxPC-3, the human colon cancer

cell line SW480, the human lung cancer cell line Calu-3, the human breast cancer cell line MBA-MD-468 and the human ovarian cancer cell line SK-OV-3 are more preferable.

[0077] Moreover, the anti-hTROP-2 antibody of the present invention has a dissociation constant (Kd value) of preferably 1.0×10^{-10} M or less, more preferably 1.0×10^{-11} M or less, and further preferably 1.0×10^{-12} M or less. Herein, the binding ability (affinity) of the antibody can be measured in the form of a dissociation constant (Kd value), a dissociation rate constant (Kdiss [1/Sec]) or an association rate constant (Kass [1/M.Sec]), for example, by Scatchard analysis or surface plasmon resonance sensor called Biacore. As such Biacore apparatuses, Biacore 3000, Biacore 2000, Biacore X, Biacore J and Biacore Q (all of which were manufactured by Biacore) may be used, for example. It is preferable that the antibody have a dissociation constant (Kd value) that is as small as possible because it could have high binding ability (affinity). The Kd value is determined based on the two parameters of Kdiss and Kass, and it can be expressed in the formula: Kd[M] = Kdiss/Kass. As a method of calculating the Kd value, the method described in the Examples as described later (specifically, Example 10) can be preferably adopted.

(4) Genetically recombinant antibody and antibody fragment

(4-1) Genetically recombinant antibody

[0078] In a preferred embodiment of the anti-hTROP-2 antibody of the present invention, there is provided a genetically recombinant antibody. The type of such a genetically recombinant antibody is not limited. Examples include a chimeric antibody, a humanized antibody, and a human antibody.

[0079] A chimeric antibody (that is, a humanized chimeric antibody) is an antibody formed by ligating (conjugating) the variable region of a mouse-derived antibody to the constant region of a human-derived antibody (please refer to Proc. Natl. Acad. Sci. U.S.A. 81, 6851-6855, (1984), etc.). When such a chimeric antibody is produced, the thus ligated antibody can be easily constructed by a genetic recombination technique.

[0080] When a humanized antibody is produced, a complementarity determining region (CDR) is transplanted from the variable region of a mouse antibody into the variable region of a human antibody, so as to produce a reconstructed variable region, in which a framework region (FR) is derived from the human and CDR is derived from the mouse (what is called CDR grafting (CDR transplantation)). Subsequently, the thus humanized, reconstructed human variable region is ligated to a human constant region. Such a method for producing a humanized antibody is well known in the present technical field (please refer to see Nature, 321, 522-525 (1986); J. Mol. Biol., 196, 901-917 (1987); Queen C et al., Proc. Natl. Acad. Sci. USA, 86: 10029-10033 (1989); JP Patent Publication (Kohyo) No. 4-502408 A (1992) (Japanese Patent No. 2828340; Queen et al.), etc.).

[0081] Herein, the type of a CDR sequence derived from the mouse, which can be used in the humanized anti-hTROP-2 antibody of the present invention, is not particularly limited. For example, the amino acid sequences shown in SEQ ID NOS: 36 to 38 or the amino acid sequences shown in SEQ ID NO: 66 to 68 are preferably used as the CDR 1 to 3 of the H chain V region (VH), respectively. The amino acid sequences shown in SEQ ID NOS: 41 to 43 or the amino acid sequences shown in SEQ ID NO: 71 to 73 are preferably used as the CDR 1 to 3 of the L chain V region (VL), respectively.

[0082] In addition, preferred examples of the amino acid sequence of an H chain V region in a humanized reconstructed human variable region include: the amino acid sequence shown in SEQ ID NO: 92 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 36 to 38; the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 75); the amino acid sequence shown in SEQ ID NO: 98 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 36 to 38; the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 97); the amino acid sequence shown in SEQ ID NO: 94 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 66 to 68; the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 79); and the amino acid sequence shown in SEQ ID NO: 95 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 66 to 68; the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 81). Herein, the above-mentioned amino acid sequence of the H chain V region shown in SEQ ID NO: 98 is a modified amino acid sequence in which the arginine (R) at position 44 of the above-mentioned amino acid sequence of the H chain V region shown in SEQ ID NO: 92 is substituted with a glycine (G).

[0083] Likewise, preferred examples of the amino acid sequence of an L chain V region in a humanized reconstructed human variable region include: the amino acid sequence shown in SEQ ID NO: 93 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 41 to 43; and the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 77); and the amino acid sequence shown in SEQ ID NO: 96 (comprising CDR 1 to 3 consisting of the amino acid sequences shown in SEQ ID NOS: 71 to 73; the amino acid sequence further comprising a signal peptide is shown in SEQ ID NO: 83).

[0084] Herein, preferred examples of the humanized anti-hTROP-2 antibody of the present invention include: (i) a humanized anti-hTROP-2 antibody, in which the amino acid sequence of the H chain V region is shown in SEQ ID NO:

92 and the amino acid sequence of the L chain V region is shown in SEQ ID NO: 93; and (ii) a humanized anti-hTROP-2 antibody, in which the amino acid sequence of the H chain V region is shown in SEQ ID NO: 98 and the amino acid sequence of the L chain V region is shown in SEQ ID NO: 93. In particular, the humanized anti-hTROP-2 antibody described in (ii) above, in which the amino acid sequence of the H chain V region is shown in SEQ ID NO: 98, has an improved avidity (that is flexibility of the movement of two antigen-binding arms) and has high antigen-binding activity, and thus, this antibody is particularly preferable.

[0085] Also, other preferred examples of the humanized anti-hTROP-2 antibody of the present invention include: (iii) a humanized anti-hTROP-2 antibody, in which the amino acid sequence of the H chain V region is shown in SEQ ID NO: 94 and the amino acid sequence of the L chain V region is shown in SEQ ID NO: 96; and (ii) a humanized anti-hTROP-2 antibody, in which the amino acid sequence of the H chain V region is shown in SEQ ID NO: 95 and the amino acid sequence of the L chain V region is shown in SEQ ID NO: 96.

[0086] In general, in the case of a human antibody (a complete human antibody), its structure comprising a Hyper Variable region that is the antigen-binding site of a V region, other parts of the V region, and a constant region is the same as the structure of the antibody of a human. However, such a Hyper Variable site may also be derived from other animals. A technique of producing a human antibody is publicly known, and a method for producing gene sequences that are common in humans by genetic engineering has been established. A human antibody can be obtained, for example, by a method using a human antibody-producing mouse that has human chromosomal fragments comprising the genes of the H chain and L chain of the human antibody (please refer to Tomizuka, K.et al., Nature Genetics, (1977) 16, 133-143; Kuroiwa, Y. et. al., Nuc. Acids Res., (1998) 26, 3447-3448; Yoshida, H. et. al., Animal Cell Technology: Basic and Applied Aspects, (1999) 10, 69-73 (Kitagawa, Y., Matsuda, T. and Iijima, S. eds.), Kluwer Academic Publishers; Tomizuka, K. et. al., Proc. Natl. Acad. Sci. USA, (2000) 97, 722-727, etc.), or by a method of obtaining a phage display-derived human antibody selected from a human antibody library (please refer to Wormstone, I. M. et. al, Investigative Ophthalmology & Visual Science., (2002) 43 (7), 2301-8; Carmen, S. et. al., Briefings in Functional Genomics and Proteomics,(2002) 1 (2), 189-203; Siriwardena, D. et. al., Opthalmology, (2002) 109 (3), 427-431, etc.).

[0087] In the case of the aforementioned chimeric antibody, humanized antibody and human antibody, the N-glycoside-linked sugar chain in the antibody Fc region is preferably a sugar chain, in which fucose does not bind to N-acetylglucosamine at the reducing terminal thereof. A specific example is an antibody consisting of genetically recombinant antibody molecules, which has, in the Fc region of the antibody molecules, a sugar chain in which the position 1 of the fucose does not bind to the position 6 of the N-acetylglucosamine at the reducing terminal of the N-glycoside-linked sugar chain via an α bond. Such an antibody is able to significantly improve ADCC activity. This point (the characteristics of the N-glycoside-linked sugar chain in the antibody Fc region) is preferable also for the aforementioned polyclonal antibody and monoclonal antibody.

(4-2) Antibody fragment

[0088] The anti-hTROP-2 antibody fragment (partial fragment) of the present invention is included in the antibody of the present invention. Herein, the antibody fragment of the present invention has binding activity to hTROP-2 (namely, it is able to bind to hTROP-2) and also has anti-tumor *activity in vivo,* as in the case of the anti-hTROP-2 antibody of the present invention.

[0089] The fragment of the antibody means a region of a portion of an anti-hTROP-2 polyclonal antibody or anti-hTROP-2 monoclonal antibody (namely, an antibody fragment derived from the anti-hTROP-2 antibody of the present invention). Examples of such an antibody fragment include peptides comprising, as at least a portion thereof, Fab, Fab', $F(ab')_2$, Fv (variable fragment of antibody), a single-stranded antibody (an H chain, an L chain, an H chain V region, and an L chain V region, etc.), scFv, diabody (scFv dimer), dsFv (a disulfide-stabilized V region), and a complementarity determining region (CDR).

[0090] Fab is an antibody fragment with a molecular weight of approximately 50,000 having antigen-binding activity, which is formed by binding about a half on the N-terminal side of the H chain to the entire L chain via a disulfide bond, among fragments obtained by treating antibody molecules with a protease, papain. In addition, it is also possible to produce such Fab by inserting DNA encoding the Fab of an antibody into a prokaryote expression vector or a eukaryote expression vector, and then introducing the vector into a prokaryote or a eukaryote so as to allow the DNA to express therein.

[0091] $F(ab')_2$ is an antibody fragment with a molecular weight of approximately 100,000 having antigen-binding activity, whose size is slightly greater than Fab that binds to Fab via disulfide bond in the hinge region, among fragments obtained by treating antibody molecules with a protease, pepsin. In addition, it is also possible to produce such $F(ab')_2$ by the thioether bond or disulfide bond of Fab, as described later.

[0092] Fab' is an antibody fragment with a molecular weight of approximately 50,000 having antigen-binding activity, which is formed by cleaving the disulfide bond in the hinge region of the aforementioned $F(ab')_2$. In addition, it is also possible to produce such Fab' by inserting DNA encoding the Fab' fragment of an antibody into a prokaryote expression

vector or a eukaryote expression vector, and then introducing the vector into a prokaryote or a eukaryote so as to allow the DNA to express therein.

[0093] scFv is an antibody fragment having antigen-binding activity, which is a VH-P-VL or VL-P-VH polypeptide formed by ligating a single H chain V region (VH) to a single L chain V region (VL) using a suitable peptide linker (P). Such scFv can be produced by obtaining cDNA encoding the VH and VL of an antibody, constructing DNA encoding scFv, inserting the DNA into a prokaryote expression vector or a eukaryote expression vector, and then introducing the vector into a prokaryote or a eukaryote so as to allow the DNA to express therein.

[0094] Diabody is an antibody fragment formed by dimerization of scFv, which has divalent antigen-binding activities. Such divalent antigen-binding activities may be identical to each other, or they may also be different from each other. Such diabody can be produced by obtaining cDNA encoding the VH and VL of an antibody, constructing DNA encoding scFv such that the length of the amino acid sequence of P is 8 residues or less, inserting the DNA into a prokaryote expression vector or a eukaryote expression vector, and then introducing the vector into a prokaryote or a eukaryote so as to allow the DNA to express therein.

[0095] dsFv is an antibody fragment formed by binding polypeptides, in which one amino acid residue in each of VH and VL has been substituted with a cysteine residue, to each other via a disulfide bond between the cysteine residues. The amino acid residue to be substituted with cysteine residues can be selected based on estimation of the three-dimensional structure of the antibody according to the method of Reiter et al. (Protein Engineering, 7, 697-704, 1994). Such dsFv can be produced by obtaining cDNA encoding the VH and VL of an antibody, constructing DNA encoding dsFv, inserting the DNA into a prokaryote expression vector or a eukaryote expression vector, and then introducing the vector into a prokaryote or a eukaryote so as to allow the DNA to express therein.

[0096] A peptide comprising a CDR comprises at least one region of CDRs of VH (CDR 1 to 3) and CDRs of VL (CDR 1 to 3). More preferred examples of such a peptide include a peptide comprising all of the CDRs of VH and a peptide comprising all of the CDRs of VL. A particularly preferred example of the peptide is a peptide comprising all of the CDRs of VH and VL (total 6 regions). Preferred examples of the amino acid sequence of such a CDR include the amino acid sequences shown in SEQ ID NOS: 36 to 38, 41 to 43, 46 to 48, 51 to 53, 56 to 58, 61 to 63, 66 to 68, and 71 to 73, as described above. A peptide comprising multiple CDRs can be bound to one another, directly or via a suitable peptide linker. Such a peptide comprising CDR can be produced by constructing DNA encoding the VH and VL of an antibody, inserting the DNA into a prokaryote expression vector or a eukaryote expression vector, and then introducing the expression vector into a prokaryote or a eukaryote so as to allow the DNA to express therein. Moreover, such a peptide comprising CDR can also be produced by chemical synthesis methods such as a Fmoc method (a fluorenylmethyloxycarbonyl method) and a tBoc method (a t-butyloxycarbonyl method).

[0097] The antibody fragment of the present invention, as is, may be an antibody fragment, which comprises a part of or the entire antibody Fc region in which fucose does not bind to N-acetylglucosamine at the reducing terminal of an N-glycoside-linked sugar chain. Otherwise, the antibody fragment of the present invention may also be a fusion protein, in which the aforementioned antibody fragment is fused with a part of or the entire antibody Fc region in which fucose does not bind to N-acetylglucosamine at the reducing terminal of an N-glycoside-linked sugar chain. Such an antibody fragment is able to significantly improve ADCC activity, and thus it is preferable.

[0098] Hereinafter, in the descriptions of the present specification, the aforementioned antibody fragments are also included in the anti-hTROP-2 antibody of the present invention.

3. Polynucleotide, recombinant vector and transformant

[0099] In the present invention, a polynucleotide (a gene, DNA) encoding the above-described anti-hTROP-2 antibody of the present invention or an antibody fragment thereof can also be provided. Specifically, the present polynucleotide is preferably a polynucleotide which comprises a nucleotide sequence encoding each amino acid sequence exemplified as the above-described anti-hTROP-2 antibody of the present invention or an antibody fragment thereof. Moreover, the polynucleotide of the present invention may be either a polynucleotide consisting of a polynucleotide alone encoding the anti-hTROP-2 antibody of the present invention or an antibody fragment thereof, or a polynucleotide which comprises the present polynucleotide as a portion thereof and also comprises known nucleotide sequences necessary for gene expression (e.g. a transcriptional promoter, an SD sequence, a Kozak sequence, a terminator, etc.). Thus, the type of the present polynucleotide is not limited.

[0100] With regard to the polynucleotide encoding the anti-hTROP-2 antibody of the present invention or an antibody fragment thereof, the codon corresponding to individual amino acids after translation is not particularly limited. The polynucleotide may comprise nucleotide DNA showing a codon commonly used in mammals such as a human (preferably, a frequently-used codon), or may also comprise nucleotide DNA showing a codon commonly used in microorganisms such as *Escherichia coli* or yeast, plants and the like (preferably, a frequently-used codon).

[0101] In the present invention, a recombinant vector comprising the above-described polynucleotide of the present invention, or a transformant comprising the recombinant vector, can also be provided.

[0102] A transcriptional promoter, an SD sequence (in a case in which the host is a prokaryotic cell) and a Kozak sequence (in a case in which the host is a eukaryotic cell) may previously be ligated to the upstream of a polynucleotide (a gene, DNA) to be incorporated into an expression vector used as a recombinant vector. Moreover, a terminator may be ligated to the downstream of the polynucleotide. Furthermore, an enhancer, a splicing signal, a poly(A) additional signal, a selective marker and the like may also be ligated to the polynucleotide. Individual elements necessary for gene expression, such as the above-described transcriptional promoter, may be comprised in the present polynucleotide from the beginning. When these elements are originally comprised in an expression vector, they may be used. Thus, the usage of individual elements is not particularly limited.

[0103] As methods of incorporating the present polynucleotide into an expression vector, various types of methods utilizing known genetic recombination techniques, such as a method using restriction enzymes or a method using topoisomerase, can be adopted. The type of an expression vector is not limited, as long as it is able to retain a polynucleotide (a gene, DNA) encoding the anti-hTROP-2 antibody of the present invention or an antibody fragment thereof, and a vector suitable for host cells to be used can be selected, as appropriate, and can be used. Examples of such an expression vector include plasmid DNA, bacteriophage DNA, retrotransposon DNA, a retrovirus vector and artificial chromosomal DNA.

[0104] Subsequently, the thus constructed recombinant vector is introduced into a host to obtain a transformant, and the obtained transformant is then cultured, so that the anti-hTROP-2 antibody of the present invention or an antibody fragment thereof can be expressed. It is to be noted that the term "transformant" is used in the present invention to mean a host into which a foreign gene has been introduced. Examples of such a transformant include: a host into which a foreign gene has been introduced by introduction of plasmid DNA or the like (transformation); and a host into which a foreign gene has been introduced by infecting the host with various types of viruses and phages (transduction).

[0105] The type of a host is not limited, as long as it is able to express the anti-hTROP-2 antibody of the present invention or an antibody fragment thereof, after the above-described recombinant vector has been introduced into the host. Thus, a host can be selected, as appropriate. Examples of such a host include known hosts such as various types of animal cells such as a human or a mouse, and various types of plant cells, bacteria, yeasts and plant cells.

[0106] When animal cells are used as host cells, examples of such animal cells include: human fibroblasts, human embryonic kidney cells, HEK293 cells, 293F cells, CHO cells, monkey COS-7 cells, Vero, mouse L cells, rat GH3 and human FL cells. Moreover, insect cells such as Sf9 cells or Sf21 cells may also be used as host cells.

[0107] When bacteria are used as hosts, examples of such bacteria include *Escherichia coli* and *Bacillus subtilis.*

[0108] When yeast is used as a host, examples of such yeast include *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.*

[0109] When plant cells are used as host cells, tobacco BY-2 cells are used for example.

[0110] The method of obtaining a transformant is not limited, and it can be selected, as appropriate, while taking into consideration a combination of the type of a host and the type of an expression vector. Preferred examples of the method of obtaining a transformant include electroporation, lipofection, a heat shock method, PEG, a calcium phosphate method, a DEAE dextran method, and a method of infecting a host with various types of viruses such as DNA virus or RNA virus.

[0111] In the obtained transformant, the codon type of a polynucleotide contained in a recombinant vector comprised may be identical to or different from the codon type of a host used. Thus, the codon type is not limited.

4. Preparation of antibody-drug conjugate

[0112] As an immunoconjugate prepared using the aforementioned anti-hTROP-2 antibody of the present invention, there can be provided an antibody-drug conjugate, which comprises the aforementioned antibody and a substance (a compound, etc.) having anti-tumor activity and/or cell-killing activity. It is to be noted that a conjugate formed by previously preparing each of the aforementioned antibody molecule and the aforementioned substance having anti-tumor activity and/or cell-killing activity, separately, and then combining them, is generally referred to as an immunoconjugate. On the other hand, a conjugate obtained by ligating a protein toxin used as such a substance having anti-tumor activity and/or cell-killing activity to an antibody gene on a gene according to a genetic recombination technique, so as to allow it to express as a single protein (a fusion protein), is generally referred to as an immunotoxin.

[0113] Examples of a substance having anti-tumor activity include doxorubicin, calicheamicin, mitomycin C, Auristatin E and radioactive isotope (RI). Examples of a substance having cell-killing activity include saporin, lysine, pseudomonas exotoxin, diphtheria toxin and radioactive isotope (RI). Of these, saporin and pseudomonas exotoxin are preferably used. The type of RI having anti-tumor activity and/or cell-killing activity is not particularly limited, and examples of such RI include ^{90}Y, ^{111}In, ^{125}I, ^{3}H ^{35}S, ^{14}C, ^{186}Re, ^{188}Re, ^{189}Re, ^{177}Lu, ^{67}Cu, ^{212}Bi, ^{213}Bi, ^{211}At, ^{198}Au, ^{224}Ac, ^{126}I, ^{133}I, ^{77}Br, ^{113m}In, ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{107}Hg, ^{203}Hg, ^{94m}Tc, ^{121m}Te, ^{122m}Te, ^{125m}Te, ^{165}Tm, ^{167}Tm, ^{168}Tm, ^{111}Ag, ^{197}Pt, ^{109}Pd, ^{32}P, ^{33}P, ^{47}Sc, ^{153}Sm, ^{177}Lu, ^{105}Rh, ^{142}Pr, ^{143}Pr, ^{161}Tb, ^{166}Ho, ^{199}Au, ^{57}Co, ^{58}Co, ^{51}Cr, ^{59}Fe, ^{18}F, ^{75}Se, ^{201}Tl, ^{225}Ac, ^{76}Br, ^{86}Y, ^{169}Yb, ^{166}Dy, ^{212}Pb and ^{223}Ra.

[0114] A method for producing an antibody-drug conjugate is not limited. For example, a method of coupling an antibody

with a drug via a disulfide bond or a hydrazone bond is applied.

[0115] The aforementioned anti-hTROP-2 antibody of the present invention is excellent in terms of internalization activity into target tumor cells that express hTROP-2. Thus, by previously combining a substance having anti-tumor activity and cell-killing activity with the anti-hTROP-2 antibody, it becomes possible to allow such a substance to directly and highly selectively act on the tumor cells. The antibody-drug conjugate of the present invention is extremely excellent in terms of ability to deliver the agent to the target tumor cells.

[0116] The internalization activity into cells can be evaluated by fluorescently labeling an antibody with rhodamine or the like and then observing the migratory behavior and localization of the antibody using a fluorescence microscope or the like.

[0117] Moreover, in the present invention, in addition to the aforementioned antibody-drug conjugate, there can also be provided an antibody fragment-drug conjugate, in which the aforementioned antibody fragment is used instead of an antibody. With regard to the details of such an antibody fragment-drug conjugate, the descriptions of the aforementioned antibody-drug conjugate can be applied, as appropriate.

[0118] Hereinafter, in the descriptions of the present specification, such an antibody fragment-drug conjugate is also included in the antibody-drug conjugate of the present invention.

5. Pharmaceutical composition

[0119] The anti-hTROP-2 antibody and antibody-drug conjugate of the present invention are useful as active ingredients contained in a pharmaceutical composition.

[0120] The pharmaceutical composition is useful as a pharmaceutical composition for treating and/or diagnosing a tumor. In particular, since the anti-hTROP-2 antibody of the present invention and an antibody-drug conjugate comprising the aforementioned antibody have excellent tumor growth inhibitory activity as such anti-tumor activity, they are preferably used in the treatment of tumor. That is to say, the anti-hTROP-2 antibody and antibody-drug conjugate of the present invention are useful as active ingredients contained in a tumor therapeutic agent and a tumor diagnostic agent. It is to be noted that the above-described treatment of tumor includes inhibition of tumor growth and suppression of tumor growth. Specifically, if it is a tumor therapeutic agent, examples of the tumor therapeutic agent include a tumor growth inhibitor and a tumor growth suppressor.

[0121] It is preferable to provide the pharmaceutical composition of the present invention in the form of a pharmaceutical composition comprising the anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention as active ingredient(s), and further comprising a pharmacologically acceptable carrier. In addition, the pharmaceutical composition of the present invention can be used in combination with known anti-tumor agents. By such a combined use, a higher anti-tumor effect can be obtained.

[0122] Target diseases (tumors), to which the pharmaceutical composition of the present invention is applied, include: the aforementioned various types of known human tumors, in which the expression of hTROP-2 has previously been confirmed. Among others, one or two or more types selected from various types of human tumors such as human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer are particularly preferable. Such target disease may be a single disease, or two or more diseases may be developed in combination. Moreover, the target tumor may be a recurrent cancer or a metastatic cancer. The pharmaceutical composition of the present invention (further, the anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention) can be effectively used as a therapeutic agent and a diagnostic agent for a recurrent cancer or a metastatic cancer.

[0123] Examples of the "pharmacologically acceptable carrier" include an excipient, a diluent, an extender, a disintegrator, a stabilizer, a preservative, a buffer, an emulsifier, an aromatic, a coloring agent, a sweetener, a thickener, a corrigent, a solubilizer and other additives. Using one or more types of such carriers, a pharmaceutical composition can be prepared in the form of an injection, a liquid agent, a capsule, a suspension, an emulsion, a syrup, etc. These pharmaceutical compositions can be administered orally or parenterally. Another form for parenteral administration is, for example, an injection comprising one or more active ingredients, which is prepared by an ordinary method. Such an injection can be produced by dissolving or suspending the present antibody in a pharmacologically acceptable carrier such as a normal saline solution or a commercially available distilled water used for injection.

[0124] In particular, when an antibody fragment derived from the anti-hTROP-2 antibody of the present invention (particularly, an antibody fragment with a low molecular weight) is administered into a living body, a colloidal dispersion system can be used in addition to the aforementioned components. Such a colloidal dispersion system is anticipated to have an effect of enhancing the stability of a compound (an antibody fragment) in a living body or an effect of efficiently transporting such a compound to a specific organ, tissue, or cell. The type of such a colloidal dispersion system is not limited, as long as it is commonly used. Examples of such a colloidal dispersion system include dispersion systems comprising, as bases, polyethylene glycol, a macromolecular conjugate, a macromolecular aggregate, a nanocapsule, microsphere, beads, and lipids including an oil in water emulsifier, micelle, mixed micelle and liposome. Preferred

examples of such a colloidal dispersion system include multiple liposomes and the vesicles of artificial membrane, which have an effect of efficiently transporting such a compound to a specific organ, tissue, or cell (Mannino et al., Biotechniques, 1988, 6, 682; Blume and Cevc, Biochem. et Biophys. Acta, 1990, 1029, 91; Lappalainen et al., Antiviral Res., 1994, 23, 119; Chonn and Cullis, Current Op. Biotech., 1995, 6, 698).

[0125] The dosage of the pharmaceutical composition of the present invention differs depending on the age, sex, body weight and symptoms of a patient, therapeutic effects, an administration method, a treatment time, the types of the anti-hTROP-2 antibody and antibody-drug conjugate of the present invention contained in the pharmaceutical composition, etc. In general, the present pharmaceutical composition may be administered within the range between 600 μg and 6,000 mg per adult per administration. However, the dose is not limited to the aforementioned range.

[0126] In a case in which the pharmaceutical composition is administered in the form of an injection, for example, it may be administered at a dosage of 100 μg to 100 mg, per administration, per body weight of a human patient, once or divided over several administrations, as an average daily dose. Preferably, the pharmaceutical composition may be administered once every three days, once a week, once every ten days, or once every two weeks, or by a single administration (wherein the total number of administrations is 1). Examples of the dosage form include intravenous injection, subcutaneous injection, intradermal injection, intramuscular injection and intraperitoneal injection. Of these, intravenous injection is preferable. In addition, such an injection may be prepared in the form of a nonaqueous diluent (e.g. polyethylene glycol, vegetable oil such as olive oil, alcohols such as ethanol, etc.), a suspension, or an emulsion. Such an injection can be sterilized by mechanical sterilization using a filter, the mixing of a microbicide, etc. The injection can be produced in the form of an injection to be prepared before using. That is, a sterilized solid composition is prepared by a freeze-drying method or the like, and the composition is then dissolved in sterilized distilled water used for injection or other solvents before it is used, so that it can be then used.

[0127] The present invention provides a use of the aforementioned anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention in the production of a pharmaceutical agent (a drug) for treating and/or diagnosing a tumor. In addition, the present invention provides the aforementioned anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention, which are used for treating and/or diagnosing a tumor.

[0128] Moreover, the present invention provides a method for treating and/or diagnosing a tumor, which is characterized in that it comprises using (namely, administering to patients) the aforementioned anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention. Furthermore, the present invention also provides the use of the aforementioned anti-hTROP-2 antibody and/or antibody-drug conjugate of the present invention in the treatment and/or diagnosis of tumor.

6. Method for detecting tumor

[0129] The method for detecting a tumor of the present invention is characterized in that it comprises allowing the aforementioned anti-hTROP-2 antibody of the present invention to react with a sample collected from a living body (hereinafter referred to as a biological sample), and detecting a signal(s) of the reacted antibody.

[0130] As described above, hTROP-2 has been confirmed to be specifically expressed in various types of tumor cells. Thus, hTROP-2, and particularly, free hTROP-2 (an extracellular region portion of hTROP-2) can be used as a marker for various types of tumors. In particular, such hTROP-2 can be preferably used as a marker for human pancreatic cancer, human prostate cancer, human colorectal cancer and human breast cancer.

[0131] Hence, the anti-hTROP-2 antibody of the present invention is allowed to react with a biological sample, and a signal of the reacted antibody is then detected, so as to detect a tumor. The obtained antibody signal can be used as an indicator of the amount of an antigen in the biological sample (that is, an hTROP-2 amount or a free hTROP-2 amount). In detection of a tumor using the antibody of the present invention, first, a biological sample collected as an analyte from a subject, such as a tissue section or blood used as a test target, is allowed to bind to the antibody of the present invention by an antigen-antibody reaction. Subsequently, based on the measurement results of the amount of the bound antibody, the amount of an antigen of interest contained in the biological sample is measured. This measurement may be carried out in accordance with known immunoassay methods. For example, an immunoprecipitation method, an immunoagglutination method, radioimmunoassay, immunonephelometry, a Western blot method, flow cytometry and the like can be used. In radioimmunoassay, a labeled antibody is used, and thus an antibody signal is expressed as the amount of the labeled antibody that is directly detected. Otherwise, an antibody whose concentration or antibody titer has been known may be used as a standard solution, and thus a signal of the target antibody may be expressed as a relative value. That is, both the standard solution and the analyte may be measured using a measurement device, and an antibody signal in a biological sample may be expressed as a value relative to the value of the standard solution used as a criterion. Examples of such radioimmunoassay include the ELISA method, the EI method, the RIA method, fluorescence immunoassay (FIA), and luminescence immunoassay. Of these, the ELISA method is particularly preferable in that it is simple and highly sensitive.

[0132] In the present invention, the state of a tumor can be evaluated or diagnosed, using the detection result obtained by the aforementioned detection method as an indicator. For example, when the detection result exceeds a predetermined

standard value, the state of a tumor is defined as tumor positive, and when the detection result is less than the predetermined standard value, it is defined as tumor negative. In the case of tumor positive, it is determined that a certain type of tumor could have been developed, and thus the tumor state can be evaluated. The term "the state of a tumor" is used herein to mean the presence or absence of the development of a tumor, or the progression degree thereof. Thus, specific examples of the state of a tumor include the presence or absence of the development of a tumor, the progression degree thereof, the degree of malignancy, the presence or absence of metastasis, and the presence or absence of recurrence.

[0133] In the aforementioned evaluation, as a state of a tumor to be evaluated, only one state may be selected from the aforementioned examples, or multiple examples may be combined and selected. The presence or absence of a tumor can be evaluated by determining whether or not the tumor has been developed, with reference to the predetermined standard value used as a boundary, based on the obtained detection result. The degree of malignancy is used as an indicator that indicates the progression degree of a cancer. Based on the detection result, the target tumor can be classified into a certain disease stage and it can be evaluated. Otherwise, an early cancer and an advanced cancer can be distinguished from each other, and then they can be evaluated. For example, it is also possible to determine the target tumor as an early cancer or an advanced cancer, using the detection result as an indicator. The metastasis of tumor can be evaluated by determining whether or not neoplasm has appeared at a site apart from the position of the initial lesion, using the detection result as an indicator. The recurrence can be evaluated by determining whether or not the detection result has exceeded the predetermined standard value again after interval stage or remission.

7. Kit for detecting or diagnosing tumor

[0134] The anti-hTROP-2 antibody of the present invention can be provided in the form of a kit for detecting or diagnosing a tumor. The kit of the present invention comprises a labeling substance, a solid-phase reagent on which the antibody or the labeled antibody has been immobilized, etc., as well as the aforementioned antibody. A labeling substance that labels the antibody means a substance labeled with an enzyme, a radioisotope, a fluorescent compound, a chemiluminescent compound, etc. The kit of the present invention may also comprise other reagents used for carrying out the detection of the present invention, in addition to the aforementioned constitutional elements. For example, when such a labeling substance is an enzyme labeling substance, the kit of the present invention may comprise an enzyme substrate (a chromogenic substrate, etc.), an enzyme substrate-solving solution, an enzyme reaction stop solution, a diluent used for analytes, etc. Moreover, the present kit may further comprise various types of buffers, sterilized water, various types of cell culture vessels, various types of reactors (an Eppendorf tube, etc.), a blocking agent (a serum component such as bovine serum albumin (BSA), skim milk, or goat serum), a washing agent, a surfactant, various types of plates, an antiseptic such as sodium azide, an experimental operation manual (instruction), etc.

[0135] The kit of the present invention can be effectively used to carry out the aforementioned detection method of the present invention, and thus it is extremely useful.

[0136] Hereinafter, the present invention will be more specifically described in the following examples. However, these examples are not intended to limit the scope of the present invention.

[Example 1]

[Cloning of hTROP-2 gene]

[0137] A full-length hTROP-2 gene was isolated from human fetal liver (10-week-old embryo) according to an RT-PCR method. First, the following PCR primers were designed based on the sequence of an hTROP-2 gene (Genbank accession No. NM_ 002353).

Forward primer: 5'-ttcctccgccccaccatggc-3' (SEQ ID NO: 3)

Reverse primer: 5'-ctcgagcaagctcggttcctttctc-3' (SEQ ID NO: 4)

[0138] When these primers were designed, a *Xho*I restriction enzyme-digested sequence except for a stop codon was added to the reverse primer. cDNA was synthesized from total RNA (TAKARA) prepared from human fetal liver (10-week-old embryo). Using this cDNA as a template, a PCR reaction was carried out with the aforementioned primers. Thereafter, development by agarose gel electrophoresis and extraction of a band of interest were carried out, and it was then cloned into a pCRII vector (Invitrogen) (pCRII-hTROP-2). The cloned hTROP-2 cDNA was confirmed by sequencing.

[0139] An expression vector was constructed by cleaving a *Eco*RI/*Xho*I fragment comprising an hTROP-2 gene from pCRII-hTROP-2, and then inserting the fragment into the *Eco*RI/*Xho*I site of a pcDNA4/*myc*-His© A vector (Invitrogen) (pcDNA4-hTROP-2-*myc*/His). Moreover, a *Hind*III/*Pme*I fragment comprising an hTROP-2 gene was cut out of pcDNA4-hTROP-2-*myc*/His (wherein the *Hind*III cleavage portion was blunt-ended), and the fragment was then inserted into a *Pme*I site of a cDNA3.1(+) vector (Invitrogen), so as to construct an expression vector comprising a neomycin resistance gene (pcDNA3.1-hTROP-2-*myc*/His).

[Example 2]

[Construction of cell line capable of stably expressing hTROP-2 gene]

[0140] The expression vector (pcDNA3.1-hTROP-2-*myc*/His) encoding the full-length cDNA of hTROP-2, which had been produced by the above-described method, was introduced into HEK293 cells (RIKEN), HuH-7 cells (HSRRB), 7E2-C cells (described in WO 2005/052156) and CHO-K1 cells (HSRRB), using a lipofectamine 2000 reagent (Invitrogen), and selection was then carried out using an antibiotic G418 (geneticin; GIBCO BRL). Thereafter, a cell line, which stably expressed hTROP-2, was established and obtained.

[Example 3]

[Production of recombinant protein of hTROP-2 extracellular region]

[0141] A gene fragment encoding a portion of the extracellular region of hTROP-2 (specifically, a region consisting of amino acids at positions 1 to 263 from the amino acid sequence shown in SEQ ID NO: 2) was amplified by a PCR method. The following primers were used in the amplification.
Forward primer: 5'-ttcctccgccccaccatggc-3' (SEQ ID NO: 3)
Reverse primer: 5'-ctcgagctcgtccaggtaatagatgagcg-3' (SEQ ID NO: 5)

[0142] In this operation, a *Xho*I restriction enzyme-digested sequence was added to the reverse primer. The DNA fragment amplified by the PCR method was developed by agarose gel electrophoresis, and it was then purified using QIAquick (registered trademark) Gel Extraction Kit (QIAGEN). The purified DNA fragment was subcloned into a pCR Blunt vector (Invitrogen) (pCRB-hTROP-2 EC), and the gene sequence was confirmed. Subsequently, a *Eco*RI/*Xho*I fragment comprising the gene fragment encoding the extracellular region of hTROP-2 was cut out of the pCRB-hTROP-2 EC, and it was then inserted into the *Eco*RI/*Xho*I site of a pcDNA4/*myc*-His© A vector (Invitrogen) (pcDNA4mH-hTROP-2 EC). Further, in order to produce a *Nru*I restriction enzyme cleavage site, the following oligonucleotides were associated and inserted into the *Bam*HI/*Eco*RI site of the pcDNA4mH-hTROP-2 EC.
Oligonucleotide 1: 5'-gatccactagtcgcgagtggtgg-3' (SEQ ID NO: 6)
Oligonucleotide 2: 5'-aattccaccactcgcgactagtg-3' (SEQ ID NO: 7)

[0143] Likewise, a pBgl II linker (TAKARA) was inserted into the *Pme*I site of the pcDNA4mH-hTROP-2 EC (pcDNA4mH-NB-hTROP-2 EC). In order to produce a recombinant protein using baculovirus, a *Nru*I/*Bgl*II fragment comprising the gene fragment encoding the extracellular region of hTROP-2 was cut out of the pcDNA4mH-NB-hTROP-2 EC, and it was then inserted into the *Nru*I/*Bgl*II site of a pPSC8 vector (Nosan Corporation) (pPSC8-hTROP-2 EC). The production of the recombinant protein of the extracellular region of hTROP-2 using baculovirus was delegated to Nosan Corporation.

[0144] The recombinant protein of the extracellular region of hTROP-2 was purified as follows. Ni Sepharose 6 Fast Flow (GE Healthcare Biosciences) was added to a culture supernatant comprising the recombinant protein, so that they were allowed to bind to each other at 4°C for 2 hours. Thereafter, the resultant was washed with a phosphate buffer containing 20 mM imidazole, employing EconoColumn (BIO RAD), and it was then eluted with a phosphate buffer containing 300 mM imidazole, so that it was purified.

[Example 4]

[Isolation of human EpCAM cDNA and construction of expression vector]

[0145] A full-length human EpCAM gene was isolated from human fetal liver (10-week-old embryo) according to an RT-PCR method. First, the following PCR primers were designed based on the sequence of a human EpCAM gene (Genbank accession No. NM_002354).
Forward primer: 5'-tcctcgtgtcccactcccgg-3' (SEQ ID NO: 8)
Reverse primer: 5'-ctcgagtgcattgagttccctatgc-3' (SEQ ID NO: 9)

[0146] When these primers were designed, a *Xho*I restriction enzyme-digested sequence except for a stop codon was added to the reverse primer. cDNA was synthesized from total RNA (TAKARA) from human fetal liver (10-week-old embryo). Using this cDNA as a template, a PCR reaction was carried out with the aforementioned primers. Thereafter, development by agarose gel electrophoresis and extraction of a band of interest were carried out, and it was then cloned into a pCRII vector (Invitrogen) (pCRII-hEpCAM). The cloned human EpCAM cDNA was confirmed by sequencing.

[0147] An expression vector was constructed by cleaving a *Eco*RI/*Xho*I fragment comprising a human EpCAM gene from pCRII-hEpCAM, and then inserting the fragment into the *Eco*RI/*Xho*I site of a pcDNA4/*myc*-His© A vector (Invitrogen) (pcDNA4-hEpCAM-myc/His). Moreover, a *Hind*III/*Pme*I fragment comprising a human EpCAM gene was cut out of

pcDNA4-hEpCAM-*myc*/His (wherein the *Hind*III cleavage portion was blunt-ended), and the fragment was then inserted into the *Pme*I site of a pcDNA3.1(+) vector (Invitrogen), so as to construct an expression vector comprising a neomycin resistance gene (pcDNA3.1-hEpCAM-*myc*/His).

[Example 5]

[Production of anti-hTROP-2 monoclonal antibody]

[0148] As immunogens, there were used cell lines capable of stably expressing hTROP-2 (HEK293-hTROP-2 cells, CHO-K1-hTROP-2 cells and 7E2-C-hTROP-2 cells); human pancreatic cancer cell line endogenously expressing an hTROP-2 protein on the cell surface (PK-59, RCB1901; purchased from RIKEN cell bank); and the recombinant protein of the extracellular region of hTROP-2 produced by the above-described method.

[0149] In the case of the cell lines capable of stably expressing hTROP-2, 1×10^7 cells were used, and in the case of the recombinant hTROP-2 protein, 20 μg of the protein was used. The cell lines or the recombinant protein was mixed with an adjuvant TiterMax Gold (Funakoshi Corporation) at a mixing ratio of 1 : 1, so as to prepare an emulsion. The emulsion was then injected into the two footpads or abdominal cavity of a mouse (C57/BL6, Balb/c) (initial immunization). When immunization was carried out by injection into the two footpads for a short period of time, booster was carried out three to ten days after the initial immunization. On the day following the final immunization, lymph nodes were collected from both knees, and lymphocytes were then prepared. When immunization was carried out by injection into the abdominal cavity for a long period of time, boosters were carried out at intervals of once a week after the initial immunization (wherein boosters were carried out for 1 to 2 months). Thereafter, B cells were isolated from the spleen according to an ordinary method. In the case of immunization using cells as immunogens, a cell suspension which was PBS containing 5×10^6 cells was used for boosters. In the case of using a protein as an immunogen, 5 μg of a PBS solution was used.

[0150] The prepared lymphocytes were mixed with a mouse myeloma cell line (P3-X63-Ag8.653) at a mixing ratio of 3 : 1, and cell fusion was then carried out according to a polyethylene glycol method. Thereafter, the fused cells were cultured for 7 to 28 days in a methyl cellulose medium (trade name: ClonaCell-HY Cloning Medium D; Stem Cell), which contained HAT (hypoxanthine, aminopterin and thymidine). Single colonies of growing hybridomas were each picked up and placed on a 96-well flat-bottom plate, and using a liquid selective medium containing HAT, the hybridomas were cultured in a 5% CO_2 incubator. A culture supernatant of growing hybridomas from single colonies was subjected to a primary screening via Cell ELISA (described later) and then to a secondary screening via FACS analysis using HuH-7-hTROP-2 cells, PK-59, thereby establishing 300 types of hybridomas, which produce anti-hTROP-2 monoclonal antibodies recognizing hTROP-2 proteins expressed on the cell surface of living cells.

[Example 6]

[Primary screening using Cell ELISA]

[0151] CHO-K1 cells (hTROP-2 negative control; purchased from Japan Health Sciences Foundation) and CHO-K1-hTROP-2 cells (or HUH-7 cells (hTROP-2 negative control; purchased from Japan Health Sciences Foundation) and HuH-7-hTROP-2 cells) were alternately inoculated on a 96-well culture plate (BD Falcon) at a cell density of 3×10^4 cells/well, and the cells were then cultured in a 5% CO^2 atmosphere at 37°C for 1 to 2 days. The cell culture medium was removed by decantation. Thereafter, the cells were washed with ice-cold PBS, and were then treated with 4% paraformaldehyde-PBS for 5 minutes, so that the cells were immobilized. The cells were washed with PBS which had been cooled on ice, and an ELISA plate was then prepared. Thereafter, ELISA was carried out according to an ordinary method. Specific procedures will be described below.

[0152] First, blocking with a 2% skim milk-PBS solution was carried out at room temperature for 30 minutes to 1 hour. Subsequently, the hybridoma culture supernatant was added thereto, and they were then reacted at room temperature for 1 hour. Thereafter, the resultant was washed with a 0.1% Tween20-PBS solution three times. As a secondary antibody, Horseradish peroxidase (HRP)-labeled anti-mouse IgG (GE Healthcare Biosciences), which had been 1000 times diluted with a blocking solution, was added to the resultant, and they were then reacted at room temperature for 1 hour. Thereafter, the resultant was washed with a 0.1% Tween20-PBS solution three times. A TMB (3,3',5,5'-tetramethylbenzidine: SIGMA) substrate solution was added to the reaction solution to carry out a color reaction, and the reaction was then terminated by adding 1 M sulfuric acid. Thereafter, absorbance (405 nm) was measured using Microplate reader Model 550 (BIO RAD). Hybridomas corresponding to a hybridoma culture supernatant exhibiting a high absorbance value to the negative control were subjected to a large-scale culture on a 24-well flat-bottom plate, and were then subjected to a secondary screening using FACS analysis.

[Example 7]

[Secondary screening using FACS analysis]

[0153] Hybridomas, which were found positive in the above-described primary screening using Cell ELISA, were subjected to a secondary screening using FACS analysis. In the evaluation of Hybridoma cells, HuH-7 cells, which were human liver cancer cells which did not express hTROP-2, were used as negative control cells and the reactivity with HuH-7-hTROP-2 cells, which were stably expressing hTROP-2, was used as an indicator. Then, the evaluation was carried out based on the reactivity with PK-59 cells (RCB1901; purchased from RIKEN cell bank), which were human pancreatic cancer cells endogenously expressing an hTROP-2 protein on the cell surface.

[0154] The cells were removed from the culture dish by a trypsin treatment, and a cell suspension was then prepared (cell density: 2×10^6 cells/mL). The hybridoma culture supernatant, which exhibited positive in the primary screening using Cell ELISA, was reacted with 100 μL of the cell suspension at 4°C for 20 minutes. The reaction mixture was washed with PBS, and it was then reacted with PE-labeled mouse IgG (BD Pharmingen) (0.1 μg) (4°C, 30 minutes). Thereafter, the reaction mixture was analyzed using FACSCalibur (Becton, Dickinson and Company).

[0155] Eventually, approximately 300 types of hybridomas, which produce an anti-hTROP-2 monoclonal antibody recognizing an hTROP-2 protein expressed on the cell surface of living cells, were established.

[Example 8]

[Identification of isotype]

[0156] The isotype of the produced anti-hTROP-2 monoclonal antibody was identified using MOUSE MONOCLONAL ANTIBODY ISOTYPING TEST KIT (Serotec) in accordance with a method included with the above-mentioned kit.

[Example 9]

[Ascites formation and purification of TROP-2 antibody]

[0157] The hybridoma clones produced by the above-described method were administered at a density of 3×10^6 clones into the abdominal cavity of a BALB/c nude mouse, to which 2,6,10,14-tetramethylpentadecane (pristane) had previously (seven days before) been administered. Two weeks later, ascites was collected. Moreover, this ascites was subjected to caprylic acid precipitation, and then to affinity purification using a protein G column (HiTrap protein G; GE Healthcare Biosciences) or a protein A column (HiTrap protein A; GE Healthcare Biosciences), so as to obtain anti-hTROP-2 monoclonal antibodies from individual hybridoma clones.

[Example 10]

[Measurement of antigen binding affinity (measurement of Kd value)]

[0158] The antigen binding affinity (Kd value) of the generated anti-hTROP-2 monoclonal antibody was calculated by a method using ELISA (Djavadi-Ohaniance L. et al (1996), In Antibody Engineering, Chapter 4, pp. 77-97. IRL Press, Oxford).

[0159] Specifically, the purified recombinant hTROP-2 protein (0.1 μg/mL) was added to a 96-well culture plate (Corning) so that the plate was coated with the antigen (at room temperature for 1 hour, or at 4°C overnight). Subsequently, the resultant was washed with PBS three times, and 2% skim milk (PBS solution) was then added thereto to block it (at room temperature for 1 hour). The resultant was washed with PBS twice. Thereafter, an antigen-antibody complex which had previously been formed by mixing an antigen solution (a purified hTROP-2 protein; 50, 25, 12.5, 6.25, or 3.125 nM) with each clone (0.5 nM) of the anti-hTROP-2 monoclonal antibody and then equilibrating the mixture, was added to the above-described ELISA plate, and they were reacted (at room temperature for 1 hour). The reaction product was washed with PBS three times, and it was then reacted with HRP-labeled anti-mouse IgG (final concentration: 1 μg/mL) (GE Healthcare Biosciences) diluted with a blocking solution (at room temperature for 1 hour). Subsequently, the reaction product was washed with a 0.1% Tween20-PBS solution three times, and a TMB (3,3',5,5'-tetramethylbenzidine: SIGMA) substrate solution was then added to the resultant to carry out a color reaction. Then, 1 M sulfuric acid was added to the reaction product to terminate the reaction. Using Microplate reader Model 550 (BIO RAD), absorbance was measured.

[0160] The following calculation expressions were used to measure dissociation constant (Kd).

[0161] In accordance with the law of mass action, an antigen-antibody reaction is represented by the following expressions.

$$\text{Ag (Antigen)} + \text{Ab (Antibody)} \underset{k2}{\overset{k1}{\Leftrightarrow}} \text{Ag-Ab (Antigen-Antibody complex)} \quad \cdots\cdot(1)$$

$$Kd = k2/k1 = Agf \times Abf / Ag\text{-}Ab = Agf \times Abf / x \quad \cdots\cdot\cdot\cdot(2)$$

[0162] In the expression (2), Agf represents the concentration of a free antigen, Abf represents the concentration of a free antibody, and Ag-Ab represents the concentration of an antigen-antibody complex. If Ag-Ab = x, the free antibody concentration is represented by the following expression.

$$Abf = Abt - x \quad \cdots\cdot\cdot\cdot(3)$$

[0163] The above expression (2) therefore can be

$$Kd = Agf \times (Abt - x) / x \quad \cdots\cdot\cdot\cdot(4)$$

[0164] If both terms of the expression (4) are multiplied by x / Kd×Abt,

$$x / Abt = Agf \times (1 - x/Abt) \times 1/Kd$$

$$x/Abt \times 1/Agf = (1 - x/Abt) \times 1/Kd \quad \cdots\cdot\cdot\cdot(5)$$

[0165] If X = x/Abt and Y = x/Abt×Agf in the expression (5),

$$Y = (1\text{-}X) \times 1/Kd \quad \cdots\cdot\cdot\cdot(6)$$

[0166] Based on the expression (6), the Kd value was calculated.

[0167] The Kd values of the generated 300 anti-hTROP-2 monoclonal antibody clones were measured by the above-described method. As a result, there were 133 clones exhibiting a Kd value of 1×10^{-10} (M) or less, 59 clones exhibiting a Kd value of 1×10^{-11} (M) or less, and 2 clones exhibiting a Kd value of 1×10^{-12} (M) or less.

[0168] Among the anti-hTROP-2 monoclonal antibodies, which exhibited tumor growth inhibitory *activity in vivo,* the Kd values of K5-70 (mouse IgG2a), T6-16 (mouse IgG2a), K5-107 (mouse IgG1), K5-116-2-1 (mouse IgG2a) and T5-86 (mouse IgG1) were found to be 6.8×10^{-12} (M), 4.3×10^{-12} (M), 4.7×10^{-12} (M), 2.69×10^{-11} (M) and 8.49×10^{-11} (M), respectively (Figure 1 and Table 1).

Table 1

Kd values of anti-hTROP-2 monoclonal antibodies

Clone No.	K5-70	T6-16	K5-107	K5-116-2-1	T5-86
Kd ($\times 10^{-12}$M)	6.8	4.3	4.7	26.9	84.9

[Example 11]

[Reactivity of anti-hTROP-2 monoclonal antibodies with human cancer cell lines]

[0169] The human cancer cell lines (human tumor cell lines) used in this studies were acquired from Health Science Research Resources Bank (HSRRB), RIKEN cell bank (RIKEN), ATCC (American Type Culture Collection), ECACC (European Collection of Cell Cultures) and DSMZ (German Collection of Microorganisms and Cell Cultures). Specifically, the following cancer cell lines were used.

[0170] huH-1 (HSRRB), HUH-6 (HSRRB), HuH-7 (HSRRB), JHH-5 (HSRRB), JHH-6 (HSRRB), JHH-7(HSRRB), HLE (HSRRB), HLF (HSRRB), HepG2 (HSRRB), Alexander (HSRRB), KP-1N (HSRRB), KP-1NL (HSRRB), KP-2 (HSRRB),

KP-3 (HSRRB), KP-3L (HSRRB), PK-1 (RIKEN), PANC-1 (RIKEN), MIA PaCa-2 (HSRRB), PK-59 (RIKEN), PK-45H (RIKEN), PK-45P (RIKEN), BxPC-3 (ATCC), SUIT-2 (HSRRB), TCC-PAN2 (HSRRB), SW480 (ATCC), DLD-1 (HSRRB), LoVo (HSRRB), COLO-320 (RIKEN), CACO-2 (RIKEN), CW-2 (RIKEN), HCT 116 (ATCC), HCC-56 (HSRRB), MCF-7 (HSRRB), JIMT-1 (DSMZ), HCC1143 (ATCC), A549 (HSRRB), DU145 (RIKEN) and PC-3 (HSRRB).

[0171] Cancer cells were removed from a culture dish by a trypsin treatment, and a cell suspension was then prepared (cell density: 2×10^6 cells/mL). An anti-hTROP-2 monoclonal antibody (0.1 μg) was added to 100 μL of the cell suspension, and they were then reacted at 4°C for 20 minutes. The reaction solution was washed with PBS, and it was then reacted with PE-labeled anti-mouse IgG (BD Biosciences Pharmingen) (0.1 μg) (at 4 °C for 30 minutes). Thereafter, the resultant was analyzed by FACSCalibur (Becton, Dickinson and Company).

[0172] All of the generated anti-hTROP-2 antibodies did not bind to a human liver cancer cell line HuH-7, which did not endogenously express hTROP-2. On the other hand, the anti-hTROP-2 antibodies bound to HuH-7-hTROP-2 cells, in which an hTROP-2 gene was stably expressed (Figure 2). Subsequently, the reactivity of the generated anti-hTROP-2 monoclonal antibodies with human cancer cell lines (in which an hTROP-2 protein was endogenously expressed on the cell surface) was examined by FACS analysis. As a result, the generated 300 types of anti-hTROP-2 monoclonal antibodies all bound to human pancreatic cancer cell lines (PK-59 and BxPC-3). In particular, K5-70, T6-16, K5-107, K5-116-2-1 and T5-86 antibodies, which exhibited tumor growth inhibitory *activity in vivo,* all bound to human cancer cell lines at high levels. For example, when compared with a case in which cancer cell lines were reacted with only PE-labeled anti-mouse IgG (BD Biosciences Pharmingen), the aforementioned antibodies exhibited the following binding ability to PK-59 cells and to BxPC-3 cells at mean fluorescence intensity: K5-70 (44 times), T6-16 (59 times), K5-107 (89 times), K5-116-2-1 (122 times) and T5-86 (15 times) (to PK-59 cells; Figure 3); and K5-70 (45 times), T6-16 (25 times), K5-107 (90 times), K5-116-2-1 (121 times) and T5-86 (10 times) (to BxPC-3 cells; Figure 4).

[0173] With regard to human cancer cell lines other than PK-59 and BxPC-3, among 12 types of pancreatic cancer cell lines, the anti-hTROP-2 monoclonal antibodies bound to KP-2, KP-3L, PK-1, PK-45H, SUIT-2 and TCC-PAN2, and did not bind to KP-1N, KP-1NL, KP-3, PANC-1 and MIA-PaCa2 (Figure 5). Among human colon cancer cell lines, the anti-hTROP-2 monoclonal antibodies bound to CACO-2, SW480, DLD-1 and HCT 116, and did not bind to COLO-320 and CW-2 (Figure 6). Furthermore, the anti-hTROP-2 monoclonal antibodies bound to JIMT-1 and HCC1143 (which were both human breast cancer cell lines) and to PC-3 and DU145 (which were both human prostate cancer cell lines). Thus, they recognized hTROP-2 proteins endogenously expressing on the cell surface of many types of human cancer cell lines (Figure 6).

[Example 12]

[Cross-reactivity with mouse TROP-2 protein and human TROP-1/EpCAM protein]

[0174] For the purpose of examining the specificity of the generated anti-hTROP-2 monoclonal antibodies, the reactivity of the antibodies with a mouse TROP-2 protein showing homology of 80% at the amino acid sequence level with the hTROP-2 protein, and with a human TROP-1/EpCAM protein showing homology of 50% at the amino acid sequence level with the hTROP-2 protein, was examined by FACS analysis.

[0175] Specifically, each of an expression vector (mouse TROP-2-pcDNA3.1(+), furnished by the Institute of Molecular and Cellular Biosciences, the University of Tokyo) comprising the full-length cDNA of a mouse TROP-2 gene (GenBank accession No. NM_020047, Y08830), and an expression vector (pcDNA3.1-hEpCAM-*myc*/His) comprising the full-length cDNA of a human TROP-1/EpCAM gene (GenBank accession No. NM_002354), was transiently introduced into CHO-K1 cells, using Lipofectamine2000 reagent (Invitrogen). Thereafter, 24 to 48 hours later, the cells were removed from a culture dish by treating them with trypsin, and a cell suspension was then prepared. The thus prepared cell suspension was successively reacted with the produced anti-hTROP-2 monoclonal antibody (0.1 μg) and with PE-labeled anti-mouse IgG, and it was then analyzed by FACSCalibur.

[0176] A T2-102 antibody (mouse IgG1) used as a positive control, which showed cross-reactivity with mouse TROP-2, exhibited high binding ability to the CHO-K1 cells in which the mouse TROP-2 gene was transiently expressed. On the other hand, K5-70, T6-16, K5-107, K5-116-2-1 and T5-86 antibodies did not show such cross-reactivity with mouse TROP-2 (Figure 7).

[0177] Similarly, an anti-human EpCAM monoclonal antibody (BD Biosciences Pharmingen) used as a positive control exhibited high binding ability to the CHO-K1 cells in which the human EpCAM/TROP-1 was transiently expressed. On the other hand, K5-70, T6-16, K5-107, K5-116-2-1 and T5-86 antibodies did not show such cross-reactivity with human EpCAM/TROP-1 (Figure 8).

[0178] The aforementioned results demonstrated that the generated anti-hTROP-2 monoclonal antibodies, and in particular, K5-70, T6-16, K5-107, K5-116-2-1 and T5-86 antibodies, which exhibited anti-tumor *activity in vivo,* specifically bound to hTROP-2.

[Example 13]

[Measurement of cell growth inhibitory activity]

[0179] As a method of examining the activity of the anti-hTROP-2 monoclonal antibody to inhibit the function of hTROP-2, the influence of the antibody on the cell growth of human cancer cells, which endogenously express hTROP-2 on the cell surface, was evaluated by measuring the number of living cells using TetraColor ONE (Seikagaku Corporation). Specifically, PK-59 cells were suspended in an RPMI1640 medium containing 0.5% fetal bovine serum (manufactured by BioWest) at a cell concentration of 2×10^5 cells/mL, and 100 μL of the prepared cell suspension was then added to each well of a 96-well culture plate. Subsequently, mouse IgG (negative control) and anti-hTROP-2 monoclonal antibodies (final concentrations: 0.1 and 1 μg/mL) were added to the wells, and the mixtures were then cultured at 37°C in a 5% CO_2 incubator for 72 hours. As a control, a commercially available anti-hTROP-2 monoclonal antibody (clone YY01, Santa Cruz) was used. TetraColor ONE (Seikagaku Corporation) was added to the wells, and they were then reacted in a 5% CO_2 incubator for 1 to 2 hours. After completion of the reaction, the 96-well culture plate was directly subjected to the measurement of absorbance at a wavelength of 490 nm (control wavelength: 655 nm), using Microplate Reader. The experiment was carried out using 3 wells for each group. A significant difference test was carried out according to Student's t-test, and $P < 0.05$ was determined to be statistically significant.

[0180] Among anti-hTROP-2 monoclonal antibodies, which had been generated by our own company so far, approximately 160 clones were examined by the above-described method, in terms of their effect on the cell growth of PK-59 cells. As a result, T6-16, T5-86, K5-70 and K5-107, which had exhibited tumor growth inhibitory *activity in vivo,* were confirmed to have cell growth inhibitory activity of 20% to 40%, when compared with mouse IgG (negative control). It became clear that these anti-hTROP-2 antibodies have activity to bind to hTROP-2 proteins, which were expressed on the surface of human cancer cells, to neutralize the hTROP-2 proteins, and to inhibit the growth of the cancer cells (Figure 9).

[Example 14]

[Scratch assay]

[0181] The effect of an anti-hTROP-2 monoclonal antibody on the migratory ability of human cancer cells was evaluated by a scratch assay. PK-59 cells were suspended in an RPMI1640 medium containing 10% fetal bovine serum at a cell concentration of 3×10^5 cells/mL, and 100 μL of the prepared cell suspension was then added to each well of a 96-well culture plate. When the cells became confluent, a portion of the monolayer-cultured cells was peeled, such that the plate was scratched in a longitudinal direction with the end of a tip. An anti-hTROP-2 monoclonal antibody and mouse IgG used as a negative control were added to the medium to final concentrations of 0.1 and 1 μg/mL, respectively, and culture was then carried out for 24 hours. Before addition of the antibody (Day 0) and 24 hours after the culture (Day 1), the cell peeled region was photographed, and the distance between the cells was then measured. Moreover, the area of such a peeled region was quantified using Scion Image software. The experiment was carried out using 8 wells for each group. A significant difference test was carried out according to Student's t-test, and $P < 0.05$ was determined to be statistically significant.

[0182] The effect of an hTROP-2 antibody on the migratory ability of the cells invading the scratch region was examined. As with the cell growth inhibition assay, antibodies having beneficial effects were evaluated. As an evaluation method, the cells were photographed on Day 0 (when the antibody was added) and on Day 1 (24 hours after the addition of the antibody), and the migratory distance (μm) and the area of a scratch region were determined by image analysis. As a result, as shown in Figure 10, clear differences were observed in terms of the migratory ability of the cells. The antibodies T6-16 and K5-70, which were used in the present test, had significant cell growth inhibitory activity, when compared with the control. Even in a reproducibility test, the same tendency was observed. Particularly, T6-16 had a result of $P < 0.01$ (by Student's t-test), and there was found correlation with the *in vivo* test.

[Example 15]

[Evaluation of beneficial effects of anti-hTROP-2 monoclonal antibody on tumor-bearing mice]

Prevention model

[0183] Pancreatic cancer cell lines (PK-59 and BxPC-3), which expressed hTROP-2, were harvested by treatment with trypsin, and PBS was added to them to prepare a cell suspension having a concentration of 1×10^8 cells/mL. The thus prepared cell suspension was mixed with an equal amount of Matrigel (BD Biosciences Pharmingen) on ice. Using

a 26 G syringe, 100 μL of the obtained mixture (5×10^6 cells) was injected into the subcutis of the right flank of each of 6-week-old female nude mouse (Balb/c, nu/nu). On the day of the transplantation of the cancer cells (Day 1), the mice were divided into groups, and administration of the antibody (1, 5 or 10 mg/kg body weight, intraperitoneal administration) was initiated. Thereafter, administration of the antibody was continued at intervals of once every three days. Anti-tumor activity was evaluated based on tumor formation frequency and tumor volume. The tumor volume was calculated by the following formula.

$$\text{Tumor volume } (mm^3) = (\text{minor axis})^2 \times (\text{major axis}) \times \pi/6$$

Treatment model

[0184] Pancreatic cancer cell lines (PK-59 and BxPC-3), which expressed hTROP-2, were harvested by treatment with trypsin, and PBS was added to them to prepare a cell suspension having a concentration of 1×10^8 cells/mL. The thus prepared cell suspension was mixed with an equal amount of Matrigel (BD Biosciences Pharmingen) on ice. Using a 26 G syringe, 100 μL of the obtained mixture (5×10^6 cells) was injected into the subcutis of the right flank of each of 6-week-old female nude mouse (Balb/c, nu/nu). Five to six days after the transplantation of the cancer cells, mice whose tumor volume had increased to 50 to 150 mm^3 (mean value: approximately 100 mm^3) were divided into groups. The day on which the mice were divided into groups was defined as a first day (Day 1), and administration of the antibody was initiated. The antibody was intraperitoneally administered at intervals of once every three days (10 mg/kg body weight). Anti-tumor activity was evaluated by measuring tumor volume. A significant difference test was carried out according to Student's t-test, and $P < 0.05$ was determined to be statistically significant.

[Example 16]

[Analysis of *in vivo* anti-tumor activity of anti-hTROP-2 monoclonal antibody on human pancreatic cancer cell xenograft model]

[0185] It is essential for an antibody used for the treatment of cancer, which targets hTROP-2, to have the activity of specifically killing tumor tissues expressing hTROP-2 or inhibiting the growth of tumor in a xenograft model.

[0186] Anti-hTROP-2 monoclonal antibodies (approximately 160 clones), which were newly produced in the present invention, were evaluated using the xenograft treatment models of a pancreatic cancer cell line PK-59. The PK-59 cells express, on the surface thereof, EpCAM (Figure 11A) acting as a pancreatic cancer stem cell marker (Chenwei Li, et al. Cancer Res 2007; 67: (3). 1030-1037), and also express P-glycoprotein/MDR1 (Figure 11B) and ABCG2/CDw338 (Figure 11C) (Chen, C. J. et al. Cell 47 (3), 381-389 (1986), Allikmets, R., et al. Hum. Mol. Genet. 5 (10), 1649-1655 (1996)), which are ABC transporters associated with drug resistance. In addition, the PK-59 cells contain a cell fraction (8.93%) (Figure 11D) positive for both CD24 and CD44, which is characteristic for pancreatic cancer stem cells, and they are assumed to be a highly malignant human pancreatic cancer cell line (Chenwei Li, et al. Cancer Res 2007; 67: (3). 1030-1037, Jane E. Visvader and Geoffrey J. Lindeman. Nat Rev Cancer. Vol. 8(10):755-68, 2008).

[0187] Most of the newly generated approximately 160 clones did not exhibit beneficial effects on the xenograft treatment models of PK-59 cells. Among such clones, clones exhibiting significant tumor growth inhibitory activity, namely clones K5-70, T6-16, K5-107, T5-86 and K5-116-2-1 could be obtained.

[0188] In a clone K5-70 (mouse IgG2a) administration group, tumor growth rate is statistically significantly inhibited. On the 21st day after initiation of the administration (day 21), the tumor volume of a control group (N = 14) was 1200.8 $\pm$ 377.3 mm^3, whereas the tumor volume of the clone K5-70 administration group was 748.7 $\pm$ 175.0 mm^3 ($P < 0.01$ by Student's t-test) (Figure 12A). When the tumor volume at the time of initiation of the administration of the antibody was defined as 1.0, the tumor volume on the 21st day (Day 21) was 7.8 in the clone K5-70 administration group, whereas the tumor volume of the control group was 12.5 (Figure 12A). The weight of the tumor excised was 0.43 $\pm$ 0.14 g ($P < 0.01$ by Student's t-test) in the clone K5-70 administration group, whereas that of the control group was 0.73 $\pm$ 0.26 g. Thus, the clone K5-70 exhibited inhibitory activity of approximately 60% (Figure 12B).

[0189] Similarly, tumor growth rate was statistically significantly inhibited even in a clone K5-107 (mouse IgG1) administration group (N = 8), a clone T6-16 (mouse IgG2a) administration group (N = 8), a clone T5-86 (mouse IgG1) administration group and a clone K5-116-2-1 (mouse IgG2a) administration group (N = 8). On the 17th day after initiation of the administration (Day 17), the tumor volumes of the clone K5-107 administration group (N = 8) and the clone T6-16 administration group (N = 8) were 698.2 $\pm$ 175.9 mm^3 ($P < 0.05$ by Student's t-test) and 707.2 $\pm$ 254.5 mm^3 ($P < 0.05$ by Student's t-test), respectively, whereas the tumor volume of the control group was 1039.3 $\pm$ 271.6 mm^3. Likewise, on the 16th day after initiation of the administration (Day 16), the tumor volume of the clone K5-116-2-1 administration

group (N = 8) was 508.5 ± 225.2 mm^3 (P < 0.05 by Student's t-test), whereas the tumor volume of the control group (N = 8) was 797.0 ± 172.9 mm^3 (Figure 13).

[0190] On the other hand, in the case of the clone T5-86, on the 15th day after initiation of the administration (Day 15), the tumor of the clone T5-86 administration group (N = 8) was 744.1 ± 289.1 mm^3, whereas the tumor volume of the control group (N = 8) was 1033.2 ± 319.4 mm^3. Thus, there was found no significant difference in terms of tumor volume. However, in the comparison of tumor weight, which was performed on the same day, the tumor weight of the clone T5-86 administration group was 0.44 ± 0.13 g (P < 0.05 by Student's t-test), whereas the tumor weight of the control group was 0.62 ± 0.14 g. Thus, the clone T5-86 exhibited significant inhibitory activity.

[0191] Moreover, in terms of both tumor volume and tumor weight, the ratio (T/C) of each clone antibody administration group to the control group on the final day of experiment is shown in Table 2 below. As shown in Table 2, each clone antibody exhibited significant inhibitory activity (T/C = 62% to 72%) on each clone antibody administration group.

Table 2

Group	N (number of mice)	Tumor volume T/C (%)	Tumor weight T/C (%)
K5-70	14	62.3**	58.8**
K5-107	8	67.2*	65.0*
T6-16	8	68.0*	64.7*
T5-86	8	72.0	70.5*
K5-116-2-1	8	63.8*	60.5*

* P < 0.05, ** P < 0.01 (by Student's t-test)

[0192] Furthermore, the anti-tumor activity of each of the clones K5-70, T6-16 and K5-116-2-1 on the xenograft prevention models of the pancreatic cancer cell line PK-59 was analyzed. After completion of the administration of each antibody clone, tumor growth was inhibited in all individuals (N = 8). On the 18th day after initiation of the administration (Day 18), the tumor volume of the clone K5-70 administration group (10 mg/kg body weight) was 62.4 ± 80.4 mm^3 (P < 0.01 by Student's t-test), whereas the tumor volume of the control group (N = 8) was 880.8 ± 206.4 mm^3. Thus, the clone K5-70 exhibited tumor growth inhibitory activity of 92.9%. On the 28th day after initiation of the administration (Day 28), the tumor volume of the clone T6-16 administration group (10 mg/kg body weight) was 152.14 ± 122.3 mm^3 (P < 0.01 by Student's t-test), whereas the tumor volume of the control group (N = 8) was 992.3 ± 250.8 mm^3. Thus, the clone T6-16 exhibited tumor growth inhibitory activity of 84.6%. On the 20th day after initiation of the administration (Day 20), the tumor volume of the clone K5-116-2-1 administration group (10 mg/kg body weight) was 207.7 ± 319.2 mm^3 (P < 0.01 by Student's t-test), whereas the tumor volume of the control group (N = 8) was 1159.4 ± 413.3 mm^3. Thus, the clone K5-116-2-1 exhibited tumor growth inhibitory activity of 82.1% (Figure 14 and Table 3). Moreover, in all of the experiments, there was no significant difference between the control group and each anti-hTROP-2 antibody administration group in terms of a change in mean body weight throughout the test period.

[0193] In terms of both tumor volume and tumor weight, the ratio (T/C) of each clone antibody administration group to the control group on the final day of experiment is shown in Table 3 below. As shown in Table 3, significant tumor growth inhibition was observed in each clone antibody administration group, and in particular, a significant effect such as T/C = 10% or less was confirmed in the clone K5-70 administration group.

Table 3

Group	N (number of mice)	Tumor volume T/C (%)	Tumor weight T/C (%)
K5-70	8	7.1**	5.8**
T6-16	8	15.3**	10.5**
K5-116-2-1	8	23.2**	21.5**

** P < 0.01 (by Student's t-test)

[0194] The known anti-TROP-2 antibody AR47A6.4.2 (U.S. Patent No. 7420041) has exhibited the effect of inhibiting tumor growth, at a dosage of 20 mg/kg, on xenograft prevention models using various human cancer cell lines. This anti-TROP-2 antibody AR47A6.4.2 has inhibited the tumor growth of a human pancreatic cancer cell line PL45 at a percentage of almost 100%. However, this antibody has had the effect of inhibiting tumor on a pancreatic cancer cell line BxPC-3 at a percentage of approximately 50%, on a prostate cancer cell line PC-3 at a percentage of approximately 40%, on a breast cancer cell line MCF-7 at a percentage of approximately 60%, and on a colon cancer cell line Colo205

at a percentage of approximately 40%. In contrast, the anti-hTROP-2 antibody of the invention of the present application has exhibited a higher tumor growth inhibitory effect at a dosage of half the aforementioned dose (10 mg/kg body weight).

[Example 17]

[Analysis of anti-tumor activity on xenograft models (prevention models and treatment models) of human pancreatic cancer cell line BxPC-3]

[0195] As in the case of using the above-described xenograft treatment models of the human pancreatic cancer cell line PK-59, the anti-tumor activity of the clone K5-70 on xenograft prevention models and xenograft treatment models of a human pancreatic cancer cell line BxPC-3 was analyzed.

[0196] When compared with a control group (N = 8), the tumor growth of the clone K-70 administration group was significantly inhibited. On the 52nd day (Day 52), the tumor volume of the clone K5-70 administration group (N = 8) was 236.0 $\pm$ 136.4 mm^3, whereas the tumor volume of the control group (N = 8) was 616.3 $\pm$ 266.8 mm^3. Thus, the clone K-70 exhibited a tumor growth inhibitory effect of 61.7% ($P < 0.01$ by Student's t-test) (Figure 15).

[0197] From the aforementioned results, it became clear that the anti-hTROP-2 monoclonal antibody exhibits significant tumor growth inhibitory activity *in vivo* on at least two cancer cell species.

[Example 18]

[Dose-dependent anti-tumor activity of anti-hTROP-2 antibody (clone K5-70) on xenograft prevention models of hTROP-2-expressing pancreatic cancer cell line (PK-59 cells)]

[0198] For the purpose of analyzing more in detail the tumor growth inhibitory activity *in vivo* of the anti-hTROP-2 antibody, a dose-dependent test was carried out. As shown in Figure 16, the tumor growth of PK-59 cells was dose-dependently inhibited by administration of the K5-70 antibody. On the 21st day after administration of the antibody (Day 21), the tumor volume of the control group (N = 8) was 937.8 $\pm$ 295.3 mm^3. On the other hand, the tumor volume of the K5-70 antibody (1 mg/kg body weight) administration group (N = 8) was 493.5 $\pm$ 305.1 mm^3, showing an inhibitory rate of 50%, and the tumor volume of the K5-70 antibody (5 mg/kg body weight) administration group (N = 8) was 124.7 $\pm$ 89.0 mm^3, showing an inhibitory rate of 90%. Thus, it became clear that, when compared with the known anti-TROP-2 antibody AR47A6.4.2 (U.S. Patent No. 7420041), the anti-hTROP-2 antibody of the present invention exhibits *in vivo* a tumor growth inhibitory effect equivalent to that of the anti-TROP-2 antibody AR47A6.4.2 at a dosage of one-twentieth the anti-TROP-2 antibody AR47A6.4.2, and that it exhibits a higher inhibitory effect of 90% at a dosage of one-fourth thereof.

[Example 19]

[Epitope assay]

Preparation of human/mouse chimeric TROP-2 protein

[0199] A human/mouse TROP-2 gene was prepared according to a PCR method. PCR primers as shown below were designed based on a human TROP-2 gene sequence and a mouse TROP-2 gene sequence (Genbank accession No. NM_020047).

Human/mouse TROP-2-C primers

Y606 (forward side): 5'-cctgagcctacgctgcgacgaagtggtgcg-3' (SEQ ID NO: 10)
Y607 (reverse side): 5'-cgcaccacttcgtcgcagcgtaggctcagg-3' (SEQ ID NO: 11)

Human/mouse TROP-2-A primers

Y612 (forward side): 5'-gactgctccacgctgacttccaagtgcctg-3' (SEQ ID NO: 12)
Y613 (reverse side): 5'-caggcacttggaagtcagcgtggagcagtc-3' SEQ ID NO: 13)

Human/mouse TROP-2-B primers

Y614 (forward side): 5'-ctcgtggacaacgatggcctctacgacccg-3' (SEQ ID NO: 14)

Y615 (reverse side): 5'-cgggtcgtagaggccatcgttgtccacgag-3' (SEQ ID NO: 15)

Mouse/human TROP-2-D primers

Y608 (forward side): 5'-ccaaagcctgcgctgcgatgagctggtgcgc-3' (SEQ ID NO: 16)
Y609 (reverse side): 5'-gcgcaccagctcatcgcagcgcaggctttgg-3' (SEQ ID NO: 17)

Mouse/human TROP-2-E primers

Y616 (forward side): 5'-agcttcctatccgcggtgcactacgagcag-3' (SEQ ID NO: 18)
Y617 (reverse side): 5'-ctgctcgtagtgcaccgcggataggaagct-3' (SEQ ID NO: 19)

Mouse/human TROP-2-F primers

Y618 (forward side): 5'-gacattaaaggcgagtctctattccagggc-3' (SEQ ID NO: 20)
Y619 (reverse side): 5'-gccctggaatagagactcgcctttaatgtc-3' (SEQ ID NO: 21)

Mouse TROP-2 primers

Forward primer: 5-ctactccaccccaccctggcg-3' (SEQ ID NO: 22)
Reverse primer: 5'-ctcgagcaagctaggttcgcttctc-3' (SEQ ID NO: 23)

[0200] To the mouse TROP-2 reverse primer, a *Xho*I restriction enzyme-digested sequence except for a stop codon was added. A schematic view of the prepared human/mouse chimeric TROP-2 proteins is shown in Figure 17.

[0201] The hmTROP-2-A chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 69 of the hTROP-2 protein and a polypeptide ranging from the amino acid at position 64 to the C-terminus of the mouse TROP-2 protein. The hmTROP-2-B chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 101 of the hTROP-2 protein and a polypeptide ranging from the amino acid at position 96 to the C-terminus of the mouse TROP-2 protein. The hmTROP-2-C chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 145 of the hTROP-2 protein and a polypeptide ranging from the amino acid at position 140 to the C-terminus of the mouse TROP-2 protein. The mhTROP-2-D chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 139 of the mouse TROP-2 protein and a polypeptide ranging from the amino acid at position 146 to the C-terminus of the hTROP-2 protein. The mhTROP-2-E chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 187 of the mouse TROP-2 protein and a polypeptide ranging from the amino acid at position 194 to the C-terminus of the hTROP-2 protein. The mhTROP-2-F chimeric protein is a chimeric protein, which consists of a polypeptide ranging from the N-terminus to the amino acid at position 227 of the mouse TROP-2 protein and a polypeptide ranging from the amino acid at position 234 to the C-terminus of the hTROP-2 protein.

[0202] Expression vectors used in the preparation of the above-described chimeric proteins were specifically constructed by the following methods. In order to prepare an hmTROP-2-A chimeric gene, the hTROP-2 gene was used as a template, and PCR was carried out using the hTROP-2 forward primer and the human/mouse TROP-2-A primer Y613. Likewise, the mouse TROP-2 gene was used as a template, and PCR was carried out using the human/mouse TROP-2-A primer Y612 and the mouse TROP-2 reverse primer. A DNA fragment amplified by the PCR was developed using acrylamide gel, and a band of interest was then recovered by extraction. Subsequently, the extracted two types of DNA fragments were mixed to prepare a template, and PCR was then carried out using the hTROP-2 forward primer and the mouse TROP-2 reverse primer. A PCR product was developed by agarose gel electrophoresis, and a DNA fragment of interest was then extracted. The extracted DNA fragment was cloned into a pCR (registered trademark)-Blunt vector (Invitrogen) (pCRB-hmTROP-2-A), and a gene sequence was then confirmed. An expression vector for animal cells was produced by removing the hTROP-2 gene from pcDNA3.1-hTROP-2-*myc*/His by *Eco*RI/*Xho*I digestion, and then inserting therein an *Eco*RI/*Xho*I fragment containing an hmTROP-2-A chimeric gene prepared from pCRB-hmTROP-2-A (pcDNA3.1-hmTROP-2-A-*myc*/His). Additionally, the following chimeric genes were prepared by the same method as described above, and expression vectors were constructed: hmTROP-2-B (using a human TROP-2 forward primer, a human/mouse TROP-2-B primer Y615, a human/mouse TROP-2-B primer Y614 and a mouse TROP-2 reverse primer), hmTROP-2-C (using a human TROP-2 forward primer, a human/mouse TROP-2-C primer Y607, a human/mouse TROP-2-C primer Y606 and a mouse TROP-2 reverse primer), mhTROP-2-D (using a mouse TROP-2 forward primer, a mouse/human TROP-2-D primer Y609, a mouse/human TROP-2-D primer Y608 and a human TROP-2 reverse primer), mhTROP-2-E (using a mouse TROP-2 forward primer, a mouse/human TROP-2-E primer Y617, a mouse/human TROP-

2-E primer Y616 and a human TROP-2 reverse primer), mhTROP-2-F (using a mouse TROP-2 forward primer, a mouse/human TROP-2-F primer Y619, a mouse/human TROP-2-F primer Y618 and a human TROP-2 reverse primer) (pcDNA3.1-hmTROP-2-B-*myc*/His, pcDNA3.1-hmTROP-2-C-*myc*/His, pcDNA3.1-mhTROP-2-D-*myc*/His, pcDNA3.1-mhTROP-2-E-*myc*/His, and pcDNA3.1-mhTROP-2-F-*myc*/His).

Establishment of HEK293 cell lines, which constitutively express hTROP-2, human/mouse TROP-2-C and mouse/human TROP-2-D chimeric proteins

[0203] The above-described expression vectors pcDNA3.1-hTROP-2-*myc*/His, pcDNA3.1-hmTROP-2-C-*myc*/His and pcDNA3.1-mhTROP-2-D-*myc*/His were each introduced into HEK293 cells. Selection was carried out using an antibiotic G418 (Calbiochem), and HEK293 cell lines constitutively expressing the hTROP-2 protein, the hmTROP-2-C chimeric protein and the mhTROP-2-D chimeric protein were established.

[0204] The binding regions of the anti-hTROP-2 monoclonal antibodies K5-70, T5-86, K5-107, T6-4, T6-16 and K5-116-2-1, which had exhibited beneficial effects on the xenograft treatment models of the pancreatic cancer cell line PK-59, were identified. First, the reactivity of the anti-hTROP-2 monoclonal antibodies exhibiting beneficial effects with HEK293 cells, which constantly express the chimeric proteins hmTROP-2-C and mhTROP-2-D, was examined by FACS analysis (Figure 18). As a result, it was found that the K5-70, K5-107, T5-86 and K5-116-2-1 antibodies reacted with hmTROP-2-C, but that these antibodies did not react with mhTROP-2-D. On the other hand, the T6-4 and T6-16 antibodies reacted with mhTROP-2-D, but they did not react with hmTROP-2-C. From these results, the binding region of each of the K5-70, K5-107, T5-84 and K5-116-2-1 antibodies was limited to a region ranging from the N-terminus to the amino acid at position 145 of hTROP-2, and the binding region of each of the T6-4 and T6-16 antibodies was limited to a region ranging from the amino acid at position 146 to the C-terminal region of hTROP-2 (Figure 18).

[0205] In order to analyze the binding regions more in detail, vectors used in the expression of hmTROP-2-A, hmTROP-2-B, mhTROP-2-E and mhTROP-2-F chimeric proteins were prepared, and the reactivity of the chimeric proteins with the anti-hTROP-2 monoclonal antibodies exhibiting beneficial effects was analyzed (Figure 19). The newly prepared expression vectors, which were to be used in the expression of the chimeric proteins, were each introduced into HEK293 cells, and FACS analysis was then carried out, using the cells which transiently expressed the chimeric proteins. The K5-70, K5-107, T5-86 and K5-116-2-1 antibodies reacted with hmTROP-2-A, but did not react with mhTROP-2-B. The examined 6 types of monoclonal antibodies all reacted with hTROP-2. These results clearly showed that the binding region of the K5-70, K5-107, T5-86 and K5-116-2-1 antibodies is present in a region ranging from the N-terminus to the amino acid at position 69 of hTROP-2. Moreover, the T6-4 and T6-16 antibodies reacted with neither mhTROP-2-E nor mhTROP-2-F. This suggested that these antibodies recognize a region ranging from the amino acid at position 146 to the amino acid at position 193 of hTROP-2.

[Example 20]

[Immunohistochemistry]

<Materials/Method>

[0206] The following normal and cancer tissue arrays were used in immunohistostaining.

[0207] Human normal tissue arrays:

Human, normal organs in duplicates (Catalog No.: AB1, Super Bio Chips)
Normal tissues more than single spots (Catalog No.: A103(VI), ISU ABXIS) Lung cancer tissue arrays:

Human lung cancer-metastasis-normal (Catalog No.: CCA3, Super Bio Chips)
Human lung carcinoma tissue with margin tissue, 2 location cores (Catalog No.: OD-CT-RsLug03-002, Shanghai Outdo Biotech)

[0208] Pancreatic cancer tissue array:

Human pancreas carcinoma tissue with mono-pathological type from 60 cases, 2 location cores (Catalog No.: OD-CT-DgPan03-001, Shanghai Outdo Biotech) Liver cancer tissue arrays:

Hepatocellular carcinoma, grades I to III with normal tissue controls, 63 cases tissue arrays (Catalog No.: CS03-01-002U, Cybrdi)

[0209] Human liver carcinoma tissue with mono-pathological type from 30 cases, 2 location cores (Catalog No.: OD-CT-DgLiv02-002, Shanghai Outdo Biotech) Colorectal cancer tissue arrays:

Human, colorectal cancer (Catalog No.: CD3, Super Bio Chips)
Human colon carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgCol03-002, Shanghai Outdo Biotech)

[0210] Colorectal cancer lymph node metastasis and liver metastasis tissue arrays:

Colorectal (colon and rectum) cancer with matched lymph node metastasis tissue array, 44 cases/99 cores, trial slide (Catalog No.: CO991t, Biomax us)
Colorectal (colon and rectum) cancer with matched lymph node metastasis and normal adjacent tissue array, 43 cases/99 cores (Catalog No.: CO992, Biomax us)
Colon cancer tissues_liver metastasis (Catalog No.: A203(IV), ISU ABXIS) Breast cancer tissue arrays:

Human, breast cancer-metastasis-normal (Catalog No.: CBA3, Super Bio Chips)
Human breast carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-RpBre03-002, Shanghai Outdo Biotech)

Stomach cancer tissue arrays:

Human, stomach cancer (Catalog No.: CQ1, Super Bio Chips)
Human gastric carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgStm03-002, Shanghai Outdo Biotech)

Esophagus cancer tissue array:

Human, esophagus cancer (Catalog No.: CR1, Super Bio Chips)
Human esophagus carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgEso03-002, Shanghai Outdo Biotech)

Ovarian cancer tissue array:

Human, ovary cancer (Catalog No.: CJ1, Super Bio Chips) Prostate cancer tissue array:
Human, prostate cancer-normal (Catalog No.: CA3, Super Bio Chips)

Bladder cancer tissue array:

Bladder carcinoma / transitional cell carcinoma, grades I to III with normal tissue arrays (Catalog No.: CC12-01-001U, Cybrdi)

[0211] Patient information and clinical information regarding the above-described tissue arrays were obtained from data sheets attached herewith and the homepages of individual companies.

Immunohistostaining method

[0212] After completion of a deparaffinization treatment, the tissue array slides of human normal tissues and cancer tissues were subjected to a protease treatment with pepsin at 37°C for 5 minutes. Thereafter, the sections were used in immunostaining using an anti-hTROP-2 monoclonal antibody. A color reaction was carried out using DAB(3,3'-diaminobenzidine) as a substrate, and as a counter staining, nuclear staining was then carried out using hematoxylin.

[0213] More specifically, these operations were carried out as follows. A paraffin-embedded section was subjected to a deparaffinization treatment, and was then subjected to a protease treatment with pepsin (DAKO) at 37°C for 5 minutes. After activation of the antigen, the section was treated at room temperature for 20 minutes using a solution prepared by adding a hydrogen peroxide solution to methanol to a final concentration of 0.3%, so that endogenous peroxidase activity was eliminated. The resultant was washed with PBS at room temperature for 5 minutes twice, and it was then blocked at room temperature for 30 minutes using a PBS solution containing 1.5% normal horse serum (DAKO), so as to carry out an operation to block non-specific binding in the tissues. Subsequently, the resultant was reacted with anti-hTROP-2 monoclonal antibody clone K5-63-17 (final concentration: 10 μg/ml), which had been diluted with a PBS solution

containing 1.5% normal horse serum, at room temperature for 1 hour, and was then washed with PBS at room temperature for 5 minutes three times. Thereafter, a biotinylated anti-mouse IgG antibody (Vector), which had been 200 times diluted with a PBS solution containing 1.5% normal horse serum, was reacted at room temperature for 30 minutes. The reaction product was washed with PBS at room temperature for 5 minutes three times, and a reagent of Vectastain ABC kit (Vector) was mixed in accordance with the instruction manual included therewith, so as to prepare an ABC complex. This ABC complex was reacted at room temperature for 30 minutes. The reaction product was washed with PBS at room temperature for 5 minutes three times, and color development was then carried out using Histofine Peroxidase Substrate Simple Stain DAB solution (Nichirei Biosciences). After completion of the color development, the reaction product was washed with deionized water for 5 minutes, and the nucleus was stained with Mayer's hematoxylin solution (Wako Pure Chemical Industries, Ltd.). Thereafter, dehydration was carried out with alcohol, followed by penetration with xylene and mounting in Entellan New (Merck Japan).

<Results>

Expression of hTROP-2 in human normal tissues

[0214] The expression pattern of hTROP-2 in human normal tissues was analyzed using the anti-hTROP-2 monoclonal antibody clone K5-63-17. A human normal tissue array (Catalog No.: AB1, Super Bio Chips) was deparaffinized, and was then subjected to a hydrophilic treatment. Thereafter, the antigen was activated with a protease, pepsin, and immunostaining was then carried out using the anti-hTROP-2 monoclonal antibody clone K5-63-17 (Figure 20). As a result, staining was observed in the skin, esophagus, kidney (cortex and medulla), pancreas, prostate, bladder and tonsil. A majority of stained images localized in the cell membrane (Figure 20A, B, C, D, F, G and H), but hTROP-2 expression was partially observed even in the cytoplasm (Figure 20E and H). On the other hand, such staining was not observed in the heart, liver, stomach, small intestine, large intestine, skeletal muscle, lung, spleen, thymus gland and the like (Figure 20I and J).

Expression of hTROP-2 in human cancer tissues

[0215] In order to examine the expression of hTROP-2 (hTROP-2-positive rate) in human cancer tissues, the cancer tissue arrays of various human cancer species were immunostained using the anti-hTROP-2 monoclonal antibody clone K5-63-17. A tissue section, in which 10% or more of cancer cells were stained, was defined as hTROP-2-positive. The staining results are shown in Table 4.

Table 4

Cancer tissues	number of TROP-2-positive cases / total number of cases	TROP-2-positive rate (%)
Breast cancer	**32/80**	**40**
Lung cancer	**53/81**	**65.4**
Esophagus cancer	**69/90**	**76.7**
Stomach cancer	**25/90**	**27.8**
Colon cancer	**29/178**	**16.3**
Pancreatic cancer	**26/62**	**41.9**
Liver cancer	**7/92**	**7.61**
Bladder cancer	**42/59**	**71.2**
Prostate cancer	**35/38**	**92.1**
Ovary cancer	**14/58**	**24.1**

[0216] Representative stained images are shown in Figure 21. Among cancer species, regarding which the expression of hTROP-2 had been analyzed, prostate cancer had the highest positive rate (92.1%), and also, lung cancer (65.4%), esophagus cancer (76.7%), bladder cancer (71.2%) and the like had high positive rates. Liver cancer had the lowest positive rate (7.61%). It was observed from stained images that, as with in normal cells, hTROP-2 was highly localized in the cell membrane even in the case of cancer cells (Figure 21A to F, H and I). In addition, hTROP-2 was also localized in the cytoplasm in some cases (Figure 21A, B, E and G).

[0217] The hTROP-2-positive rate in pancreatic cancer was 41.9%. The relationship between this hTROP-2-positive rate and the grade (degree of differentiation) of pancreatic cancer was analyzed. As a result, hTROP-2 was expressed at high frequency in pancreatic cancer with a high grade, namely, with a low degree of differentiation (Table 5).

Table 5

Pancreatic cancer hTROP-2-positive spots 26/62 (41.94%)

Grade	-	+	Positive rate	
I	8	0	0%	
I - II	5	0	0%	
II	19	21	52.5%	
II - III	4	5	55.6%	p<0.01
total	36	26		

[Example 21]

[Anti-tumor activity of K5-70 antibody by single administration on xenograft prevention models of human pancreatic cancer cell line PK-59]

[0218] Strong anti-tumor *activity in vivo* of a clone K5-70 (mouse IgG2a) was exhibited even by a single administration of K5-70 at a dosage of 10 mg/kg body weight to xenograft prevention models using a human pancreatic cancer cell line PK-59. In a control group (mouse IgG, 10 mg/kg body weight, N = 3), tumor formation was observed in all of the individuals, and the tumor volume on the 28th day after cell transplantation (Day 28) was 781.7 $\pm$ 74.5 mm^3. On the other hand, in a group in which the K5-70 antibody was administered only once on the day of transplantation of the cancer cells (Day 1) (10 mg/kg body weight, N = 3), the tumor volume on Day 28 was 144.4 $\pm$ 176.9 mm^3 ($P < 0.05$ by Student's t-test), showing tumor growth inhibitory activity of 81.5% (Figure 22A). With regard to tumor weight, the tumor weight of the control group on Day 28 was 0.59 $\pm$ 0.06 g. In contrast, the tumor weight of the clone K5-70 antibody administration group was 0.07 $\pm$ 0.10 g ($P < 0.01$ by Student's t-test), showing an inhibitory activity of 88% (Figure 22B). With regard to both tumor volume and tumor weight, tumor formation was completely inhibited in 2 out of 3 individuals in the K5-70 antibody administration group at a dosage of 10 mg/kg body weight per administration (Figure 22C).

[Example 22]

[Anti-tumor activity of anti-hTROP-2 monoclonal antibody on xenograft treatment models of human colon cancer cell line SW480]

[0219] The anti-tumor activity of each of anti-hTROP-2 monoclonal antibodies (clones K5-70, K5-116-2-1, and T6-16) was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 6-week-old female NOD-scid mouse (Day 1). When the mean tumor volume reached 100 mm^3, grouping was carried out (Day 7 or Day 10). From Day 7 or Day 10, intraperitoneal administration of the antibody was carried out at administration intervals of once every three days. The anti-tumor activity of the clone K5-70 antibody and the anti-tumor activities of the clone K5-116-2-1 antibody and the clone T6-16 antibody were evaluated by independent studies, separately. In the study of evaluating the anti-tumor activity of the K5-70 antibody, the tumor volume of a control group (mouse IgG (10 mg/kg body weight), N = 8) on the 44th day after cancer cell transplantation (Day 44) was 365.4 $\pm$ 214.6 mm^3. On the other hand, the tumor volume of a K5-70 antibody (10 mg/kg body weight) administration group was 27.4 $\pm$ 29.4 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited in the K5-70 administration group (inhibitory rate: 92.5%) (Figure 23A). With regard to tumor weight, the tumor weight of the control group was 0.11 $\pm$ 0.07 g, whereas the tumor weight of the K5-70 antibody administration group was 0.005 $\pm$ 0.007 (g) ($P < 0.01$ by Student's t-test), showing an inhibitory rate of 95.5% (Figure 23B). In particular, in two out of the eight individual mice in the K5-70 antibody administration group, tumor formation was completely inhibited, and the presence of tumor could not be confirmed.

[0220] In the study of evaluating the anti-tumor activities of the K5-116-2-1 antibody and the T6-16 antibody, which was carried out separately, the tumor volume of the control group on Day 42 was 713.8 $\pm$ 354.5 mm^3 (N = 8). In contrast, the tumor volume of the K5-116-2-1 antibody administration group (10 mg/kg body weight) was 188.9 $\pm$ 97.4 mm^3 (N = 8, $P < 0.01$ by Student's t-test) (Figure 24A), and the tumor volume of the T6-16 antibody administration group (10 mg/kg body weight) was 292.8 $\pm$ 199.7 mm^3 (N = 8, $P < 0.05$ by Student's t-test) (Figure 25A). Thus, the two above

administration groups showed inhibitory rates of 73.5% and 59.0%, respectively. With regard to tumor weight as well, the tumor weight of the control group was 0.39 ± 0.19 g. In contrast, the tumor weight of the K5-116-2-1 antibody administration group was 0.10 ± 0.07 g ($P < 0.01$ by Student's t-test), and the tumor weight of the T6-16 antibody administration group was 0.17 ± 0.14 g ($P < 0.05$ by Student's t-test). Thus, the two above administration groups showed inhibitory rates of 72.2% and 56.4%, respectively (Figure 24B and Figure 25B).

[Example 23]

[Dose-dependent anti-tumor activity of clone K5-70 on xenograft treatment models using human colon cancer cell line SW480]

[0221] Subsequently, the dose-dependent anti-tumor activity of the K5-70 antibody was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 6-week-old female NOD-scid mouse. Ten days after the transplantation (Day 10) at which the mean tumor volume reached 100 mm^3, the mice were divided into a control group (mouse IgG, 10 mg/kg body weight administration group, N = 8, 104.4 ± 17.6 mm^3), a K5-70 antibody (1 mg/kg body weight) administration group (N = 8, 104.3 ± 16.1 mm^3), a K5-70 antibody (5 mg/kg body weight) administration group (N = 8, 104.6 ± 15.9 mm^3), and a K5-70 antibody (10 mg/kg body weight) administration group (N = 8, 104.8 ± 14.9 mm^3). Then, intraperitoneal administration was carried out at administration intervals of once every three days. On Day 42, the tumor volume of the control group was 713.8 ± 354.5 mm^3. On the other hand, in the K5-70 antibody administration groups, dose-dependent tumor formation inhibitory activity was observed. That is, the tumor volume of the 1 mg/kg body weight administration group was 485.0 ± 207.3 mm^3 (inhibitory rate: 32.1%), the tumor volume of the 5 mg/kg body weight administration group was 339.5 ± 253.2 mm^3 (inhibitory rate: 52.4%), and the tumor volume of the 10 mg/kg body weight administration group was 355.4 ± 202.8 mm^3 (inhibitory rate: 50.2 %, $P < 0.05$ by Student's t-test) (Figure 26A). Likewise, with regard to tumor weight on Day 42, the tumor weight of the control group was 0.39 ± 0.19 g. On the other hand, the tumor weight of the K5-70 antibody (1 mg/kg body weight) administration group was 0.24 ± 0.11 g (inhibitory rate: 37.8%), the tumor weight of the 5 mg/kg body weight administration group was 0.17 ± 0.14 g (inhibitory rate: 55.8%, $P < 0.05$ by Student's t-test), and the tumor weight of the 10 mg/kg body weight administration group was 0.20 ± 0.13 g (inhibitory rate: 47.1 %). Thus, dose-dependent anti-tumor activity was confirmed (Figure 26B).

[Example 24]

[Analysis of administration intervals of K5-70 antibody to xenograft treatment models using human colon cancer cell line SW480]

[0222] Subsequently, in order to analyze optimal administration intervals of the K5-70 antibody, the anti-tumor activity of the clone K5-70 when it was administered once a week (once every 7 days) was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 6-week-old female NOD-scid mouse. Ten days after the transplantation (Day 10) at which the mean tumor volume reached 100 mm^3, the mice were divided into a control group (mouse IgG, 10 mg/kg body weight, N = 8, 104.42 ± 15.1 mm^3) and a K5-70 antibody administration group (10 mg/kg body weight, N = 8, 104.3 ± 16.1 mm^3). Then, intraperitoneal administration was carried out once every 7 days. On Day 42, the tumor volume of the control group was 713.8 ± 354.5 mm^3, whereas the tumor volume of the K5-70 antibody administration group (administered once a week) was 332.3 ± 239.9 mm^3 (inhibitory rate: 55%, $P < 0.05$ by Student's t-test) (Figure 27A). Moreover, when the administration interval was increased to once every 10 days and to once every two weeks, the tumor volume of the control group on Day 39 was 956.9 ± 367.8 mm^3. On the other hand, the tumor volume of the K5-70 antibody administration group (administered once every 10 days) on Day 39 was 525.4 ± 180.6 mm^3 (inhibitory rate: 45.1%, $P < 0.01$ by Student's t-test), and the tumor volume of the K5-70 antibody administration group (administered once every 2 weeks) was 459.4 ± 217.6 mm^3 (inhibitory rate: 52.0%, $P < 0.01$ by Student's t-test) (Figure 27B). In the prior arts (US 7420040 and US 7420041), when antibodies were administered to xenograft treatment models using a pancreatic cancer cell line (BxPC-3) at a dosage of 20 mg/kg body weight, three times a week (at administration intervals of 2 days), the antibodies exhibited anti-tumor activity at an inhibitory rate of 50% to 60%. In contrast, the K5-70 antibody exhibited anti-tumor activity equivalent to those of the prior arts, at a dosage of half of the prior arts (10 mg/kg body weight), once every 2 weeks (at administration intervals of 13 days). Accordingly, it became clear that that the K5-70 antibody exhibited significant anti-tumor activity at a total dosage of at least one twelfth of those of the prior-arts.

[Example 25]

[Dose-dependent anti-tumor activity of T6-16 antibody on xenograft treatment models using human colon cancer cell line SW480]

[0223] Subsequently, the dose-dependent anti-tumor activity of the T6-16 antibody was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 6-week-old female NOD-scid mice. Ten days after the transplantation (Day 10) at which the mean tumor volume reached 100 mm^3, the mice were divided into a control group (mouse IgG, 10 mg/kg body weight, N = 8, 105.8 ± 9.9 mm^3), a T6-16 antibody administration group (1 mg/kg body weight, N = 8, 104.4 ± 13.3 mm^3), a T6-16 antibody administration group (5 mg/kg body weight, N = 8, 104.7 ± 13.0 mm^3), and a T6-16 antibody administration group (10 mg/kg body weight, N = 8, 104.8 ± 12.4 mm^3). Then, intraperitoneal administration was carried out at administration intervals of once every three days. On Day 43, the tumor volume of the control group was 473.5 ± 137.0 mm^3. On the other hand, in the T6-16 antibody administration groups, dose-dependent tumor formation inhibitory activity was observed. That is, the tumor volume of the 1 mg/kg body weight administration group was 397.9 ± 97.5 mm^3 (inhibitory rate: 16.0%), the tumor volume of the 5 mg/kg body weight administration group was 195.9 ± 89.7 mm^3 (inhibitory rate: 58.7 %, $P < 0.01$ by Student's t-test), and the tumor volume of the 10 mg/kg body weight administration group was 190.2 ± 56.5 mm^3 (inhibitory rate: 59.8 %, $P < 0.01$ by Student's t-test) (Figure 28A). Likewise, with regard to tumor weight on Day 43, the tumor weight of the control group was 0.19 ± 0.07 g. On the other hand, the tumor weight of the T6-16 antibody (1 mg/kg body weight) administration group was 0.20 ± 0.08 g, the tumor weight of the T6-16 antibody (5 mg/kg body weight) administration group was 0.08 ± 0.04 g (inhibitory rate: 57.9%, $P < 0.01$ by Student's t-test), and the tumor weight of the T6-16 antibody (10 mg/kg body weight) administration group was 0.09 ± 0.04 g (inhibitory rate: 52.6 %, $P < 0.01$ by Student's t-test). Thus, dose-dependent anti-tumor activity was confirmed (Figure 28B).

[Example 26]

[Analysis of administration intervals of T6-16 antibody to xenograft treatment models using human colon cancer cell line SW480]

[0224] Subsequently, in order to analyze optimal administration intervals of the T6-16 antibody, the anti-tumor activity of the clone T6-16 when it was administered at administration intervals of once a week (once every 7 days) and once every 10 days was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 6-week-old female NOD-scid mouse. Ten days after the transplantation (Day 10) at which the mean tumor volume reached 100 mm^3, the mice were divided into a control group (mouse IgG, 10 mg/kg body weight, N = 8, 105.8 ± 9.9 mm^3), a T6-16 antibody (once a week) administration group (10 mg/kg body weight, N = 8, 105.0 ± 11.6 mm^3), and a T6-16 antibody (once every 10 days) administration group (10 mg/kg body weight, N = 5, 130.8 ± 2.4 mm^3). Then, administration was initiated. On Day 43, the tumor volume of the control group was 473.5 ± 137.0 mm^3. On the other hand, the tumor volume of the T6-16 antibody (once a week) administration group was 243.7 ± 65.3 mm^3 (inhibitory rate: 48.5%, $P < 0.01$ by Student's t-test), and the tumor volume of the T6-16 antibody (once every 10 days) administration group was 297.8 ± 54.4 mm^3 (inhibitory rate: 37.1%, $P < 0.05$ by Student's t-test) (Figure 29). In the prior arts (US 7420040 and US 7420041), when antibodies were administered to xenograft treatment models using a pancreatic cancer cell line (BxPC-3) at a dosage of 20 mg/kg body weight, three times a week (at administration intervals of 2 days), the antibodies exhibited anti-tumor activity at an inhibitory rate of 50% to 60%. In contrast, the T6-16 antibody exhibited significant anti-tumor activity, when it was administered at a dosage of half of the prior arts and at a frequency of once every 10 day (at administration intervals of 9 days). Accordingly, it became clear that that the T6-16 antibody exhibited significant anti-tumor activity at a total dosage of at least one eighth of those of the prior-arts.

[Example 27]

[Analysis of anti-tumor activity of clone K5-70 on xenograft prevention models using human prostate cancer cell line DU-145]

[0225] The anti-tumor activity of clone K5-70 on human prostate cancer was evaluated with xenograft prevention models using DU-145 cells (RIKEN Cell Bank, RCB2143). DU-145 cells (5×10^6 cells) were subcutaneously transplanted into each of 6-week-old female nude mouse (Balb/c, nu/nu). The day on which the transplantation was carried out was defined as Day 1. The mice were divided into a control group (mouse IgG) (N = 8) and a K5-70 antibody administration

group (N = 8). From Day 1, the K-70 antibody and the control antibody were intraperitoneally administered to the mice at a frequency of once every 3 days at a dosage of 10 mg/kg body weight. On Day 40, the tumor volume of the control group was 368.2 ± 307.8 mm^3. On the other hand, the tumor volume of the K5-70 antibody administration group was 30.6 ± 29.6 mm^3 ($P < 0.05$ by Student's t-test), showing a tumor formation inhibitory activity of approximately 90% (Figure 30A). With regard to tumor weight, further significant anti-tumor activity was observed. The tumor weight of the control group on Day 40 was 0.18 ± 0.18 g. In contrast, in the K5-70 antibody administration group, tumors disappeared from all of the 8 individual mice, and thus, tumor formation was completely inhibited (Figure 30B). From the above-mentioned results, it became clear that the anti-human TROP-2 monoclonal antibody clone K5-70 shows strong anti-tumor activity even on human prostate cancer cells.

[Example 28]

[Metastasis-inhibitory activity of K5-70 antibody on liver metastasis models using human pancreatic cancer cell line PK-59]

[0226] Cancer metastasis is an important factor that influences clinical prognosis in the treatment of gastrointestinal cancer. The control of metastasis is therapeutically significantly important. If not only tumor formation but also the metastasis of cancer to other organs could be suppressed by administering an antibody for use in cancer therapy that targets to TROP-2, high clinical usefulness would be anticipated. Thus, this is a desired property as a cancer therapeutic antibody.

[0227] The expression of TROP-2 was confirmed in many types of carcinomas. It was reported that TROP-2 was expressed at a high level particularly in metastatic foci (Br. J. Cancer (2008); 99: 1290-1295, Clin. Cancer Res. (2006); 12: 3057-3063, Mod. Pathol. (2008); 21: 186-191). Moreover, it was also reported that, when Trop-2-gene-introduced cancer cells were transplanted into nude mice via transsplenic or transpancreastic administration, the incidence of liver metastasis increased (WO 2010/089782, Molecular Cancer (2010); 9: 253), and thus, the report suggested the importance of TROP-2 in the cancer metastasis process. However, to date, there have been no reports specifically describing that an antibody that targets TROP-2 has metastasis-inhibitory action *in vivo.*

[0228] The anti-hTROP-2 mouse monoclonal antibody K5-70, which was discovered by the present invention, exhibits high therapeutic effects on xenograft models, into the subcutis of which human pancreatic cancer cells had been transplanted. It was demonstrated by a scratch assay performed *in vitro* that the antibody K5-70 is able to suppress the migration ability of a human pancreatic cancer cell line PK-59, in addition to the effect of suppressing the growth of cancer cells. Thus, it was considered that the antibody K5-70 could inhibit cancer metastasis *in vivo.* Hence, the metastasis-inhibitory effect of an anti-hTROP-2 mouse monoclonal antibody was examined, using models in which the PK-59 cells were injected into the spleen of nude mice so that liver metastasis was developed.

[0229] On the day before cancer cell transplantation, the mice were divided into groups. Then, an anti-hTROP-2 monoclonal antibody (K5-70) or a control antibody (purified mouse IgG) was intraperitoneally administered to the mice at a dosage of 10 mg/kg body weight. On the following day, a human pancreatic cancer cell line (PK-59) endogenously expressing hTROP-2 was harvested by treatment with trypsin, and a 2×10^7 cells/mL cell suspension was then prepared with PBS. The cell suspension was preserved on ice until transplantation. Each of 6- or 7-week-old female nude mouse (Balb/c, nu/nu) was anesthetized by intraperitoneal administration of pentobarbital, and 10 to 15 mm of the left flank thereof was excised under anesthesia. The spleen was taken out of the abdominal cavity, and 50 μL of the cell suspension (1×10^6 cells) was then injected into the spleen using a 27G syringe. Four minutes after injection of the cells, the hilum of spleen was ligated with 5-0 silk sutures, and the spleen was then excised. The cut peritoneum was closed with 4-0 silk sutures, and the surgical site was then closed with Wound Clips (AUTOCLIP 9mm, Becton Dickinson). In addition, seven days after the cancer cell transplantation, the K-70 antibody and the control antibody were administered to the mice at a dosage of 10 mg/kg body weight. Four to six weeks after the cancer cell transplantation, the mice were subjected to euthanasia by cervical dislocation. Then, the liver was excised from each mouse, and the presence or absence of metastatic foci was confirmed.

[0230] In the control group in which the mouse IgG was administered to the mice, in 4 out of the 6 mice into which PK-59 cells had been transplanted, apparent metastatic foci (2 to 7 foci) were observed around the liver lobe four to six weeks after the transplantation (Figure 31A, incidence of metastasis: 67%, Table 6). In contrast, in four mice in the K5-70 antibody administration group, into which the PK-59 cells had also been transplanted, such metastatic foci were not observed in the liver of all of the mice, and thus, an incidence of metastasis was 0% (Figure 31B, Table 6).

Table 6

Metastasis-suppressing effect of clone K5-70 on liver metastasis models produced by transsplenic transplantation of PK-59 cells into nude mice

Administration group	Weeks after transplantation	Individual No.	Number of metastatic foci	Determination of metastasis
Control group	4W	C-1	0	-
	4W	C-2	5	++
	6W	C-3	7	++
	6W	C-4	7	++
	6W	C-5	2	+
	6W	C-6	0	-
		Average number of metastatic foci	3.5	Incidence of metastasis 67%
K5-70 administration group	4W	K-1	0	-
	6W	K-2	0	-
	6W	K-3	0	-
	6W	K-4	0	-
		Average number of metastatic foci	0	Incidence of metastasis 0%

[0231] From these results, it became clear that the anti-hTROP-2 antibody K5-70 has extremely strong inhibitory action on the liver metastasis of the human pancreatic cancer cell line PK-59.

[Example 29]

[Anti-tumor activity of K5-70 antibody on xenograft models using human colon cancer cell line SW480, which are recurrent cancer models after administration of irinotecan hydrochloride]

[0232] In recent years, many chemotherapeutical drugs for suppressing the growth of cancer cells have been developed as cancer therapeutic drugs. These drugs have achieved certain treatment results. However, these chemotherapeutical drugs have been problematic in terms of side effects associated with the growth suppressive action thereof on normal cells other than cancer cells and the recurrence of cancer after suspension of the treatment. Accordingly, if tumor recurrence after completion of the treatment with chemotherapeutical drugs could be suppressed by administration of a cancer therapeutic antibody targeting to TROP-2, high clinical usefulness would be anticipated. Thus, this is a desired property as a cancer therapeutic antibody.

[0233] As therapeutic agents for colorectal cancer, in addition to 5-FU and platinum-containing drugs, irinotecan hydrochloride (Topotecin, Daiichi Sankyo Co., Ltd.) having a topoisomerase inhibitory effect has been recently applied to clinical sites. With regard to animal models as well, the anti-tumor effect of irinotecan hydrochloride on mouse models, into which various types of human tumor cells including colon cancer as a typical example had been transplanted, has been reported (Cancer Chemother Pharmacol. (1991); 28(3): 192-8). Thus, the recurrence-preventing effect of the anti-hTROP-2 antibody clone K5-70 (mouse IgG2a) on recurrent tumor after administration of irinotecan hydrochloride has been examined with xenograft models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of 8-week-old female NOD-scid mice. Eleven days after the transplantation (Day 11) at which the mean tumor volume reached 100 mm^3, the mice were divided into a non-treat group (normal saline administration group, N = 8, 130.7 $\pm$ 16.2 mm^3) and an irinotecan hydrochloride (CPT-11, Topotecin, Daiichi Sankyo Co., Ltd.) administration group (N = 16, 123.0 $\pm$ 21.4 mm^3). Thereafter, irinotecan hydrochloride was intraperitoneally administered to the mice at a dosage of 40 mg/kg body weight, once every 3 days, total 3 times (Days 11, 14, and 17). On the third day after the final administration of irinotecan hydrochloride (Day 20), the tumor volume of the non-treat group reached 232.1 $\pm$ 21.1 mm^3. On the other hand, the tumor volume of the irinotecan hydrochloride administration

group was 126.6 ± 26.6 mm^3 ($P < 0.01$ by Student's t-test), and thus, an apparent tumor-suppressing effect was observed. At this stage, the irinotecan hydrochloride administration group was divided into two groups based on tumor size. One group was defined as a K5-70 antibody administration group (10 mg/kg body weight, N = 8, tumor volume on Day 20: 126.0 ± 28.0 mm^3), and the other group was defined as a mouse IgG administration group (10mg/kg body weight, N = 8, tumor volume on Day 20: 127.2 ± 27.0 mm^3). Intraperitoneal administration of the antibodies and the measurement of a tumor volume were carried out on each group once every 3 days, so that the recurrence of a tumor was evaluated (Figure 32). In the mouse IgG administration group, from the 18th day after the final administration of irinotecan hydrochloride (Day 35), several mice having an apparent recurrent tumor with a tumor volume of greater than 300 mm^3 were observed. On the 30th day after the final administration of irinotecan hydrochloride (Day 47), a tumor with a tumor volume of greater than 300 mm^3 was observed in 5 out of the 8 mice (mean tumor volume: 401.7 ± 172.7 mm^3). In contrast, in the K5-70 antibody administration group, tumor recurrence was significantly suppressed, and the mean tumor volume was 180.5 ± 142.1 mm^3 ($P < 0.05$ by Student's t-test) (Figure 32). In particular, in the K5-70 antibody administration group, the tumor volume on Day 47 became smaller than the tumor volume when the mice were divided into groups (126.0 ± 28.0 mm^3). The tumor volume became less than 100 mm^3 in 4 out of the 8 mice. From these results, it became clear that the anti-hTROP-2 antibody K5-70 has extremely strong suppressive action even on recurrent tumor after administration of irinotecan hydrochloride.

[Example 30]

[Epitope mapping using CLIPS technology]

<Materials and methods>

Peptide synthesis

[0234] 15-mer and 30-mer of linear peptides derived from TROP-2 extracellular domains, which were used in the present experiment, were obtained by solid-phase synthesis according to a Fmoc (9-Fluorenylmethoxycarbonyl) method. In addition, for discontinuous epitope analysis, a 17-mer peptides derived from a TROP-2 extracellular domain, to both ends of which cysteine residues had been added, was synthesized, and a conformation having one or two loop structures was reconstructed by CLIPS technology (Chemically Linked Peptides on Scaffolds technology). When another cysteine residue was present close to the added cysteine residue, it was substituted with alanine.

Epitope screening ELISA

[0235] 5034 types of the synthesized peptides were covalently bound to PEPSCAN cards (455 peptides/card), and the binding of the synthesized peptides to antibodies was then analyzed by the ELISA method. The PEPSCAN cards were allowed to react with anti-human TROP-2 monoclonal antibodies (K5-70, K5-107, K5-116-2-1, T5-86, and T6-16) that had been diluted to a concentration of 1 μg/mL with a blocking buffer (a phosphate buffer containing 4% horse serum, 5% ovalbumin, and 1% Tween). After washing, the resultant was allowed to react with a 1000-fold diluted peroxidase-secondary antibody complex at 25°C for 1 hour. After washing, a substrate solution (a solution containing 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 μL of 3% hydrogen peroxide solution) was added to the reaction solution, followed by a chromogenic reaction for 1 hour. The binding activity of the antibodies was quantified by photographing with a CCD camera and then performing an image analysis.

<Results>

[0236] The anti-hTROP-2 monoclonal antibodies K5-70, K5-107, K5-116-2-1, T5-86 and T6-16, which exhibited beneficial effects, were subjected to epitope analysis using CLIPS (Chemically Linked Peptides on Scaffolds) technology. It is to be noted that the term "amino acid number" is used in the present examples to mean the amino acid number in the amino acid sequence shown in SEQ ID NO: 2 (hTROP-2 protein (323 amino acid residues)).

[0237] The result of analysis for the K5-70 antibody is shown in Table 7 below. As a result, it was found that 33 peptides exhibit strong binding activity to the K5-70 antibody. In these 33 peptides, a sequence comprising VCSPDGPGGRCQCRALGSGMAVD (amino acid numbers 43-65) (peptide Nos. 1-7 and 9 shown in Table 7), a sequence comprising HHILIDLRHRPTAG (amino acid numbers 152-165) (peptide Nos. 14, 22-24 and 28 shown in Table 7), a sequence comprising VHYEQPTIQIELRQ (amino acid numbers 193-206) (peptide Nos. 8, 10, 12, 13, 18, 20, 21, 23, 26, 28, 30 and 32 shown in Table 7), and a sequence comprising DLDAELRRLFRER (amino acid numbers 171-183) (peptide Nos. 11, 16, 18, 19, 21, 22, 29 and 31 shown in Table 7) appeared repeatedly. The K5-70 antibody particularly strongly bound to the sequence comprising VCSPDGPGGRCQCRALGSGMAVD. From these results, it was suggested that, in the

hTROP-2 protein, the aforementioned 4 types of peptide sequence regions are likely to be epitopes of the K5-70 antibody.

Table 7

Binding of K5-70 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of K5-70
1	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2742
2	TVCSPDGPGGRCQCRALGSGMAVDCSTLTS	2604
3	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2562
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2402
5	KMTVCSPDGPGGRCQCRALGSGMAVDCSTL	1770
6	PTNNKMTVCSPDGPGGRCQCRALGSGMAVDC	1391
7	VCSPDGPGGRCQCRALGSGMAVDCSTLTSK	932
8	CAAVHYEQPTIQIELRCAAVHYEQPTIQIELRC	876
9	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	839
10	CVHYEQPTIQIELRQNCVHYEQPTIQIELRQNC	825
11	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	725
12	RLFRERYRLHPKFVAAVHYEQPTIQIELRQ	687
13	AVHYEQPTIQIELRQ	642
14	CAGAFNHSDLDAELRRCHHILIDLRHRPTAGAC	624
15	CPKFVAAVHYEQPTIQCGLDLRVRGEPLQVERC	579
16	CHSDLDAELRRLFRERCGLDLRV	538
17	FQGRGGLDLRVRGEP	538
18	CVHYEQPTIQIELRQNCDLDAELRRLFRERYRC	524
19	CHSDLDAELRRLFRERCRGEPLQ	519
20	CTIQIELRQNTSQKAACVHYEQPTIQIELRQNC	513
21	CVHYEQPTIQIELRQNCHSDLDAELRRLFRERC	511
22	CHHILIDLRHRPTAGACHSDLDAELRRLFRERC	489
23	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	489
24	CHHILIDLRHRPTAGACGLDLRVRGEPLQVERC	488
25	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	483
26	CVHYEQPTIQIELRQNC	483
27	CAFNHSDLDAELRRLFCVHYEQPTIQIELRQNC	478
28	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	473
29	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	472
30	VHYEQPTIQIELRQNCGLDLRVRGEPLQVERC	470
31	CDELVRTHHILIDLRHCDLDAELRRLFRERC	469
32	AVHYEQPTIQIELRQCAVHYEQPTIQIELRQC	468
33	CHSDLDAELRRLFRERCDELVRTHHILIDLRHC	466

[0238] The result of analysis for the K5-107 antibody is shown in Table 8 below. As a result, it was found that a sequence comprising VCSPDGPGGRCQCRALGSGMAVD (amino acid numbers 43-65) was comprised in 10 out of the 20 peptides (peptide Nos. 1-6, 8, 9, 14 and 17 shown in Table 8) (Table 8).

[0239] Accordingly, it was suggested that, in the hTROP-2 protein, the aforementioned peptide sequence region consisting of VCSPDGPGGRCQCRALGSGMAVD may be an epitope of the K5-107 antibody.

Table 8

Binding of KS-107 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of K5-107
1	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2763
2	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2761
3	KMTVCSPDGPGGRCQCRALGSGMAVDCSTL	2752
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2726

(continued)

Binding of KS-107 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of K5-107
5	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	2723
6	TVCSPDGPGGRCQCRALGSGMAVDCSTLTS	2720
7	TCPTNKMTVCSPDGPGGRCQCRALGSGMAV	2716
8	VCSPDGPGGRCQCRALGSGMAVDCSTLTSK	2689
9	CSPDGPGGRCQCRALGSGMAVDCSTLTSKC	2655
10	CTCPTNKMTVCSPDGPGGRCQCRALGSGMA	2655
11	NCTCPTNKMTVCSPDGPGGRCQCRALGSGM	2207
12	DNCTCPTNKMTVCSPDGPGGRCQCRALGSG	1816
13	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	1525
14	CTVCSPDGPGGRCQCRALGSGMAVDASTLTSKC	1118
15	QDNCTCPTNKMTVCSPDGPGGRCQCRALGS	874
16	SPDGPGGRCQCRALGSGMAVDCSTLTSKCL	561
17	CTNKMTVCSPDGPGGRCQCRALGSGMAVDASTC	380
18	TVCSPDGPGGRCQCR	312
19	CAPKNARTLVRPSEHACARTLVRPSEHALVDNC	284
20	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	272

[0240] The result of analysis for the K5-116-2-1 antibody is shown in Table 9 below. In this analysis, three types of peptide sequences, namely, a sequence comprising VCSPDGPGGRCQCRALGSGMAVD (amino acid numbers 43-65) (peptide Nos. 1-7, 15 and 25 shown in Table 9), a sequence comprising HHILIDLRHRPTAG (amino acid numbers 152-165) (peptide Nos. 8-11, 16, 17, 19, 20, 22-24, and 27-29 shown in Table 9), and a sequence comprising DLDAE-LRRLFRER (amino acid numbers 171-183) (peptide Nos. 11-14, 17, 19, 21, 23 and 29 shown in Table 9) appeared several times (Table 9). Accordingly, it was suggested that, in the hTROP-2 protein, these three types of peptide sequence regions may be epitopes of the K5-116-2-1 antibody.

Table 9

Binding of K5-116-2-1 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of K5-116-2-1
1	TVCSPDGPGGRCQCRALGSGMAVDCSTLTS	2672
2	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2613
3	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2482
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2440
5	KMTVCSPDGPGGRCQCRALGSG MAVDCSTL	2423
6	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	2136
7	PTNKMTVCSPDGPGGRCQCRALGSGMAVDC	1723
8	CAGAFNHSDLDAELRRCHHILIDLRHRPTAGAC	1643
9	CTHHILIDLRHRPTAGC	1586
10	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	1504
11	CHHILIDLRHRPTAGCHSDLDAELRRLFRERC	1475
12	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	1467
13	CDAELRRLFRERYRLHCHSDLDAELRRLFRERC	1462
14	CDAELRRLFRERYRLHCPK	1442
15	VCSPDGPGGRCQCRALGSGMAVDCSTLTSK	1432
16	DLSLRCDELVRTHHILIDLRHRPTAGAFNH	1421
17	CDELVRTHHILIDLRHCDLDAELRRLFRERYRC	1392
18	CFQGRGGLDLRVRGEPC	1376
19	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	1366
20	CGLDLRVRGEPLQVERCHHILIDLRHRPTAGAC	1342
21	CHSDLDAELRRLFRERCHSDLDAELRRLFRERC	1331

(continued)

Binding of K5-116-2-1 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of K5-116-2-1
22	CDELVRTHHILIDLRHCHHILIDLRHRPTAGAC	1323
23	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	1266
24	CHHILIDLRHRPTAGACRGEPLQVERTLIYYLC	1229
25	CSPDGPGGRCQCRAL	1227
26	CTVASPDGPGGRAQARACVHYEQPTIQIELRQNC	1223
27	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	1222
28	LSLRCDELVRTHHILIDLRHRPTAGAFNHS	1220
29	CDELVRTHHILIDLRHCHSDLDAELRRLFRERC	1205

[0241] The results of analysis for the T5-86 and T6-16 antibodies are shown in Table 10 and Table 11 below, respectively. In these analyses, the antibodies strongly bound to a peptide comprising a sequence consisting of DPEGRFKARQCN (amino acid numbers 109-120). The above-mentioned peptide sequence was comprised in 22 out of the 26 peptides binding to the T5-86 antibody (peptide Nos. 1-4, 6-8, 10-13, 15-19, and 21-26 shown in Table 10), and it was comprised in 4 out of the 26 peptides binding to the T6-16 antibody (peptde Nos. 1, 2, 9 and 13 shown in Table 11) (Table 10 and Table 11). Moreover, in the analysis regarding the T5-86 antibody, other than the sequence comprising DPEGRFKARQCN (amino acid numbers 109-120), a sequence comprising VCSPDGPGGRCQCRA (amino acid numbers 43-57) (peptide Nos. 5, 14 and 20 shown in Table 10) appeared several times. Furthermore, in the analysis regarding the T6-16 antibody as well, another sequence comprising HHILIDLRHRPTAG (amino acid numbers 152-165) (peptide Nos. No. 4-8, 10-12, 19, 21, 23, 25 and 26 shown in Table 11) was found several times. Accordingly, it was suggested that, in the hTROP-2 protein, two types of peptide sequence regions, namely, DPEGRFKARQCN (amino acid numbers 109-120) and VCSPDGPGGRCQCRA (amino acid numbers 43-57), may be epitopes of the K5-86 antibody. It was also suggested that, in the hTROP-2 protein, two types of peptide sequence regions, namely, DPEGRFKARQCN (amino acid numbers 109-120) and HHILIDLRHRPTAG (amino acid numbers 152-165), may be epitopes of the T6-16 antibody.

Table 10

Binding of T5-86 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of T5-86
1	CYDPDADPEGRFKARQCADPEGRFKARQANQTC	2306
2	PDCDPEGRFKARQCN	2292
3	CADPEGRFKARQANCPDADPEGRFKARQANC	2287
4	VCSPDGPGGRCQCRA	2263
5	CYDPDADPEGRFKARQCPDADPEGRFKARQANC	2260
6	CADPEGRFKARQANQTCTDPDADPEGRFKARQC	2240
7	CADPEGRFKARQANQTCYDPDADPEGRFKARQC	2208
8	DCDPEGRFKARQCNQ	2150
9	CTVASPDGPGGRAQARCHSDLDAELRRLFRERC	2086
10	CDADPEGRFKARQANQCDADPEGRFKARQANQC	2035
11	DGRFKARQANQTSVAWCARTLVRPSEHALVDNC	2019
12	DADPEGRFKARQANQTCPDADPEGRFKARQANC	1980
13	CPDADPEGRFKARQANCPDADPEGRFKARQANC	1950
14	CSPDGPGGRCQCRAL	1946
15	CEGRFKARQANQTSVACEGRFKARQANQTSVAC	1895
16	CTVASPDGPGGRAQARCPDADPEGRFKARQANC	1890
17	CGLYDPDADPEGRFKACPDADPEGRFKARQANC	1857
18	DPDCDPEGRFKARQCNCQTSVCWCVNSVGVR	1850
19	CPEGRFKARQANQTSVCDELVRHHILIDLRHC	1841
20	CPDGPGGRAQARALGSCHSDLDAELRRLFRERC	1830
21	CTLVRPSEHALVDNDGCGRFKARQANQTSVAWC	1820
22	CPDADPEGRFKARQANCYDPDADPEGRFKARQC	1795

(continued)

Binding of T5-86 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of T5-86
23	CGLYDPDADPEGRFKACPEGRFKARQANQTSVC	1793
24	YDPDCDPEGRFKARQ	1775
25	CPDADPEGRFKARQANCADPEGRFKARQANQTC	1773
26	CDPEGRFKARQCNQT	1772

Table 11

Binding of T6-16 antibody to CLIPS peptides derived from human TROP-2 extracellular domains

number	peptide	binding of T6-16
1	CVNSVGVRRTDKGDLSCPDCYDPDADPEGRFKARQC	1072
2	CSVGVRRTDKGDLSLRCYDPDADPEGRFKARQC	786
3	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	714
4	CDELVRTHHILIDLRHCDLDAELRRLFRERYRC	713
5	CVNSVGVRRTDKGDLSLRCDELVRTHHILI	688
6	VRRTDKGDLSLRCDELVRTHHILIDLRHRP	670
7	CVERTLIVYLDEIPPKCHHILIDLRHRPTAGAC	626
8	CHHILIDLRHRPTAGACHSDLDAELRRLFRERC	620
9	CVNSVGVRRTDKGDLSCPDADPEGRFKARQANC	611
10	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	602
11	VGVRRTDKGDLSLRCDELVRTHHILIDLRH	601
12	CAGAFNHSDLDAELRRCHHILIDLRHRPTAGAC	592
13	CSVGVRRTDKGDLSLRCPDADPEGRFKARQANC	585
14	CVRPSEHALVDNDGLYCSVGVRRTDKGDLSLRC	573
15	CDAELRRLFRERYRLHCHSDLDAELRRLFRERC	566
16	CSVGVRRTDKGDLSLRCNDGLYDPDADPEGRFC	559
17	CVNSVGVRRTDKGDLSCGLYDPDADPEGRFKAC	553
18	CDLDAELRRLFRERYRCHSDLDAELRRLFRERC	534
19	CDELVRTHHILIDLRHCHHILIDLRHRPTAGAC	534
20	CAGAFNHSDLDAELRRCDLDAELRRLFRERYRC	529
21	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	527
22	CVHYEQPTIQIELRQNCDLDAELRRLFRERYRC	526
23	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	524
24	CGVRRTDKGDLSLRADCGVRRTDKGDLSLRADC	524
25	CGLDLRVRGEPLQVERCHHILIDLRHRPTAGAC	521
26	CDLDAELRRLFRERYRCDELVRTHHILIDLRHC	516

[Example 31]

[Sequencing of variable regions of antibody genes of mouse anti-hTROP-2 antibodies (clones K5-70, K5-107, K5-116-2-1 and T6-16)]

[0242] Total RNA was extracted from 3×10^6 mouse anti-TROP-2 monoclonal antibody-producing hybridomas, using TRIzol reagent (Invitrogen). With regard to the clone K5-70, clone K5-107 and clone K5-116-2-1, cDNA was synthesized employing SMARTer™ RACE cDNA Amplification kit (Clontech) according to the method included with the kit, using a mouse IgG H chain-specific primer (5'-TCCAKAGTTCCA-3' (SEQ ID NO: 24)) and a mouse IgG L chain-specific primer (5'-GCTGTCCTGATC-3' (SEQ ID NO: 25)). With regard to the clone T6-16, cDNA was synthesized employing GeneRacer kit (Invitrogen) according to the method included with the kit, using an oligo dT primer. Genes encoding the variable regions (VH, VL) of the H and L chains of clone K5-70 (mouse IgG2a), clone K5-107 (mouse IgG1) and clone K5-116-2-1 (mouse IgG2a) were each cloned by a PCR method using the above-synthesized cDNA as a template. In this operation,

10 × Universal Primer A Mix (UPM) included with SMARTer™ RACE cDNA Amplification kit was used as a 5'-primer. On the other hand, as a 3'-primer for VH amplification, a primer having a sequence specific to the mouse IgG H chain was used, and as a 3'-primer for VL amplification, a primer having a sequence specific to the mouse IgG L chain was used.

5'-Primer (10 × Universal Primer A Mix (UPM)):

Long (0.4 μM)

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' (SEQ ID NO: 26)

Short (2 μM)
5'-CTAATACGACTCACTATAGGGC-3' (SEQ ID NO: 27)

3'-Primer (R primer):

VH: 5'-GGGAARTARCCCTTGACCAGGCA-3' (SEQ ID NO: 28) 5'-GGGAARTAGCCTTTGACAAGGCA-3' (SEQ ID NO: 29)
VL: 5'-CACTGCCATCAATVCTCCACTTGACA-3' (SEQ ID NO: 30)

[0243] Using each of the above-described primers, PCR was carried out under the following composition of reaction solution and reaction conditions. In addition, a R primer for amplification of VH cDNA was prepared by mixing the two above sequences with each other at an equimolar ratio and was then used.

<Composition of reaction solution>

[0244]

Template cDNA:	2.5 μL
5 × PrimeSTAR buffer (Mg^{2+} plus):	10 μL
2.5 mM dNTP:	4 μL
PrimeSTAR HS DNA polymerase (2.5 U/μL):	0.5 μL
10 × Universal Primer A Mix (UPM):	5 μL
R primer (10 μM):	1 μL
Sterilized water:	27 μL
Total:	50 μL

<Reaction conditions>

[0245] A reaction was carried out at 94°C (10 sec), and thereafter, a cycle consisting of "heat denaturation/dissociation at 98°C (10 sec) → annealing at 60°C (5 sec) → synthesis/elongation at 72°C (60 sec)" was carried out 30 times in total. Finally, a reaction was carried out at 72°C (3 min).

[0246] The synthesized VH and VL cDNAs were subcloned into a pMD20-T vector (Takara Bio Inc.), and the nucleotide sequences thereof were determined. The nucleotide sequences of a plurality of VH clones and VL clones were decoded, and nucleotide sequences specific to the variable regions of mouse H chain and L chain were identified. Figure 33 and Figure 34 show the consensus cDNA nucleotide sequences of the VH and VL of K5-70, and putative amino acid sequences. Figure 35 and Figure 36 show the consensus cDNA nucleotide sequences of the VH and VL of K5-107, and putative amino acid sequences. Figure 37 and Figure 38 show the consensus cDNA nucleotide sequences of the VH and VL of K5-116-2-1, and putative amino acid sequences.

[0247] Genes encoding the variable regions (VH, VL) of the H and L chains of clone T6-16 were cloned by a PCR method using the above-synthesized cDNA as a template. In this operation, a primer included with GeneRacer kit was used as a 5'-primer. On the other hand, as a 3'-primer for VH amplification, a primer having a sequence specific to the mouse IgG H chain was used, and as a 3'-primer for VL amplification, a primer having a sequence specific to the mouse IgG L chain was used.

5'-Primer (F primer):

5'-CGACTGGAGCACGAGGACACTGA-3' (SEQ ID NO: 31) 3'-Primer (R primer):

VH: 5'- GCCAGTGGATAGACAGATGG-3' (SEQ ID NO: 32)
VL: 5'- GATGGATACAGTTGGTGCAGC-3' (SEQ ID NO: 33)

[0248] Using each of the above-described primers, PCR was carried out under the following composition of reaction solution and reaction conditions.

<Composition of reaction solution>

[0249]

Template cDNA:	1.0 μL
5 × PrimeSTAR buffer (Mg^{2+} plus):	10 μL
2.5 mM dNTP:	4 μL
PrimeSTAR HS DNA polymerase (2.5 U/μL):	0.5 μL
F primer (10 μM):	3 μL
R primer (10 μM):	1.0 μL
Sterilized water:	30.5 μL
Total:	50 μL

<Reaction conditions>

[0250] A cycle consisting of "heat denaturation/dissociation at 98°C (10 sec) → annealing at 57°C (10 sec) → synthesis/elongation at 72°C (60 sec)" was carried out 35 times in total.

[0251] The synthesized VH and VL cDNAs were subcloned into a pCR4Blunt-TOPO vector (Invitrogen), and the nucleotide sequences thereof were determined. The nucleotide sequences of a plurality of VH clones and VL clones were decoded, and nucleotide sequences specific to the variable regions of mouse H chain and L chain were identified. Figure 39 and Figure 40 show the consensus cDNA nucleotide sequences of the VH and VL of T6-16, and putative amino acid sequences.

[Example 32]

[Design of humanized K5-70 antibody]

[0252] Humanization of the variable regions (VH, VL) of the K5-70 antibody prepared in the previous Examples was carried out as follows according to the method of Queen et al. (Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989). First, the molecular modeling of the three-dimentional structure of each K5-70 antibody variable region was carried out using a computer. Subsequently, homology search was performed with the variable region sequences of human antibody genes. As a result, a cDNA sequence (DA980192 VH) with GenBank accession number of DA980102 was selected as an acceptor that provides a framework region (FR) necessary for humanization of the K5-70 VH (Genome Res. 16:55-65, 2006). Likewise, a cDNA sequence (L41174 VL) with GenBank accession number of L41174 was selected as an acceptor that provides a framework region (FR) necessary for humanization of the K5-70 VL (J. Biol. Chem. 270:12457-12465, 1995).

[0253] For humanization of the K5-70 VH, the CDR sequence of the K5-70 VH was substituted with the corresponding position of the DA980102 VH used as an acceptor. As a result of the analysis of the three-dimensional structure according to computer modeling, with regard to amino acid residues (isoleucine (I) at position 48, lysine (K) at position 66, alanine (A) at position 67, valine (V) at position 71 and threonine (T) at position 93) that are adjacent to the CDR of the K5-70 VH and are assumed to play important roles for the maintenance of the structure, those of the K5-70 VH were retained, and the residual FR region was substituted with the acceptor sequence. The amino acid residue position numbers in VH and VL were used in accordance with the definitions of Kabat et al. (Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991).

[0254] Moreover, the methionine (M) as an amino acid residue at position 82 of the acceptor sequence DA980102 VH was highly likely to be a special amino acid residue caused by somatic hypermutation. Thus, in order to decrease

potential antigenicity, the aforementioned methionine was substituted with leucine (L) that is most common as an amino acid residue at position 82. An alignment of the amino acid sequences of the thus designed humanized K5-70 VH (HuK5-70 VH), K5-70 VH and DA980102 VH is shown in Figure 41.

[0255] With regard to the design of humanized K5-70 VL as well, the same transplantation of a CDR sequence as described above was carried out. As an amino acid residue (lysine (K) at position 49) important for the maintenance of the structure of CDR, that of the K5-70 VL was retained, and the residual FR region was substituted with the acceptor sequence (HuK5-70 VL). An alignment of the amino acid sequences of the HuK5-70 VL, K5-70 VL and L41174 VL is shown in Figure 42.

[Example 33]

[Design of humanized T6-16 antibody]

[0256] Humanization of the variable regions (VH, VL) of the T6-16 antibody prepared in the previous Examples was carried out as follows according to the method of Queen et al. (Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989). First, the molecular modeling of the three-dimentional structure of each T6-16 antibody variable region was carried out using a computer. Subsequently, homology search was performed with the variable region sequences of human antibody genes. As a result, a cDNA sequence (DA935238 VH) with GenBank accession number of DA935238 was selected as an acceptor that provides a framework region (FR) necessary for humanization of the T6-16 VH (Genome Res. 16:55-65, 2006). Likewise, a cDNA sequence (M99608 VL) with GenBank accession number of M99608 was selected as an acceptor that provides a framework region (FR) necessary for humanization of the T6-16 VL (J. Immunol. 149:2518-2529, 1992).

[0257] For humanization of the T6-16 VH, the CDR sequence of the T6-16 VH was substituted with the corresponding position of the DA935238 VH used as an acceptor. As a result of the analysis of the three-dimensional structure according to computer modeling, with regard to amino acid residues (isoleucine (I) at position 48, alanine (A) at position 67 and lysine (K) at position 73) that are adjacent to the CDR of the T6-16 VH and are assumed to play important roles for the maintenance of the structure, those of the T6-16 VH were retained, and the residual FR region was substituted with the acceptor sequence (HuT6-16 VH1). In addition, the lysine residue (K) at position 73 was unlikely to have influence on appropriate formation of an antigen-binding site, and thus, an amino acid sequence, in which the lysine residue of the HuT6-16 VH1 was substituted with threonine (T) as a more generous amino acid residue, was designed, separately (HuT6-16 VH2). An alignment of the amino acid sequences of the thus designed humanized T6-16 VH (HuT6-16 VH1 and HuT6-16 VH2), T6-16 VH and DA980102 VH is shown in Figure 43.

[0258] With regard to the design of humanized T6-16 VL as well, the same transplantation of a CDR sequence as described above was carried out. Amino acid residues important for the maintenance of the structure of CDR were retained also in the acceptor sequence, and as a FR sequence, the same sequence as the acceptor sequence was used (HuT6-16 VL). An alignment of the amino acid sequences of the HuT6-16 VL, T6-16 VL and L41174 VL is shown in Figure 44.

[Example 34]

[Synthesis of humanized K5-70 VH and VL genes]

[0259] Genes encoding HuK5-70 VH and HuK5-70 VL were prepared as follows. The gene were synthesized based on an amino acid sequence in which a signal peptide sequence derived from K5-70 VH or VL had been added to the N-terminal side of each of the above-designed HuK5-70 VH and VL (Operon). In this gene synthetic operation, a Kozak sequence (ACC ACC) was added to the 5'-terminal side of the gene sequence of each of the synthesized HuK5-70 VH and HuK5-70 VL. In addition, an EcoRI site (GAA TTC) was added as a restriction enzyme site to the 5' end of the HuK5-70 VH, and an NheI site (GCT AGC) was added to the 3' end thereof. Likewise, an AgeI site (ACC GGT) was added as a restriction enzyme site to the 5' end of the HuK5-70 VL, and a BsiWI site (CGT ACG) was added to the 3' end thereof. The synthesized HuK5-70 VH gene and HuK5-70 VL gene were incorporated into a pCR2.1 vector (Invitrogen) according to TA cloning. The gene sequences of the HuK5-70 VH and VL prepared by the gene synthesis are shown in Figures 45 and 46, respectively.

[Example 35]

[Synthesis of humanized T6-16 VH1, T6-16 VH2 and T6-16 VL genes]

[0260] Genes encoding HuT6-16 VH1, HuT6-16 VH2 and HuT6-16 VL were prepared as follows. The gene were

synthesized, based on an amino acid sequence in which a signal peptide sequence derived from T6-16 VH had been added to the N-terminal side of each of the above-designed HuT6-16 VH1 and HuT6-16 VH2, and an amino acid sequence in which a signal peptide sequence derived from T6-16 VL had been added to the N-terminal side of the HuT6-16 VL (Operon). In this gene synthetic operation, a Kozak sequence (ACC ACC) was added to the 5'-terminal side of the gene sequence of each of the synthesized HuT6-16 VH1, HuT6-16 VH2 and HuT6-16 VL. In addition, an EcoRI site (GAA TTC) was added as a restriction enzyme site to the 5' end of each of the HuT6-16 VH1 and HuT6-16 VH2, and an NheI site (GCT AGC) was added to the 3' end thereof. Likewise, an AgeI site (ACC GGT) was added as a restriction enzyme site to the 5' end of the humanized T6-16VL, and a BsiWI site (CGT ACG) was added to the 3' end thereof. The synthesized HuT6-16 VH1 gene, HuT6-16 VH2 gene and HuT6-16 VL gene were incorporated into a pCR2.1 vector (Invitrogen) according to TA cloning.

[0261] The gene sequences of the HuT6-16 VH1, HuT6-16 VH2 and HuT6-16 VL prepared by the gene synthesis are shown in Figures 47 to 49, respectively.

[Example 36]

[Construction of gene expression vectors for humanized K5-70 VH and VL genes]

[0262] The HuK5-70 VH and VL genes, which had been each incorporated into the pCR2.1 vector (Invitrogen), were digested with the restriction enzymes EcoRI and NheI, and AgeI and BsiWI, respectively, and gene fragments were then recovered. Subsequently, the cleaved HuK5-70 VH gene was inserted into the EcoRI/NheI site of a pFUSE-CHIg-hG1 vector (InvivoGen) as an animal cell expression vector for the expression of a human IgG1 form (pFUSE-CHIg-HuK5-70), whereas the HuK5-70 VL gene was inserted into the AgeI/BsiWI site of a pFUSE2-CLIg-hk vector (InvivoGen) as a human Igκ form expression vector (pFUSE2-CLIg-HuK5-70). Thus, each construct was completed.

[Example 37]

[Construction of gene expression vectors for humanized T6-16 VH1, T6-16 VH2 and T6-16 VL genes]

[0263] The T6-16 VH1 and T6-16 VH2 genes, which had been each incorporated into the pCR2.1 vector (Invitrogen), were digested with the restriction enzymes EcoRI and NheI, and the T6-16 VL gene was digested with the restriction enzymes AgeI and BsiWI. Then, gene fragments were recovered. Subsequently, the cleaved T6-16 VH1 gene was inserted into the EcoRI/NheI site of a pFUSE-CHIg-hG1 vector (InvivoGen) (pFUSE-CHIg-HuT6-16-1), and the T6-16 VH2 gene was also inserted into the EcoRI/NheI site of a pFUSE-CHIg-hG1 vector (pFUSE-CHIg-HuT6-16-2). The T6-16 VL gene was inserted into the AgeI/BsiWI site of a pFUSE2-CLIg-hk vector (InvivoGen) (pFUSE2-CLIg-HuT6-16). Thus, each construct was completed.

[Example 38]

[Establishment of 293F cell line capable of stably expressing HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody]

[0264] 293F cells (Invitrogen) were maintained and cultured in FreeStyle 293 Expression Medium (Invitrogen). Genes were introduced into the 293F cells using a 293 fectin reagent (Invitrogen) in accordance with protocols included therewith. That is, pFUSE-CHIg-HuK5-70 and pFUSE2-CLIg-HuK5-70 were both introduced into the 293F cells, and drug selection was then carried out using Zeocin (InvivoGen) and Blasticidin (InvivoGen), so as to establish a cell line capable of stably expressing a HuK5-70 antibody. Also, pFUSE-CHIg-HuT6-16-1 and pFUSE2-CLIg-HuT6-16 were both introduced into the 293F cells, and the above-mentioned drug selection was then carried out, so as to establish a cell line capable of stably expressing a HuT6-16-1 antibody. Also, pFUSE-CHIg-HuT6-16-2 and pFUSE2-CLIg-HuT6-16 were both introduced into the 293F cells, and the above-mentioned drug selection was then carried out, so as to establish a cell line capable of stably expressing a HuT6-16-2 antibody.

[Example 39]

[Purification of HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody proteins]

[0265] The established antibody-expressing cell lines were each inoculated on FreeStyle 293 Expression Medium (Invitrogen) at a cell density of 1 to 2.5 $\times$ 10^5 cells/ml, and thereafter, roller bottle culture was carried out for 6 to 8 days. Thereafter, a culture supernatant was recovered, and each humanized antibody was then purified using rProtein A

Sepharose Fast Flow (GE Healthcare) according to an ordinary method.

[0266] Figure 50 shows the results obtained by confirming by Western blotting the expression of each humanized antibody protein in a culture supernatant of the 293F cells, in which a HuK5-70 antibody, a HuT6-16-1 antibody and a HuT6-16-2 antibody were expressed. Specifically, after completion of SDS-PAGE, each protein was transferred on a PVDF membrane (Immobilon-P, Millipore, IPVH00010). The membrane was blocked at room temperature for 30 minutes using TBS (Tris-buffered saline) containing 5% skim milk. The resultant was washed with 0.1% TBST (TBS containing 0.1% Tween 20) for 5 minutes three times, and was then allowed to react with a primary antibody.

[0267] Lane 1 indicates a culture supernatant of 293F cells into which no genes had been introduced (negative control), and lane 2 indicates a culture supernatant of 293F cells into which pFUSE-CHIg-HuK5-70 and pFUSE2-CLIg-HuK5-70 had been introduced. For detection of the heavy chain and light chain proteins of the HuK5-70 antibody, a biotin-labeled anti-human IgG $F(ab')_2$ antibody (Rockland) was used. Lane 3 indicates a culture supernatant of 293F cells into which pFUSE-CHIg-HuT6-16-1 and pFUSE2-CLIg-HuT6-16 had been introduced, and lane 4 indicates a culture supernatant of 293F cells into which pFUSE-CHIg-HuT6-16-2 and pFUSE2-CLIg-HuT6-16 had been introduced. The heavy chain proteins of the HuT6-16-1 antibody and the HuT6-16-2 antibody were detected with a biotin-labeled anti-human IgG Fc antibody (Rockland), whereas the light chain proteins of the HuT6-16-1 antibody and the HuT6-16-2 antibody were detected with a biotin-labeled anti-human IgG $F(ab')_2$ antibody (Rockland).

[0268] As a result, in all cases of the HuK5-70 antibody, the HuT6-16-1 antibody and the HuT6-16-2 antibody, the expression of heavy chain and light chain proteins was confirmed in each culture supernatant.

[0269] Moreover, Figure 51 shows the results obtained by loading the purified HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody on SDS-PAGE and then staining them with CBB. In all of the cases, a heavy chain of approximately 50 kD and a light chain of approximately 25 kD were detected under reducing conditions, and bands were confirmed in the same positions as the heavy chain and light chain detected by the above-described Western blotting. From these results, it was confirmed that a HuK5-70 antibody protein, a HuT6-16-1 antibody protein and a HuT6-16-2 antibody protein were generated.

[Example 40]

[Antigen affinity of humanized K5-70 antibody (HuK5-70) and humanized T6-16 antibodies (HuT6-16-1 and HuT6-16-2)]

[0270] The antigen affinity of the purified HuK5-70 antibody, HuT6-16-1 antibody and HuT6-16-2 antibody was examined by methods using FACS and ELISA.

[0271] FACS was carried out, using HEK293-hTROP-2 cells in which a full-length human TROP-2 gene was stably expressed in HEK293 cells, and a pancreatic cancer cell line PK-59 which endogenously expressed a human TROP-2 protein on the cell surface. 100 μl of an antibody solution used as a primary antibody, which had been diluted to 1 μg/ml with a medium containing 10% FCS, was added to a suspension (5×10^5 cells) of cells (HEK293-hTROP-2 cells or PK-59 cells), which had been removed from a culture dish by treatment with trypsin, and the obtained mixture was then incubated at 4°C for 20 minutes. Thereafter, the resultant was washed with 1 ml of a medium containing 10% FCS. Then, secondary antibody (100 μl each), in which a phycoerythrin (PE)-labeled anti-mouse IgG antibody (BD Pharmingen) or a biotin-labeled anti-human IgG Fc antibody (Rockland) was diluted up to 200 times or 2000 times, respectively, was added to the resultant. The obtained mixture was incubated at 4°C for 20 minutes, and was then washed with 1 ml of a medium containing 10% FCS again. In a case in which the biotin-labeled anti-human IgG Fc antibody was used as a secondary antibody, 100 μl of a labeling solution, in which streptavidin-labeled PE (BD Pharmingen) was diluted up to 400 times, was added thereto as a fluorescence labeling reagent. Thereafter, the obtained mixture was incubated at 4°C for 20 minutes, and was then washed with 1 ml of a medium containing 10% FCS. Subsequently, the sample containing labeled cells was suspended in 1 ml of PBS containing 1% FCS and 2 mM EDTA, and the obtained suspension was then analyzed using FACSCalibur (Becton Dickinson). As a result, it was found that the HuK5-70 antibody showed reactivity equivalent to that of a mouse K5-70 antibody both in the HEK293-hTROP-2 cells and in the PK-59 cells. Likewise, the HuT6-16-1 antibody and the HuT6-16-2 antibody showed reactivity equivalent to that of a T6-16 antibody (Figure 52).

[0272] Furthermore, antigen affinity was also examined by an ELISA method. ELISA was carried out using an ELISA plate which was coated with the recombinant protein of the hTROP-2 extracellular region as described in Example 3. Specifically, a 96-well plate (BD FALCON) was coated with a 50 μl/well recombinant protein of a hTROP-2 extracellular region that had been diluted with PBS to 0.5 μg/ml (at 4°C overnight). Thereafter, the resultant was washed with a washing buffer (PBS containing 0.05% Tween 20), and a blocking buffer (PBS containing 2% skim milk and 0.05% Tween 20) was then added thereto (200 μl/well) to block it (at room temperature for 1 hour). The resultant was washed with a washing buffer. Thereafter, a HuK5-70 antibody, a HuT6-16-1 antibody, a HuT6-16-2 antibody, a K5-70 antibody and a T6-16 antibody were diluted with an ELISA buffer (PBS containing 1% skim milk and 0.025% Tween 20) in a concentration range from 3.05×10^{-4} to 5 μg/ml, so as to prepare a series of two-fold dilution samples. The obtained

dilution samples were each added in an amount of 50 μl/well to the above-described ELISA plate (at room temperature for 2 hours). The reaction product was washed with a washing buffer, and thereafter, a HRP-labeled goat anti-human κ chain antibody (SouthernBiotech) or a HRP-labeled sheep anti-mouse IgG antibody (GE Healthcare), each of which had been diluted to 2000 times with an ELISA buffer, was added (50 μl/well) as a detection antibody to the reaction product (at room temperature for 1 hour). After the mixture had been washed with a washing buffer, a TMB (3,3',5,5'-tetramethylbenzidine: SIGMA) substrate solution was added (50 μl/well) to the resultant to carry out a color reaction. Then, 1 M sulfuric acid (25 μl/well) was added to the reaction product to terminate the reaction. Using Microplate reader Model 550 (BioRad), an absorbance at 450 nm was measured with an absorbance at 655 nm used as a reference. As a result, the reaction curves of K5-70 and HuK5-70 were almost overlapped with each other, and the EC50 values thereof were 27 ng/ml and 22 ng/ml, respectively (Figure 53). Also, the reaction curves of T6-16, HuT6-16-1 and HuT6-16-2 were almost overlapped with one another, and the EC50 values thereof were 30 ng/ml, 27 ng/ml and 27 ng/ml, respectively (Figure 54). From these results, it became clear that all of the HuK5-70 antibody, the HuT6-16-1 antibody and the HuT6-16-2 antibody exhibit antigen affinity equivalent to that of the K5-70 or T6-16 antibody that is a parent antibody before humanization.

[Example 41]

[Anti-tumor activity of humanized anti-hTROP-2 antibody (HuK5-70) *in vivo*]

[0273] Subsequently, the anti-tumor activity of a HuK5-70 antibody *in vivo* was examined with xenograft treatment models using a human colon cancer cell line SW480, which endogenously expresses human TROP-2 on the cell surface. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female NOD-scid mice (Day 1). When the mean tumor volume reached 100 mm^3, grouping was carried out (Day 9). From Day 9, intraperitoneal administration of the antibody was carried out at administration intervals of once every three days. On the 39th day after the cancer cell transplantation (Day 39), the tumor volume of a control group (PBS administration, N = 8) was 824.3 ± 188.8 mm^3. On the other hand, the tumor volume of a HuK5-70 antibody administration group (10 mg/kg body weight, N = 8) was 455.5 ± 208.6 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited in the HuK5-70 antibody administration group (inhibitory rate: 44.7%) (Figure 55A). With regard to tumor weight, the tumor weight of the control group was 0.509 ± 0.161 g. In contrast, in the HuK5-70 antibody administration group, the tumor weight of the control group was 0.272 ± 0.162 g ($P < 0.05$ by Student's t-test), showing an inhibitory rate of 46.6% (Figure 55B).

[Example 42]

[Dose-dependent anti-tumor activity of humanized anti-hTROP-2 antibodies (HuK5-70 and HuT6-16-2) on xenograft treatment models using human colon cancer cell line SW480]

[0274] The dose-dependent anti-tumor activity of HuK5-70 and HuT6-16-2 antibodies was examined with xenograft treatment models using a human colon cancer cell line SW480. SW480 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female NOD-scid mice (Day 1). On the 9th day after the cancer cell transplantation (Day 9) at which the mean tumor volume reached 100 mm^3, the mice were divided into a control group (PBS administration group, N = 8, 101.65 ± 8.35 mm^3), a 1 mg/kg body weight HuK5-70 antibody administration group (N = 8, 103.18 ± 9.86 mm^3), a 5 mg/kg body weight HuK5-70 antibody administration group (N = 8, 101.34 ± 8.94 mm^3), a 10 mg/kg body weight HuK5-70 antibody administration group (N = 8, 101.53 ± 8.98 mm^3), a 1 mg/kg body weight HuT6-16-2 antibody administration group (N = 8, 103.18 ± 9.86 mm^3), a 5 mg/kg body weight HuT6-16-2 antibody administration group (N = 8, 101.34 ± 8.94 mm^3), and a 10 mg/kg body weight HuT6-16-2 antibody administration group (N = 8, 101.53 ± 8.98 mm^3). Then, from Day 9, intraperitoneal administration of each antibody was carried out at administration intervals of once every three days. On the 48th day after the cancer cell transplantation (Day 48), the tumor volume of the control group was 754.67 ± 276.05 mm^3. On the other hand, in the HuK5-70 antibody administration groups, the tumor volume of the 1 mg/kg body weight administration group was 521.81 ± 183.45 mm^3 (inhibitory rate: 30.9%), the body volume of the 5 mg/kg body weight administration group was 258.78 ± 137.02 mm^3 (inhibitory rate: 65.7%, $P < 0.01$ by Student's t-test), and the tumor volume of the 10 mg/kg body weight administration group was 314.60 ± 152.89 mm^3 (inhibitory rate: 58.3 %, $P < 0.01$ by Student's t-test) (Figure 56A). In the HuT6-16-2 antibody administration groups, the tumor volume of the 1 mg/kg body weight administration group was 600.41 ± 319.84 mm^3 (inhibitory rate: 20.4%), the tumor volume of the 5 mg/kg body weight administration group was 315.32 ± 189.02 mm^3 (inhibitory rate: 58.2%, $P < 0.01$ by Student's t-test), and the tumor volume of the 10 mg/kg body weight administration group was 270.79 ± 266.71 mm^3 (inhibitory rate: 64.1 %, $P < 0.01$ by Student's t-test) (Figure 57A). With regard to tumor weight on Day 48, the tumor weight of the control group was 0.422 ± 0.201 g. On the other hand, in the HuK5-70 antibody administration

groups, the tumor weight of the 1 mg/kg body weight administration group was 0.301 ± 0.160 g (inhibitory rate: 28.7%), the tumor weight of the 5 mg/kg body weight administration group was 0.115 ± 0.083 g (inhibitory rate: 72.7%, $P < 0.01$ by Student's t-test), and the tumor weight of the 10 mg/kg body weight administration group was 0.244 ± 0.181 g (inhibitory rate: 42.2%) (Figure 56B). In the HuT6-16-2 antibody administration groups, the tumor weight of the 1 mg/kg body weight administration group was 0.422 ± 0.255 g (inhibitory rate: 0%), the tumor weight of the 5 mg/kg body weight administration group was 0.247 ± 0.151 g (inhibitory rate: 41.5%), and the tumor weight of the 10 mg/kg body weight administration group was 0.190 ± 0.190 g (inhibitory rate: 53.1%, $P < 0.01$ by Student's t-test) (Figure 57B). From these results, it was confirmed that the HuK5-70 and HuT6-16-2 antibodies have dose-dependent anti-tumor activity.

[Example 43]

[Anti-tumor activity of anti-hTROP-2 mouse monoclonal antibodies K5-70 and T6-16 on xenograft treatment models using human ovarian cancer cell line SK-OV-3]

[0275] The anti-tumor activity of K5-70 and T6-16 antibodies as parent antibodies *in vivo* was examined with xenograft treatment models using a human ovarian cancer cell line SK-OV-3, which endogenously expresses hTROP-2 on the cell surface. SK-OV-3 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female nude mice (Day 1). On the 11th day after the cancer cell transplantation (Day 11), individuals mice, in which clear tumor formation was observed (mean tumor volume: approximately 50 mm^3), were divided into groups. Then, from Day 11, intraperitoneal administration of each antibody was carried out at administration intervals of twice a week. On the 56th day after the cancer cell transplantation (Day 56), the tumor volume of a control group (PBS administration, N = 8) was 652.6 ± 349.1 mm^3. On the other hand, the tumor volume of a K5-70 antibody administration group (10 mg/kg body weight, N = 8) was 253.7 ± 137.3 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 61.1%); and the tumor volume of a T6-16 antibody administration group (10 mg/kg body weight, N = 8) was 214.6 ± 98.6 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 67.1%) (Figure 58A). With regard to tumor weight, the tumor weight of the control group was 0.413 ± 0.218 g. In contrast, the tumor weight of the K5-70 antibody administration group was 0.194 ± 0.112 (g) ($P < 0.05$ by Student's t-test), showing an inhibitory rate of 53.0%; and the tumor weight of the T6-16 antibody administration group was 0.183 ± 0.093 (g) ($P < 0.05$ by Student's t-test), showing an inhibitory rate of 55.7% (Figure 58B).

[Example 44]

[Anti-tumor activity of anti-hTROP-2 mouse monoclonal antibodies K5-70 and T6-16 on xenograft treatment models using human breast cancer cell line MDA-MB-468]

[0276] Likewise, the anti-tumor activity of K5-70 and T6-16 antibodies *in vivo* was examined with xenograft treatment models using a human breast cancer cell line MDA-MB-468, which endogenously expresses hTROP-2 on the cell surface. MDA-MB-468 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female nude mice (Day 1). On the 12th day after the cancer cell transplantation (Day 12), individuals mice, in which clear tumor formation was observed (mean tumor volume: approximately 50 mm^3), were divided into groups. Then, from Day 12, intraperitoneal administration of each antibody was carried out at administration intervals of twice a week. On the 54th day after the cancer cell transplantation (Day 54), the tumor volume of a control group (PBS administration, N = 8) was 218.6 ± 75.5 mm^3. On the other hand, the tumor volume of a K5-70 antibody administration group (10 mg/kg body weight, N = 8) was 70.2 ± 37.4 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 67.9%); and the tumor volume of a T6-16 antibody administration group (10 mg/kg body weight, N = 8) was 88.3 ± 42.9 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 59.6%) (Figure 59A). With regard to tumor weight on Day 54, the tumor weight of the control group was 0.142 ± 0.049 g. In contrast, the tumor weight of the K5-70 antibody administration group was 0.050 ± 0.033 (g) ($P < 0.01$ by Student's t-test), showing an inhibitory rate of 64.8%; and the tumor weight of the T6-16 antibody administration group was 0.077 ± 0.046 (g) ($P < 0.05$ by Student's t-test), showing an inhibitory rate of 45.8% (Figure 59B).

[Example 45]

[Anti-tumor activity of anti-hTROP-2 mouse monoclonal antibodies K5-70 and T6-16 on xenograft treatment models using human lung cancer cell line Calu-3]

[0277] Likewise, the anti-tumor activity of K5-70 and T6-16 antibodies *in vivo* was examined with xenograft treatment models using a human lung cancer cell line Calu-3, which endogenously expresses hTROP-2 on the cell surface. Calu-

3 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female nude mice (Day 1). On the 9th day after the cancer cell transplantation (Day 9), individuals mice, in which clear tumor formation was observed (mean tumor volume: approximately 100 mm^3), were divided into groups. Then, from Day 9, intraperitoneal administration of each antibody was carried out at administration intervals of twice a week. On the 41st day after the cancer cell transplantation (Day 41), the tumor volume of a control group (PBS administration, N = 8) was 395.7 ± 221.2 mm^3. On the other hand, the tumor volume of a K5-70 antibody administration group (10 mg/kg body weight, N = 8) was 120.7 ± 125.6 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 69.5%); and the tumor volume of a T6-16 antibody administration group (10 mg/kg body weight, N = 8) was 146.3 ± 128.4 mm^3 ($P < 0.05$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 63.0%) (Figure 60A). With regard to tumor weight, the tumor weight of the control group was 0.301 ± 0.189 g. In contrast, the tumor weight of the K5-70 antibody administration group was 0.08 ± 0.085 (g) ($P < 0.01$ by Student's t-test), showing an inhibitory rate of 73.5%; and the tumor weight of the T6-16 antibody administration group was 0.106 ± 0.096 (g) ($P < 0.05$ by Student's t-test), showing an inhibitory rate of 64.9% (Figure 60B).

[Example 46]

[Anti-tumor activity of anti-hTROP-2 mouse monoclonal antibody K5-70 on xenograft prevention models using human bile duct cancer cell line TFK-1]

[0278] Likewise, the anti-tumor activity of a K5-70 antibody *in vivo* was examined with xenograft prevention models using a human bile duct cancer cell line TFK-1, which endogenously expresses hTROP-2 on the cell surface. TFK-1 cells (5×10^6 cells) were subcutaneously transplanted into the right flank of each of 7-week-old female nude mice (Day 1), and from the same day, intraperitoneal administration of the antibody was initiated at administration intervals of twice a week. On the 31st day after the cancer cell transplantation (Day 31), the tumor volume of a control group (PBS administration, N = 5) was 1000.4 ± 268.9 mm^3. On the other hand, the tumor volume of a K5-70 antibody administration group (10 mg/kg body weight, N = 5) was 197.2 ± 215.5 mm^3 ($P < 0.01$ by Student's t-test), and thus, tumor formation was significantly inhibited (inhibitory rate: 80.3%) (Figure 61A). With regard to tumor weight, the tumor weight of the control group was 0.443 ± 0.070 g. In contrast, the tumor weight of the K5-70 antibody administration group was 0.063 ± 0.052 (g) ($P < 0.01$ by Student's t-test), showing an inhibitory rate of 85.8% (Figure 61B).

[Example 47]

[Analysis of avidity of HuK5-70 and HuT6-16-2 antibodies]

[0279] The antigen-binding activity of HuK5-70 and HuT6-16-2 antibodies was examined according to an ELISA method (low-density antigen-coated ELISA) using a 96-well plate on which a low-density antigen has been coated. A recombinant hTACSTD2-Fc-His protein (Creative BioMart), which had been prepared to a concentration of 0.1 μg/mL with a 0.1 M acetate buffer (pH 5.3), was added in an amount of 50 μL/well to a 96-well plate, and coating was carried out at 4°C overnight. Thereafter, the same analysis as that in Example 40 was carried out. Test antibodies were diluted with an ELISA buffer (PBS containing 1% skim milk and 0.025% Tween 20), so as to prepare samples with a concentration range from 20 μg/mL to a series of two-fold dilutions (15 samples) and to use them. As a result, it was found that the HuT6-16-2 antibody had binding activity that was almost equivalent to that of the T6-16 antibody (wherein their EC50 values were 49 ng/mL and 41 ng/mL, respectively), but that the EC50 value of the HuK5-70 antibody was approximately 20 times higher than that of the K5-70 antibody (wherein their EC50 values were 222 ng/mL and 12 ng/mL, respectively; Figure 62).

[0280] Subsequently, the antigen-binding activity of HuK5-70 and K5-70 antibodies was examined by ELISA for analyzing a monovalent antigen-antibody reaction. Goat anti-human IgG (Fcγ specific) (Southern Biotech) which had been diluted to 1 μg/mL with a 0.1 M acetate buffer (pH 5.3) and Goat anti-mouse IgG (γ chain specific) (Southern Biotech) which had been diluted to 3 μg/mL were added in each amount of 50 μL/well to a 96-well plate. Thereafter, coating was carried out at 4°C overnight. Thereafter, the reaction product was washed with a washing buffer (PBS containing 0.05% Tween 20), and a blocking buffer (PBS containing 2% skim milk and 0.05% Tween 20) was then added thereto (200 μL/well) to block it (at room temperature for 1 hour). The resultant was washed with a washing buffer. Thereafter, the test antibody, which had been diluted to 1 μg/mL with an ELISA buffer (PBS containing 1% skim milk and 0.025% Tween 20), was added in an amount of 50 μL/well to the above-described plate. During this operation, a HuK5-70 antibody was added to a well coated with Goat anti-human IgG (Fcγ specific), and a K5-70 antibody was added to a well coated with Goat anti-mouse IgG (γ chain specific). The mixture was left at rest at room temperature for 1 hour, and the reaction product was then washed with a washing buffer. The recombinant protein of the hTROP-2 extracellular region described in Example 3 (hTROP-2 EC) was diluted with an ELISA buffer to prepare samples with a concentration range from 5

μg/mL to a series of three-fold dilutions (10 samples). The thus obtained sample was added in each amount of 50 μL/well to the plate. The mixture was left at rest at room temperature for 1 hour, and the reaction product was then washed with a washing buffer. Then, anti-His (G-18) (Santa Cruz), which had been diluted to 2 μg/mL with an ELISA buffer, was added as a primary antibody to the reaction product (50 μL/well). The obtained mixture was left at rest at room temperature for 1 hour, and it was then washed with a washing buffer. Thereafter, HRP-labeled anti-rabbit IgG (GE Healthcare), which had been diluted to 1000 times with an ELISA buffer, was added as a secondary antibody to the reaction product (50 μL/well). The obtained mixture was left at rest at room temperature for 1 hour, and it was then washed with a washing buffer. Thereafter, a TMB (3,3',5,5'-tetramethylbenzidine: SIGMA) substrate solution was added in an amount of 50 μL/well to the resultant to carry out a color reaction. Then, 1 M sulfuric acid (25 μL/well) was added to the reaction product to terminate the reaction. Using Microplate reader Model 550 (BioRad), an absorbance at 450 nm was measured with an absorbance at 655 nm used as a reference. As a result, the EC50 values calculated from the binding curves of the hTROP-2EC protein with the K5-70 and HuK5-70 antibodies were 7 ng/mL and 6 ng/mL, respectively (Figure 63). These results demonstrated that the HuK5-70 antibody and the K5-70 antibody have equivalent antigen affinity in a monovalent antigen-antibody reaction.

[0281] As described in Example 40, in an ELISA method using a 96-well plate on which a high-density antigen (0.5 μg/mL) had been coated (antigen-coated ELISA), the antigen affinity of the K5-70 antibody was equivalent to the antigen affinity of the HuK5-70 antibody (Figure 53). Thus, it was considered that the antigen-binding activity of the HuK5-70 antibody that is relatively lower than that of the K5-70 antibody in an ELISA method, in which a low-density antigen (0.1 μg/mL) has been coated on a plate, may be caused by the fact that flexibility of the movement of two antigen-binding arms, namely, "avidity" is relatively lower in the HuK7-50 antibody than that in the K5-70 antibody.

[Example 48]

[Preparation and characterization of humanized K5-70 antibody mutants]

[0282] For the purpose of improving the "avidity" of a HuK5-70 antibody, the following experiment was carried out.

[0283] Whether the above-mentioned relatively low "avidity" of the HuK5-70 antibody is caused by VH or by VL was examined by the following experiment. First, genes encoding the H chain variable region (K5-70 VH) and L chain variable region (K5-70 VL) of a K5-70 antibody as a parent antibody were prepared by gene synthesis (Operon). During the gene synthesis, a Kozak sequence (ACC ACC) was added to the 5'-terminal side of the gene sequence of each of the K5-70 VH and the K5-70 VL. Further, an EcoRI site (GAA TTC) was added as a restriction enzyme site to the 5' end of the K5-70 VH, and an NheI site (GCT AGC) was added as a restriction enzyme site to the 3' end thereof. Likewise, an AgeI site (ACC GGT) was added as a restriction enzyme site to the 5' end of the K5-70 VL, and a BsiWI site (CGT ACG) was added as a restriction enzyme site to the 3' end thereof. The thus synthesized K5-70 VH gene and K5-70 VL gene were each incorporated into a pCR2.1 vector (Invitrogen). The gene sequences of the K5-70 VH and VL prepared by the gene synthesis are shown in Figure 64 and Figure 65, respectively. The K5-70 VH and K5-70 VL genes incorporated into the pCR2.1 vector were digested with the restriction enzymes EcoRI and NheI, and AgeI and BsiWI, respectively, and gene fragments were then recovered. Subsequently, the cleaved K5-70 VH gene was inserted into the EcoRI/NheI site of a pFUSE-CHIg-hG1 vector (InvivoGen) as an expression vector for the expression of a human IgG1 form, whereas the K5-70 VL gene was inserted into the AgeI/BsiWI site of a pFUSE2-CLIg-hk vector (InvivoGen) as a human Igκ form expression vector, thereby completing mouse-human chimeric constructs (pFUSE-CHIg-MuK5-70 and pFUSE2-CLIg-MuK5-70).

[0284] The thus prepared constructs, and the HuK5-70 H chain expression vector (pFUSE-CHIg-HuK5-70) and the HuK5-70 L chain expression vector (pFUSE2-CLIg-HuK5-70), which had been prepared in Example 36, were allowed to co-express in 293F cells (Invitrogen) by combinations 1 to 4 in the following table.

	H chain	L chain	Antibody generated	Remarks
1	pFUSE-CHIg-HuK5-70	pFUSE2-CLIg-HuK5-70	HuK5-70 antibody	Humanized K5-70 antibody
2	pFUSE-CHIg-HuK5-70	pFUSE2-CLIg-MuK5-70	HuVH/MuVL antibody	Humanized K5-70 VH / Mouse K5-70 VL
3	pFUSE-CHIg-MuK5-70	pFUSE2-CLIg-HuK5-70	MuVH/HuVL antibody	Mouse K5-70 VH / Humanized K5-70 VL

(continued)

	H chain	L chain	Antibody generated	Remarks
4	pFUSE-CHIg-MuK5-70	pFUSE2-CLIg-MuK5-70	ChK5-70 antibody	Chimeric K5-70 antibody

[0285] Transfection of the expression vectors described above into 293F cells (Invitrogen) was carried out using NeoFection reagent (Astec) in accordance with the method described in instructions included therewith. After completion of the transfection, the resultant was cultured for 5 days using FreeStyle 293 Expression Medium (Invitrogen) at 37°C in a CO_2 incubator with a CO_2 concentration of 8%. Thereafter, a culture supernatant was recovered. The antibody concentration in the culture supernatant was measured by a sandwich ELISA method. Specifically, Goat anti-human IgG (Fcγ specific) (Southern Biotech), which had been diluted to 1 μg/mL with PBS, was added in an amount of 50 μL/well to a 96-well plate. Thereafter, coating was carried out at 4°C overnight. Thereafter, the reaction product was washed with a washing buffer (PBS containing 0.05% Tween 20), and a blocking buffer (PBS containing 2% skim milk and 0.05% Tween 20) was then added thereto (200 μL/well) to block it at room temperature for 1 hour. The resultant was washed with a washing buffer. Thereafter, a culture supernatant, which had been diluted to an appropriate dilution magnification with an ELISA buffer (PBS containing 1% skim milk and 0.025% Tween 20), was added in an amount of 50 μL/well to the above-described plate, and a reaction was then carried out at room temperature for 2 hours. As a standard preparation, a HuK5-70 antibody was used. The reaction product was then washed with a washing buffer. As a detection antibody, HRP-labeled Goat anti-human kappa (κ chain specific) (Southern Biotech), which had been diluted to 1,000 times with an ELISA buffer, was added in an amount of 50 μL/well to the reaction product, and a reaction was then carried out at room temperature for 1 hour. The reaction product was washed with a washing buffer, and thereafter, a TMB (3,3',5,5'-tetramethylbenzidine: SIGMA) substrate solution was added in an amount of 50 μL/well to the resultant to carry out a color reaction. Then, 1 M sulfuric acid (25 μL/well) was added to the reaction product to terminate the reaction. Using iMark Microplate reader (BioRad), an absorbance at 450 nm was measured with an absorbance at 655 nm used as a reference. The binding of the 4 types of antibodies contained in the culture supernatants to hTROP-2 was measured by the above-described low-density antigen-coated ELISA. As a result, it was found that the binding activity of a MuVH/HuVL antibody constituted with mouse K5-70 VH and HuK5-70 VL to hTROP-2 was equivalent to that of a ChK5-70 antibody, but that the binding activity of a HuVH/MuVL antibody constituted with HuK5-70 VH and mouse K5-70 VL was relatively lower than that of the ChK5-70 (Figure 66). These results suggested that HuK5-70 VH be involved in the "avidity" of HuK5-70. Hence, in order to prepare modified antibodies, in which the "avidity" of the Huk5-70 antibody has been improved, amino acid substitution was carried out on the HuK5-70 VH. As is found from a alignment of the amino acid sequences of HuK5-70 VH and K5-70 VH as shown in Figure 41, a total of 17 amino acids with amino acid numbers 5, 7, 11, 12, 13, 20, 38, 40, 44, 73, 75, 81, 82c, 83, 87, 108 and 109 are different between HuK5-70 VH and K5-70 VH (wherein the amino acid numbers are used in accordance with the definitions of Kabat et al. (1991)). The aforementioned amino acids of HuK5-70 VH were substituted with the corresponding amino acids of K5-70 VH, so that mutants were prepared according to gene synthesis. Then, an expression vector (a pFUSE-CHIg-HuK5-70 mutant) was prepared. According to the report by Landolfi et al. (J. Immunol. 166:1748, 2001), it has been reported that the amino acids at positions 11 and 38 of VH are involved in the avidity of a humanized antibody, and that avidity and biological activity are improved by substituting the two above amino acids with the corresponding amino acids derived from mouse. Hence, a double mutant comprising substitution of the amino acids at positions 11 and 38 was also prepared. The names of the thus prepared 18 types of HuK5-70 VH mutants and their amino acid sequences are shown in Figure 67.

[0286] The prepared 18 types of HuK5-70 VH mutants (expression vectors for pFUSE-CHIg-HuK5-70 mutants) were each combined with a HuK5-70 L chain expression vector (pFUSE2-CLIg-HuK5-70), and the thus obtained expression vectors were each transfected into HEK293 cells. Then, using the obtained culture supernatant, the binding activity of each amino acid substitution antibody to hTROP-2 was examined by low-density antigen-coated ELISA. As a result, among the 18 types of HuK5-70 VH mutants, an R44G mutant, in which the R (arginine) at position 44 of HuK5-70 VH had been substituted with G (glycine) (HuK5-70 R44G; which is hereafter referred to as HuK5-70-2), was observed to have an apparently improved antigen-binding activity (Figure 68). The sequence of a HuK5-70 VH R44G (hereinafter referred to as HuK5-70 VH2) gene is shown in Figure 69.

[Example 49]

[Purification and characterization of HuK5-70-2 antibody]

[0287] The HuK5-70-2 antibody that is a HuK5-70 R44G mutant antibody was purified as follows. That is, pFUSE-

CHIg-HuK5-70 R44G and pFUSE2-CLIg-HuK5-70 were transfected into 293F cells, and the resultant was then cultured for 5 days. Thereafter, a culture supernatant was recovered. The HuK5-70-2 antibody was purified from the recovered culture supernatant, using rProtein A sepharose Fast Flow (GE Healthcare). The purified HuK5-70-2 antibody was loaded on SDS-PAGE under reducing conditions. As a result, an H chain of approximately 50 kDa and an L chain of approximately 25 kDa were found. The purity of each antibody was 95% or more (Figure 70).

[0288] The binding activity of the purified HuK5-70-2 and HuK5-70 antibodies to hTROP-2 was examined by high-density antigen-coated ELISA and low-density antigen-coated ELISA. The affinity of the antibodies was examined by the high-density antigen-coated ELISA (using a 96-well plate which was coated with 1 μg/mL hTROP-2). As a result, it was found that the binding curve of the HuK5-70 antibody was almost overlapped with the binding curve of the HuK5-70-2 antibody, and that their affinity was equivalent to each other. Subsequently, the avidity of the antibodies was examined by the low-density antigen-coated ELISA (using a 96-well plate which was coated with 0.1 μg/mL hTROP-2). As a result, it was found that, as same the case with using a culture supernatant, the antigen-binding activity of the HuK5-70-2 antibody was clearly higher than the antigen-binding activity of the HuK5-70 antibody (Figure 71). Specifically, the EC_{50} value of a K5-70 antibody was 11.4 ng/mL, that of a HuK5-70 antibody was 33.4 ng/mL, and that of a HuK5-70-2 antibody was 11.4 ng/mL. Thus, it was demonstrated that the HuK5-70-2 antibody has an improved avidity in comparison with the HuK5-70 antibody, and that the HuK5-70-2 antibody has activity equivalent to that of the K5-70 antibody.

[Example 50]

[Measurement of antibody-dependent cellular cytotoxicity (ADCC) activity of humanized anti-hTROP-2 antibodies]

(1) Preparation of target cell solution

[0289] As target cells, a human colon cancer cell line SW480, a human pancreatic cancer cell line PK-59, and a human prostate cancer cell line PC-3, each of which endogenously expresses hTROP-2, were used. Target cells cultured on a 10-cm cell culture dish were harvested from the plate by treatment with trypsin, and were then suspended in an assay medium. A Leivovitz L-15 medium (for SW480 cells) or an RPMI-1640 medium (for PK-59 and PC-3 cells), to which 0.5% FBS had been added, was used for ADCC assay. After completion of centrifugation (at 1000 rpm for 3 minutes at room temperature), pellets were prepared at a cell density of 2×10^5 cells/mL with the same medium as that used above, and thus a target cell solution was prepared.

(2) Separation of human peripheral blood mononuclear cells

[0290] Healthy venous blood was collected with heparin, and was then diluted to 2 times with PBS. Thereafter, the diluted blood was layered on Lymphoprep (Daiichi Kagaku Yakuhin K.K.) and was then centrifuged (at room temperature at 750 rpm for 5 minutes, and then at 2000 rpm for 20 minutes). After completion of the centrifugation, mononuclear cells (healthy peripheral blood monocytes) were recovered from an intermediate layer fraction, and were then washed with PBS three times. Thereafter, a cell suspension was prepared with an assay medium, and the prepared cells were used as effector cells.

(3) ADCC activity of humanized anti-hTROP-2 antibodies (HuK5-70, HuK5-70-2 and HuT6-16-2 antibodies)

[0291] 100 μL (2×10^4 cells/well) of the prepared target cell solution was dispensed in a 96-well flat bottom plate (manufactured by FALCON). Subsequently, human peripheral blood mononuclear cells (effector cells) were added to the plate, so that the ratio between the effector cells and the target cells could be 40 : 1. Thereafter, humanized anti-hTROP-2 antibodies (HuK5-70, HuK5-70-2 and HuT6-16-2 antibodies) were each added as test antibodies to the plate to a final concentration of 0.1 to 30 μg/mL. The mixture was adjusted to a total amount of 200 μL, and it was then cultured in a CO_2 incubator (at 37°C in 5% CO_2) for 6 hours. After completion of the culture, the activity of lactate dehydrogenase released from the cytoplasm of the target cells damaged by the effector cells was measured using Cytotoxicity Detection Kit (LDH) (Roche, Cat. No. 11 644 793 001) in accordance with protocols included with the kit, and ADCC activity was then evaluated using the measurement result as an indicator.

[0292] As shown in Figures 72A to 72C, it became clear that the HuK5-70 and HuT6-16-2 antibodies dose-dependently exhibit ADCC activity on a human colon cancer cell line SW480 (Figure 72A), a human pancreatic cancer cell line PK-59 (Figure 72B) and a human prostate cancer cell line PC-3 (Figure 72C), each of which expresses hTROP-2 on the cell surface. In addition, as shown in Figures 72B and 72C, it became clear that the HuK5-70-2 antibody exhibits ADCC activity on the pancreatic cancer cell line PK-59 and the human prostate cancer cell line PC-3, wherein the above-mentioned ADCC activity is stronger than that of the HuK5-70 antibody. As mentioned above, the HuK5-70-2 antibody is an antibody whose binding ability (avidity) to hTROP-2 has been improved by substituting the R (arginine) at position

44 of HuK5-70 VH with G (glycine). It was demonstrated that the avidity of the HuK5-70-2 antibody, which had been improved when compared with the HuK5-70 antibody, is reflected in ADCC activity. Therefore, it is assumed that the HuK5-70-2 antibody has excellent anti-tumor activity even *in vivo,* as with the HuK5-70 antibody (wherein the HuK5-70-2 antibody can preferably have anti-tumor activity higher than that of the HuK5-70 antibody). From the above-described results, it was suggested that the HuK5-70, HuK5-70-2 and HuT6-16-2 antibodies become therapeutic antibodies useful for cancer that expresses hTROP-2 on the cell surface.

INDUSTRIAL APPLICABILITY

[0293] The present invention is able to provide an antibody, which specifically reacts with hTROP-2 and has high anti-tumor activity *in vivo,* and specifically, a monoclonal antibody having high anti-tumor activity *in vivo* at a low dose, and particularly, such an antibody, which is a humanized antibody. In addition, the present invention is able to provide a hybridoma, which produces the antibody, a fragment of the antibody, a complex of the antibody or the like and various types of drugs, a pharmaceutical composition for diagnosing or treating a tumor, a method for detecting a tumor, and a kit for detecting or diagnosing a tumor.

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Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
20 25 30

gtg agt cca gga gaa aga gtc agt ttc tcc tgc agg gcc agt cag agc 144
Val Ser Pro Gly Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Ser
35 40 45

att ggc aca agc ata cac tgg tat cag caa aga aca aat ggt tct cca 192
Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro
50 55 60

```
agg ctt ctc ata aag tat gct tct gag tct atc tct ggg atc cct tcc      240
Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65                  70                  75                  80

agg ttt agt ggc agt gga tca ggg aca gat ttt act ctt agc atc aac      288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn
                85                  90                  95

agt gtg gag tct gaa gat att gca gat tat tac tgt caa caa agt aat      336
Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
            100                 105                 110

agc tgg cca ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa          381
Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
        115                 120                 125
```

<210> 40
<211> 127
<212> PRT
<213> homo sapiens

<400> 40

```
Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1               5                   10                  15

Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
            20                  25                  30

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

Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro
    50                  55                  60

Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn
                85                  90                  95

Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
            100                 105                 110
```

Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
115 120 125

<210> 41
<211> 11
<212> PRT
<213> homo sapiens

<400> 41

Arg Ala Ser Gln Ser Ile Gly Thr Ser Ile His
1 5 10

<210> 42
<211> 7
<212> PRT
<213> homo sapiens

<400> 42

Tyr Ala Ser Glu Ser Ile Ser
1 5

<210> 43
<211> 9
<212> PRT
<213> homo sapiens

<400> 43

Gln Gln Ser Asn Ser Trp Pro Phe Thr
1 5

<210> 44
<211> 402
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (402)

<400> 44
atg gga tgg agc tgt atc atc ctc ttc ttg gta gca aca gct aca ggt 48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly

```
1               5                  10                  15

gtc cac tcc cag gtc caa ctg cag caa cct ggg tct gag ctg gtg agg      96
Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ser Glu Leu Val Arg
            20                  25                  30

cct gga gct tca gtg aag ctg tcc tgc aag gct tct ggc tac aca ttc     144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc agc tac tgg atg cac tgg gtg aag cag agg cat gga caa ggc ctt     192
Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg His Gly Gln Gly Leu
    50                  55                  60

gag tgg att gga aat att tat cct ggt ggt ggt tat act aac tac gat     240
Glu Trp Ile Gly Asn Ile Tyr Pro Gly Gly Gly Tyr Thr Asn Tyr Asp
65                  70                  75                  80

gag aag ttc aag agc aag ggc aca ctg act gta gac aca tcc tcc agc     288
Glu Lys Phe Lys Ser Lys Gly Thr Leu Thr Val Asp Thr Ser Ser Ser
                85                  90                  95

aca gcc tac atg cac ctc agc agc ctg aca tct gag gac tct gcg gtc     336
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

tat tac tgt aca aga tca tcc gtt ttt gac tac tgg ggc caa ggc acc     384
Tyr Tyr Cys Thr Arg Ser Ser Val Phe Asp Tyr Trp Gly Gln Gly Thr
        115                 120                 125

act ctc aca gtc tcc tca                                             402
Thr Leu Thr Val Ser Ser
    130
```

<210> 45
<211> 134
<212> PRT
<213> homo sapiens

<400> 45

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1               5                  10                  15

Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ser Glu Leu Val Arg
            20                  25                  30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
```

35 40 45

Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg His Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Asn Ile Tyr Pro Gly Gly Gly Tyr Thr Asn Tyr Asp
65 70 75 80

Glu Lys Phe Lys Ser Lys Gly Thr Leu Thr Val Asp Thr Ser Ser Ser
85 90 95

Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Thr Arg Ser Ser Val Phe Asp Tyr Trp Gly Gln Gly Thr
115 120 125

Thr Leu Thr Val Ser Ser
130

<210> 46
<211> 5
<212> PRT
<213> homo sapiens

<400> 46

Ser Tyr Trp Met His
1 5

<210> 47
<211> 17
<212> PRT
<213> homo sapiens

<400> 47

Asn Ile Tyr Pro Gly Gly Gly Tyr Thr Asn Tyr Asp Glu Lys Phe Lys
1 5 10 15

Ser

<210> 48
<211> 6
<212> PRT
<213> homo sapiens

<400> 48

Ser Ser Val Phe Asp Tyr
1 5

<210> 49
<211> 381
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (381)

<400> 49

```
atg gta tcc aca cct cag ttc ctt gta ttt ttg ctt ttc tgg att cca      48
Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1               5                  10                  15

gcc tcc aga ggt gac atc ttg ctg act cag tct cca gcc atc ctg tct      96
Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
            20                  25                  30

gtg agt cca gga gaa aga gtc agt ttc tcc tgc agg gcc agt cag aac     144
Val Ser Pro Gly Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Asn
        35                  40                  45

att ggc aca agc ata cac tgg ttt cag caa aga aca aat ggt tct cca     192
Ile Gly Thr Ser Ile His Trp Phe Gln Gln Arg Thr Asn Gly Ser Pro
    50                  55                  60

agg ctt ctc ata aag tat gct tct gag tct atc tct ggg atc cct tcc     240
Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65                  70                  75                  80

agg ttt agt ggc agt gga tca ggg aca gat ttt act ctt agc atc aac     288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn
                85                  90                  95

agt gtg gag tct gaa gat att gca gat tat tac tgt caa caa agt aat     336
Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
            100                 105                 110
```

agc tgg cca ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa 381
Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
115 120 125

<210> 50
<211> 127
<212> PRT
<213> homo sapiens

<400> 50

Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1 5 10 15

Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
20 25 30

Val Ser Pro Gly Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Asn
35 40 45

Ile Gly Thr Ser Ile His Trp Phe Gln Gln Arg Thr Asn Gly Ser Pro
50 55 60

Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn
85 90 95

Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
100 105 110

Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
115 120 125

<210> 51
<211> 11
<212> PRT
<213> homo sapiens

<400> 51

Arg Ala Ser Gln Asn Ile Gly Thr Ser Ile His
1 5 10

<210> 52
<211> 7
<212> PRT
<213> homo sapiens

<400> 52

Tyr Ala Ser Glu Ser Ile Ser
1 5

<210> 53
<211> 9
<212> PRT
<213> homo sapiens

<400> 53

Gln Gln Ser Asn Ser Trp Pro Phe Thr
1 5

<210> 54
<211> 396
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (396)

<400> 54

```
atg gga tgg agc tgt atc atc ctc ttc ttg gta gca aca gct aca ggt      48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1               5                  10                  15

gtc cac tcc cag gtc caa ctg cag cag cct ggg gct gag ctg gtg agg      96
Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Arg
            20                  25                  30

cct ggg gct tca gtg aag ctg tcc tgc aag gct tct ggc tac acc ttc     144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc agc tac tgg ata acc tgg gtg aag cag agg cct gga caa ggc ctt     192
```

```
Thr Ser Tyr Trp Ile Thr Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg atc gga aat att tat cct tct gat agt tat act aac tac aat      240
Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn
65                  70                  75                  80

caa aag ttc agg gac aag gcc aca ttg act gta gac aaa tcc tcc agt      288
Gln Lys Phe Arg Asp Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
                85                  90                  95

aca gcc tac atg cag ctc agc agc ccg aca tct gag gac tct gcg gtc      336
Thr Ala Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

tat tac tgt tca gcc ctc ttt gac tac tgg ggc caa ggc acc act ctc      384
Tyr Tyr Cys Ser Ala Leu Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu
        115                 120                 125

aca gtc tcc tca                                                     396
Thr Val Ser Ser
    130
```

<210> 55
<211> 132
<212> PRT
<213> homo sapiens

<400> 55

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1               5                   10                  15

Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Arg
            20                  25                  30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Ser Tyr Trp Ile Thr Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn
65                  70                  75                  80
```

Gln Lys Phe Arg Asp Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Ser Ala Leu Phe Asp Tyr Trp Gly Gln Gly Thr Thr Leu
115 120 125

Thr Val Ser Ser
130

<210> 56
<211> 5
<212> PRT
<213> homo sapiens

<400> 56

Ser Tyr Trp Ile Thr
1 5

<210> 57
<211> 17
<212> PRT
<213> homo sapiens

<400> 57

Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn Gln Lys Phe Arg
1 5 10 15

Asp

<210> 58
<211> 4
<212> PRT
<213> homo sapiens

<400> 58

Leu Phe Asp Tyr
1

<210> 59
<211> 381
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (381)

<400> 59

```
atg gta tcc aca cct cag ttc ctt gta ttt ttg ctt ttc tgg att cca      48
Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1               5                  10                  15

gcc tcc aga ggt gac atc ttg ctg act cag tct cca gcc atc ctg tct      96
Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
            20                  25                  30

gtg agt cca gga gaa aga gtc agt ttc tcc tgc agg gcc agt cag agc     144
Val Ser Pro Gly Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45

att ggc aca agc ata cac tgg tat cag caa aga aca aat ggt tct cca     192
Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro
    50                  55                  60

agg ctt ctc ata aag tat gct tct gag tct atc tct ggg atc cct tcc     240
Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65                  70                  75                  80

agg ttt agt ggc agt gga tca ggg aca gat ttt att ctt agc atc aac     288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ile Leu Ser Ile Asn
                85                  90                  95

agt gtg gag tct gaa gat att gca gat tat tac tgt caa caa agt aat     336
Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
            100                 105                 110

agc tgg cca ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa         381
Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
        115                 120                 125
```

<210> 60
<211> 127
<212> PRT
<213> homo sapiens

<400> 60

Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1 5 10 15

Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser
20 25 30

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

Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro
50 55 60

Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser
65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ile Leu Ser Ile Asn
85 90 95

Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asn
100 105 110

Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
115 120 125

<210> 61
<211> 11
<212> PRT
<213> homo sapiens

<400> 61

Arg Ala Ser Gln Ser Ile Gly Thr Ser Ile His
1 5 10

<210> 62
<211> 7
<212> PRT
<213> homo sapiens

<400> 62

Tyr Ala Ser Glu Ser Ile Ser
1 5

<210> 63
<211> 9
<212> PRT
<213> homo sapiens

<400> 63

Gln Gln Ser Asn Ser Trp Pro Phe Thr
1 5

<210> 64
<211> 423
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (423)

<400> 64

```
atg gga tgg agc tgg atc ttt ctc ttc ctc ctg tca gga act gca ggc      48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1               5                  10                  15

gtc cac tct gag gtc cag ctt cag cag tca gga cct gag ctg gtg aaa      96
Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
            20                  25                  30

cct ggg gcc tca gtg aag att tcc tgc aag gct tct gga tac aca ttc     144
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

act gac tac aat atg cac tgg gtg aag cag agc cat gga aag aac ctt     192
Thr Asp Tyr Asn Met His Trp Val Lys Gln Ser His Gly Lys Asn Leu
    50                  55                  60

gaa tgg att gga tat att tat cct tac aat ggt ggt act ggc tac aac     240
Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

cag agg ttc aag agc agg gcc aca atg act gta gac aaa tcc tcc agc     288
Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Lys Ser Ser Ser
                85                  90                  95
```

aca gcc tac atg gag ctc cgc agc ctg aca tct gat gac tct gca gtc 336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Asp Asp Ser Ala Val
100 105 110

tat tac tgt gca aga gaa gac tac ggt agt agc ccc tct tat gct atg 384
Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
115 120 125

gac tat tgg ggt caa gga acc tca gtc atc gtc tcc tca 423
Asp Tyr Trp Gly Gln Gly Thr Ser Val Ile Val Ser Ser
130 135 140

<210> 65
<211> 141
<212> PRT
<213> homo sapiens

<400> 65

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Asn Met His Trp Val Lys Gln Ser His Gly Lys Asn Leu
50 55 60

Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65 70 75 80

Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Asp Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
115 120 125

Asp Tyr Trp Gly Gln Gly Thr Ser Val Ile Val Ser Ser
130 135 140

<210> 66
<211> 5
<212> PRT
<213> homo sapiens

<400> 66

Asp Tyr Asn Met His
1 5

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

<400> 67

Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn Gln Arg Phe Lys
1 5 10 15

Ser

<210> 68
<211> 13
<212> PRT
<213> homo sapiens

<400> 68

Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met Asp Tyr
1 5 10

<210> 69
<211> 390
<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (1).. (390)

<400> 69
atg aag ttg cct gtt agg ctg ttg gtg ctg atg ttc tgg att cct gct 48
Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1 5 10 15

tcc agc agt gat gtt gtg atg acc caa act cca ctc tcc ctg cct gtc 96
Ser Ser Ser Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val
20 25 30

agt ctt gga gat cag gcc tcc atc tct tgc aga tct agt cag agc ctt 144
Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
35 40 45

gta cac ggt aat gga aac acc tat tta cat tgg tac ctg cag aag cca 192
Val His Gly Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro
50 55 60

ggc cag tct cca aag ctc ctg atc tac aaa gtt tcc aac cga ttt tct 240
Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser
65 70 75 80

ggg gtc cca gac agg ttc agt ggc agt gga tca ggg acg gat ttc aca 288
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

ctc aag atc agc aga gtg gag gct gag gat ctg gga gtt tat ttc tgc 336
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys
100 105 110

tct caa act aca cat gtt ccc acg ttc ggc tcg ggg aca aag ttg gaa 384
Ser Gln Thr Thr His Val Pro Thr Phe Gly Ser Gly Thr Lys Leu Glu
115 120 125

ata aaa 390
Ile Lys
130

<210> 70
<211> 130
<212> PRT
<213> homo sapiens

<400> 70

Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1 5 10 15

Ser Ser Ser Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val

20 25 30

Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
35 40 45

Val His Gly Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro
50 55 60

Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser
65 70 75 80

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys
100 105 110

Ser Gln Thr Thr His Val Pro Thr Phe Gly Ser Gly Thr Lys Leu Glu
115 120 125

Ile Lys
130

<210> 71
<211> 16
<212> PRT
<213> homo sapiens

<400> 71

Arg Ser Ser Gln Ser Leu Val His Gly Asn Gly Asn Thr Tyr Leu His
1 5 10 15

<210> 72
<211> 7
<212> PRT
<213> homo sapiens

<400> 72

Lys Val Ser Asn Arg Phe Ser
1 5

<210> 73
<211> 8
<212> PRT
<213> homo sapiens

<400> 73

Ser Gln Thr Thr His Val Pro Thr
1 5

<210> 74
<211> 402
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
<221> CDS
<222> (1).. (402)

<400> 74

```
atg ggg tgg agc tgc att atc ctg ttt ctt gtc gca act gca aca ggc      48
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1               5                  10                  15

gtt cac tca cag gtt cag cta gtc cag tct gga gct gag gtg aag aaa      96
Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

cca ggg gca tct gtc aaa gtg agc tgt aag gcc tct ggc tat acg ttc     144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acg ata tac tgg atc aat tgg gtg agg caa gct cct gga caa cgg ttg     192
Thr Ile Tyr Trp Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Arg Leu
    50                  55                  60

gaa tgg att ggc aac atc tat ccc tca gac tcc tac acc aac tac aat     240
Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn
65                  70                  75                  80

cag aag ttc aag gac aaa gcc act ctc acc gta gat acc agt gcc tcc     288
Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Thr Ser Ala Ser
                85                  90                  95
```

```
aca gcc tat atg gag ctg agc agt tta cgc agc gaa gat aca gcg gtg      336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110

tac tac tgc acc aga acc tcc atg gct gac tat tgg ggt cag ggt aca      384
Tyr Tyr Cys Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly Gln Gly Thr
        115                 120                 125

ctg gtg act gtg agc tcc                                              402
Leu Val Thr Val Ser Ser
    130
```

<210> 75
<211> 134
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 75

```
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1               5                   10                  15

Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Ile Tyr Trp Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Arg Leu
    50                  55                  60

Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn
65                  70                  75                  80

Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Thr Ser Ala Ser
                85                  90                  95

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
            100                 105                 110
```

Tyr Tyr Cys Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly Gln Gly Thr
115 120 125

Leu Val Thr Val Ser Ser
130

<210> 76
<211> 381
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
<221> CDS
<222> (1).. (381)

<400> 76

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atg gta agc aca ccc cag ttc ctc gtt ttc ctc ctg ttt tgg att ccc      48
Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1               5                  10                  15

gca agt aga ggg gag atc gtc ttg act cag agt cct gcc aca ctg tct      96
Ala Ser Arg Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30

ctt tca cca gga gaa agg gcg aca ctt agc tgt cga gct tct cag agc     144
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45

att ggc acg tcc ata cac tgg tat cag cag aaa ccg gga caa gct cca     192
Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60

cgg tta ctg atc aag tat gcc tcc gaa agc atc tct ggg att cct gca     240
Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala
65                  70                  75                  80

cgc ttt agc gga agc ggt agt ggt acc gac ttc act ctg acc ata tcc     288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95

tca cta gaa ccc gag gat ttt gcc gtg tac tac tgc cag cag tcc aac     336
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Asn
            100                 105                 110

tca tgg cct ttc acc ttt ggc caa ggc acc aaa gtg gag atc aag         381
```

Ser Trp Pro Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
115 120 125

<210> 77
<211> 127
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 77

Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe Trp Ile Pro
1 5 10 15

Ala Ser Arg Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
20 25 30

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

Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
50 55 60

Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala
65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
85 90 95

Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Asn
100 105 110

Ser Trp Pro Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
115 120 125

<210> 78
<211> 423
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
<221> CDS
<222> (1).. (423)

<400> 78

```
atg gga tgg tct tgg atc ttt ctc ttc ctg ctg tct ggc aca gct gga      48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1               5                  10                  15

gtg cat tcc caa gtt cag ctg gtc cag tcc gga gct gaa gtt aaa aag      96
Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

ccc ggg gcc agc gtc aaa gtc tcc tgc aag gca tcc ggg tat act ttt     144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc gat tat aac atg cac tgg gtg cgc caa gca ccc ggc cag ggc ctg     192
Thr Asp Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg att ggc tat atc tat cct tat aat gga ggg acc ggc tac aac     240
Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

cag aga ttc aag agc aga gcc acc atg aca gtg gat aaa tct acc agc     288
Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Lys Ser Thr Ser
                85                  90                  95

act gcc tac atg gag ctg aga agc ctg cgg agc gac gac aca gcc gtg     336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

tac tac tgt gcc cgc gag gat tac gga agc agc cca agc tac gcc atg     384
Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
        115                 120                 125

gat tac tgg ggc caa ggc act atg gtg acc gtg agc agc                 423
Asp Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
    130                 135                 140
```

<210> 79
<211> 141
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 79

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65 70 75 80

Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Lys Ser Thr Ser
85 90 95

Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
115 120 125

Asp Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
130 135 140

<210> 80
<211> 423
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>

<221> CDS
<222> (1).. (423)

<400> 80

```
atg ggc tgg tct tgg atc ttc ctc ttc ctg ctg agc ggg acc gct gga      48
Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1               5                  10                  15

gtc cat tct caa gtc caa ctg gtc cag tcc ggg gct gaa gtg aaa aaa      96
Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

cca gga gca tcc gtt aag gtg tcc tgt aag gca agc gga tac acc ttt     144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc gac tat aac atg cac tgg gtg agg cag gcc ccc gga cag ggg ctg     192
Thr Asp Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg atc ggc tat att tat cct tac aac ggg ggc act ggc tat aat     240
Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65                  70                  75                  80

cag aga ttt aag agc cgc gct acc atg acc gtg gac acc tcc act tct     288
Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Thr Ser Thr Ser
                85                  90                  95

aca gcc tat atg gag ctg aga agc ctg cgg agc gat gat aca gcc gtg     336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

tac tac tgc gcc aga gaa gat tac ggc agc agc ccc agc tac gcc atg     384
Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
        115                 120                 125

gac tac tgg ggc cag ggc aca atg gtt act gtg agc agc                 423
Asp Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
    130                 135                 140
```

<210> 81
<211> 141
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 81

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn
65 70 75 80

Gln Arg Phe Lys Ser Arg Ala Thr Met Thr Val Asp Thr Ser Thr Ser
85 90 95

Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met
115 120 125

Asp Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser
130 135 140

<210> 82
<211> 390
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
<221> CDS
<222> (1).. (390)

<400> 82
atg aag ctc cca gtg cgc ctc ctg gtc ctg atg ttc tgg att ccc gct 48

```
Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1               5                  10                  15

tcc tct agc gat atc gtc atg acc caa tcc cca ctg tct ctg cct gtg      96
Ser Ser Ser Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val
            20                  25                  30

aca cca ggc gaa cct gca tct att agc tgt aga agc agc cag tcc ctg     144
Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
        35                  40                  45

gtg cac gga aac gga aac acc tat ctg cac tgg tac ctg caa aaa cct     192
Val His Gly Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro
    50                  55                  60

gga cag agc ccc cag ctg ctg atc tac aaa gtc agc aat aga ttt agc     240
Gly Gln Ser Pro Gln Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser
65                  70                  75                  80

ggg gtg ccc gac agg ttt agc ggc agc ggc agc ggc aca gat ttc acc     288
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
                85                  90                  95

ctg aaa atc tcc cgg gtg gaa gcc gag gac gtt ggg gtt tac tat tgc     336
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys
            100                 105                 110

agc cag aca acc cat gtg ccc act ttc ggg ggc ggc act aag gtg gag     384
Ser Gln Thr Thr His Val Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
        115                 120                 125

atc aag                                                            390
Ile Lys
    130
```

<210> 83
<211> 130
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 83

```
Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1               5                  10                  15

Ser Ser Ser Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val
```

20 25 30

Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
35 40 45

Val His Gly Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro
50 55 60

Gly Gln Ser Pro Gln Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser
65 70 75 80

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys
100 105 110

Ser Gln Thr Thr His Val Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
115 120 125

Ile Lys
130

<210> 84
<211> 563
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (102).. (458)

<400> 84
aaccacatcc ctcctcagaa gcccccagag cacaactcct taccatggac tggacctgga 60

ggatcctctt tttggtggca gcagccacag gtgcccactc c cag gtc cag ctt gtg 116
Gln Val Gln Leu Val
1 5

cag tct ggg gct gag gtg aag aag cct ggg gcc tca gtg aag gtt tcc 164
Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val Ser
10 15 20

```
tgc aag gct tct gga tac acc ttc act aac tat gct ctg cat tgg gtg      212
Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Ala Leu His Trp Val
            25                  30                  35

cgc cag gcc ccc gga caa agg ctt gag tgg atg gga tgg atc aac act      260
Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met Gly Trp Ile Asn Thr
        40                  45                  50

ggc aat ggt aac aca aaa tat tca cag aag ttc cag ggc aga gtc acc      308
Gly Asn Gly Asn Thr Lys Tyr Ser Gln Lys Phe Gln Gly Arg Val Thr
    55                  60                  65

ctt acc agt gac aca tcc gcg agc aca gcc tac atg gag atg agc agc      356
Leu Thr Ser Asp Thr Ser Ala Ser Thr Ala Tyr Met Glu Met Ser Ser
70                  75                  80                  85

ctg aga tct gaa gac acg gct gtg tat tac tgt gcg agg agc agt ggc      404
Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Ser Ser Gly
                90                  95                  100

tgg tac gtt tgg ttc gac ccc tgg ggc cag gga acc ctg gtc acc gtc      452
Trp Tyr Val Trp Phe Asp Pro Trp Gly Gln Gly Thr Leu Val Thr Val
            105                 110                 115

tcc tca gcttccacca agggcccatc ggttttcccc ctggcgccct gctccaggag      508
Ser Ser

cacctctggg ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaa        563
```

<210> 85
<211> 119
<212> PRT
<213> Homo sapiens

<400> 85

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
            20                  25                  30

Ala Leu His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met
        35                  40                  45
```

```
Gly Trp Ile Asn Thr Gly Asn Gly Asn Thr Lys Tyr Ser Gln Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Leu Thr Ser Asp Thr Ser Ala Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Ser Ser Gly Trp Tyr Val Trp Phe Asp Pro Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115
```

<210> 86
<211> 384
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1).. (384)

<400> 86

```
atg gaa gtc ctc gtg cag ctt ctc ttc ctc ctg cta ctc tgg ctc cca      48
Met Glu Val Leu Val Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1               5                   10                  15

gat acc acc gga gaa att gtg ttg aca cag tct cca gcc acc ctg tct      96
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30

ttg tct cca ggg gaa aga gcc acc ctc tcc tgc agg gcc agt cag agt     144
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45

gtt agc agc tac tta gcc tgg tat caa cag aaa cct ggc cag gct ccc     192
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60

agg ctc ctc atc tat gat gca tcc aac agg gcc act ggc atc cca gcc     240
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80
```

```
agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc agc      288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95

agc cta gag cct gaa gat ttt gca gtt tat tac tgt cag cag cgt agc      336
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110

aac tgg cct cgg tcg ttc ggc caa ggg acc aag gtg gaa atc aaa cga      384
Asn Trp Pro Arg Ser Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
        115                 120                 125
```

<210> 87
<211> 128
<212> PRT
<213> Homo sapiens

<400> 87

```
Met Glu Val Leu Val Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1               5                   10                  15

Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30

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

Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60

Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95

Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110

Asn Trp Pro Arg Ser Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
        115                 120                 125
```

<210> 88
<211> 601
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (112).. (477)

<400> 88
acccaaaaac cacacccctc cttgggagaa tcccctagat cacagctcct caccatggac 60

tggacctgga gcatcctttt cttggtggca gcagcaacag gtgcccactc c cag gtt 117
Gln Val
1

cag ctg gtg cag tct gga gct gag gtg aag aag cct ggg gcc tca gtg 165
Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val
5 10 15

aag gtc tcc tgc aag gct tct ggt tac acc ttt acc agt tat ggt atc 213
Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Gly Ile
20 25 30

agt tgg gtg cgc cag gcc cct gga caa ggg ctt gag tgg atg gga ttg 261
Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Leu
35 40 45 50

atc agc gct tac aat ggt aac aca aac tat gca cag aag ctc cag ggc 309
Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala Gln Lys Leu Gln Gly
55 60 65

aga gtc acc atg acc gta gac acg tct acg agc aca gcc tat atg gaa 357
Arg Val Thr Met Thr Val Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
70 75 80

ctg agg agc ctg aga tct gac gac acg gcc gtc tat tac tgt gcg aga 405
Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys Ala Arg
85 90 95

gat gga ata gca gtg gcg acc aaa gat gct ttt gat atc tgg ggc caa 453
Asp Gly Ile Ala Val Ala Thr Lys Asp Ala Phe Asp Ile Trp Gly Gln
100 105 110

ggg aca atg gtc acc gtc tct tca gcacccacca aggctccgga tgtgttcccc 507
Gly Thr Met Val Thr Val Ser Ser
115 120

atcatatcag ggtgcagaca cccaaaggat aacagccctg tggtcctggc atgcttgata 567

actgggtacc acccaacgtc cgtgactgtc acct 601

<210> 89
<211> 122
<212> PRT
<213> Homo sapiens

<400> 89

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Gly Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Leu Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala Gln Lys Leu
50 55 60

Gln Gly Arg Val Thr Met Thr Val Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asp Gly Ile Ala Val Ala Thr Lys Asp Ala Phe Asp Ile Trp
100 105 110

Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

<210> 90
<211> 342
<212> DNA
<213> Homo sapiens

<220>
<221> CDS

<222> (1).. (342)

<400> 90

```
gat att gtg atg act cag tct cca ctc tcc ctg ccc gtc acc cct gga      48
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1               5                  10                  15

gag ccg gcc tcc atc tcc tgc agg tct agt cag agc ctc ctg cat agt      96
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu His Ser
            20                  25                  30

aat gga tac aac tat ttg gat tgg tac ctg cag aag cca ggg cag tct     144
Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

cca cag ctc ctg atc tat ttg ggt tct aat cgg gcc tcc ggg gtc cct     192
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60

gac agg ttc agt ggc agt gga tca ggc aca gat ttt aca ctg aaa atc     240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

agc agg gtg gag gct gag gat gtt ggg gtt tat tac tgc atg caa gct     288
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95

cta caa act ctg ggg ctc act ttc ggc gga ggg acc aag gtg gag atc     336
Leu Gln Thr Leu Gly Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
            100                 105                 110

aaa cga                                                             342
Lys Arg
```

<210> 91
<211> 114
<212> PRT
<213> Homo sapiens

<400> 91

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1               5                  10                  15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu His Ser
            20                  25                  30
```

Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
85 90 95

Leu Gln Thr Leu Gly Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
100 105 110

Lys Arg

<210> 92
<211> 115
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 92

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
20 25 30

Trp Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Ile
35 40 45

Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn Gln Lys Phe
50 55 60

Lys Asp Lys Ala Thr Leu Thr Val Asp Thr Ser Ala Ser Thr Ala Tyr

65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
100 105 110

Val Ser Ser
115

<210> 93
<211> 107
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 93

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Ser
20 25 30

Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
35 40 45

Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Asn Ser Trp Pro Phe
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

<210> 94
<211> 122
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 94

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn Gln Arg Phe
50 55 60

Lys Ser Arg Ala Thr Met Thr Val Asp Lys Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met Asp Tyr Trp
100 105 110

Gly Gln Gly Thr Met Val Thr Val Ser Ser
115 120

<210> 95
<211> 122
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 95

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Gly Tyr Asn Gln Arg Phe
    50                  55                  60

Lys Ser Arg Ala Thr Met Thr Val Asp Thr Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Glu Asp Tyr Gly Ser Ser Pro Ser Tyr Ala Met Asp Tyr Trp
            100                 105                 110

Gly Gln Gly Thr Met Val Thr Val Ser Ser
        115                 120
```

<210> 96
<211> 111
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 96

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1               5                  10                  15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Gly
            20                  25                  30
```

Asn Gly Asn Thr Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Ser Gln Thr
85 90 95

Thr His Val Pro Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 97
<211> 134
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct (recombinant protein)

<400> 97

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1 5 10 15

Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Ile Tyr Trp Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn
65 70 75 80

Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Thr Ser Ala Ser
85 90 95

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly Gln Gly Thr
115 120 125

Leu Val Thr Val Ser Ser
130

<210> 98
<211> 115
<212> PRT
<213> Artificial

<220>
<223> recombinant protein

<400> 98

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

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ile Tyr
20 25 30

Trp Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr Asn Tyr Asn Gln Lys Phe
50 55 60

Lys Asp Lys Ala Thr Leu Thr Val Asp Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

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Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
            100                 105                 110


Val Ser Ser
        115
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<210> 99
<211> 420
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
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<222> (13)..(414)

<400> 99

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gaattcacca cc atg gga tgg tcc tgc att att ctc ttt ctc gtc gcc acc      51
              Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr
              1               5                   10

gcc aca ggc gtg cac agc cag gtt caa ctg cag caa cct ggg gca gag       99
Ala Thr Gly Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu
    15                  20                  25

ctg gtt cgg cca ggg gcc tcc gtc aaa ctg tcc tgc aaa gct tct ggc      147
Leu Val Arg Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly
30                  35                  40                  45

tac act ttc acc atc tac tgg atc aac tgg gtg aag cag agg ccc ggc      195
Tyr Thr Phe Thr Ile Tyr Trp Ile Asn Trp Val Lys Gln Arg Pro Gly
                50                  55                  60

cag ggc ctg gaa tgg atc gga aat atc tat cct agc gat tct tac aca      243
Gln Gly Leu Glu Trp Ile Gly Asn Ile Tyr Pro Ser Asp Ser Tyr Thr
            65                  70                  75

aat tac aac cag aag ttc aag gac aag gct acc ctg acc gtg gac aaa      291
Asn Tyr Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys
        80                  85                  90

tct agc tcc aca gcc tac atg cag ctg agc agc ccc act agc gag gat      339
Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Pro Thr Ser Glu Asp
    95                  100                 105

agc gca gtg tat tat tgt acc aga acc agc atg gcc gac tat tgg gga      387
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Ser Ala Val Tyr Tyr Cys Thr Arg Thr Ser Met Ala Asp Tyr Trp Gly
110                 115                 120                 125

cag ggc aca act ctg acc gtg agc agc gctagc                          420
Gln Gly Thr Thr Leu Thr Val Ser Ser
                130
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<210> 100
<211> 399
<212> DNA
<213> Artificial

<220>
<223> recombinant DNA

<220>
<221> CDS
<222> (13)..(393)

<400> 100

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accggtacca cc atg gtt agc aca cct caa ttt ctg gtc ttc ctg ctc ttc     51
              Met Val Ser Thr Pro Gln Phe Leu Val Phe Leu Leu Phe
              1               5                   10

tgg att cct gcc agc aga gga gat atc ctc ctg aca caa agc ccc gca      99
Trp Ile Pro Ala Ser Arg Gly Asp Ile Leu Leu Thr Gln Ser Pro Ala
    15                  20                  25

atc ctg agc gtg tcc ccc gga gag cgc gtg agc ttt agc tgc cgg gcc     147
Ile Leu Ser Val Ser Pro Gly Glu Arg Val Ser Phe Ser Cys Arg Ala
30                  35                  40                  45

agc cag agc att gga acc agc atc cac tgg tat cag cag aga acc aac     195
Ser Gln Ser Ile Gly Thr Ser Ile His Trp Tyr Gln Gln Arg Thr Asn
                50                  55                  60

ggg tct ccc agg ctg ctg att aaa tac gct tct gag tct att tcc ggg     243
Gly Ser Pro Arg Leu Leu Ile Lys Tyr Ala Ser Glu Ser Ile Ser Gly
            65                  70                  75

atc cca agc aga ttc tcc ggc tct ggc agc ggc act gat ttt act ctg     291
Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
        80                  85                  90

tct atc aac agc gtg gag tcc gaa gac atc gcc gac tac tat tgt cag     339
Ser Ile Asn Ser Val Glu Ser Glu Asp Ile Ala Asp Tyr Tyr Cys Gln
    95                  100                 105

cag agc aat tcc tgg cca ttc acc ttt ggc agc ggc acc aag ctg gaa     387
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Gln Ser Asn Ser Trp Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu
110 115 120 125

atc aag cgtacg 399
Ile Lys

<210> 101
<211> 30
<212> PRT
<213> homo sapiens

<400> 101

Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln
1 5 10 15

Cys Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr
20 25 30

<210> 102
<211> 30
<212> PRT
<213> homo sapiens

<400> 102

Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala
1 5 10 15

Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser
20 25 30

<210> 103
<211> 30
<212> PRT
<213> homo sapiens

<400> 103

Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys
1 5 10 15

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

<210> 104
<211> 30
<212> PRT
<213> homo sapiens

<400> 104

Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg
1 5 10 15

Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr
20 25 30

<210> 105
<211> 30
<212> PRT
<213> homo sapiens

<400> 105

Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys
1 5 10 15

Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu
20 25 30

<210> 106
<211> 30
<212> PRT
<213> homo sapiens

<400> 106

Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg
1 5 10 15

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

<210> 107
<211> 30
<212> PRT
<213> homo sapiens

<400> 107

Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu
1 5 10 15

Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys
20 25 30

<210> 108
<211> 33
<212> PRT
<213> homo sapiens

<400> 108

Cys Ala Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg
1 5 10 15

Cys Ala Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg
20 25 30

Cys

<210> 109
<211> 30
<212> PRT
<213> homo sapiens

<400> 109

Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly
1 5 10 15

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

<210> 110
<211> 33
<212> PRT
<213> homo sapiens

<400> 110

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 111
<211> 32
<212> PRT
<213> homo sapiens

<400> 111

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
1 5 10 15

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
20 25 30

<210> 112
<211> 30
<212> PRT
<213> homo sapiens

<400> 112

Arg Leu Phe Arg Glu Arg Tyr Arg Leu His Pro Lys Phe Val Ala Ala
1 5 10 15

Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln
20 25 30

<210> 113
<211> 15
<212> PRT
<213> homo sapiens

<400> 113

Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln

1 5 10 15

<210> 114
<211> 33
<212> PRT
<213> homo sapiens

<400> 114

Cys Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu Arg Arg
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 115
<211> 33
<212> PRT
<213> homo sapiens

<400> 115

Cys Pro Lys Phe Val Ala Ala Val His Tyr Glu Gln Pro Thr Ile Gln
1 5 10 15

Cys Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg
20 25 30

Cys

<210> 116
<211> 23
<212> PRT
<213> homo sapiens

<400> 116

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
1 5 10 15

Cys Gly Leu Asp Leu Arg Val
20

<210> 117
<211> 15
<212> PRT
<213> homo sapiens

<400> 117

Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly Glu Pro
1 5 10 15

<210> 118
<211> 33
<212> PRT
<213> homo sapiens

<400> 118

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 119
<211> 23
<212> PRT
<213> homo sapiens

<400> 119

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
1 5 10 15

Cys Arg Gly Glu Pro Leu Gln
20

<210> 120

<211> 33
<212> PRT
<213> homo sapiens

<400> 120

Cys Thr Ile Gln Ile Glu Leu Arg Gln Asn Thr Ser Gln Lys Ala Ala
1 5 10 15

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 121
<211> 33
<212> PRT
<213> homo sapiens

<400> 121

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 122
<211> 33
<212> PRT
<213> homo sapiens

<400> 122

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 123
<211> 33
<212> PRT
<213> homo sapiens

<400> 123

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 124
<211> 33
<212> PRT
<213> homo sapiens

<400> 124

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg
20 25 30

Cys

<210> 125
<211> 33
<212> PRT
<213> homo sapiens

<400> 125

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His

1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 126
<211> 17
<212> PRT
<213> homo sapiens

<400> 126

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys

<210> 127
<211> 33
<212> PRT
<213> homo sapiens

<400> 127

Cys Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe
1 5 10 15

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 128
<211> 33
<212> PRT
<213> homo sapiens

<400> 128

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 129
<211> 33
<212> PRT
<213> homo sapiens

<400> 129

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 130
<211> 32
<212> PRT
<213> homo sapiens

<400> 130

Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn Cys
1 5 10 15

Gly Leu Asp Leu Arg Val Arg Gly Glu Pro Leu Gln Val Glu Arg Cys
20 25 30

<210> 131
<211> 33
<212> PRT
<213> homo sapiens

<400> 131

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
1 5 10 15

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 132
<211> 32
<212> PRT
<213> homo sapiens

<400> 132

Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Cys
1 5 10 15

Ala Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Cys
20 25 30

<210> 133
<211> 33
<212> PRT
<213> homo sapiens

<400> 133

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 134
<211> 30

<212> PRT
<213> homo sapiens

<400> 134

Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys
1 5 10 15

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

<210> 135
<211> 30
<212> PRT
<213> homo sapiens

<400> 135

Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln
1 5 10 15

Cys Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr
20 25 30

<210> 136
<211> 30
<212> PRT
<213> homo sapiens

<400> 136

Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys
1 5 10 15

Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu
20 25 30

<210> 137
<211> 30
<212> PRT
<213> homo sapiens

<400> 137

Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg

1 5 10 15

Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr
20 25 30

<210> 138
<211> 30
<212> PRT
<213> homo sapiens

<400> 138

Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly
1 5 10 15

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

<210> 139
<211> 30
<212> PRT
<213> homo sapiens

<400> 139

Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala
1 5 10 15

Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser
20 25 30

<210> 140
<211> 30
<212> PRT
<213> homo sapiens

<400> 140

Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly
1 5 10 15

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

<210> 141
<211> 30
<212> PRT
<213> homo sapiens

<400> 141

Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg
1 5 10 15

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

<210> 142
<211> 30
<212> PRT
<213> homo sapiens

<400> 142

Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu
1 5 10 15

Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys
20 25 30

<210> 143
<211> 30
<212> PRT
<213> homo sapiens

<400> 143

Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly
1 5 10 15

Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys Cys
20 25 30

<210> 144
<211> 30
<212> PRT
<213> homo sapiens

<400> 144

Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro
1 5 10 15

Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly Met Ala
20 25 30

<210> 145
<211> 30
<212> PRT
<213> homo sapiens

<400> 145

Asn Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly
1 5 10 15

Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly Met
20 25 30

<210> 146
<211> 30
<212> PRT
<213> homo sapiens

<400> 146

Asp Asn Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp
1 5 10 15

Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser Gly
20 25 30

<210> 147
<211> 33
<212> PRT
<213> homo sapiens

<400> 147

Cys Thr Val Ala Ser Pro Asp Gly Pro Gly Gly Arg Ala Gln Ala Arg
1 5 10 15

Cys Leu Gly Ser Gly Met Ala Val Asp Ala Ser Thr Leu Thr Ser Lys
20 25 30

Cys

<210> 148
<211> 30
<212> PRT
<213> homo sapiens

<400> 148

Gln Asp Asn Cys Thr Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro
1 5 10 15

Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser
20 25 30

<210> 149
<211> 30
<212> PRT
<213> homo sapiens

<400> 149

Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu Gly Ser
1 5 10 15

Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys Cys Leu
20 25 30

<210> 150
<211> 33
<212> PRT
<213> homo sapiens

<400> 150

Cys Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg
1 5 10 15

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

Cys

<210> 151
<211> 15
<212> PRT
<213> homo sapiens

<400> 151

Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg
1 5 10 15

<210> 152
<211> 33
<212> PRT
<213> homo sapiens

<400> 152

Cys Ala Pro Lys Asn Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala
1 5 10 15

Cys Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala Leu Val Asp Asn
20 25 30

Cys

<210> 153
<211> 32
<212> PRT
<213> homo sapiens

<400> 153

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
1 5 10 15

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
20 25 30

<210> 154
<211> 30
<212> PRT
<213> homo sapiens

<400> 154

Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala
1 5 10 15

Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser
20 25 30

<210> 155
<211> 30
<212> PRT
<213> homo sapiens

<400> 155

Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln
1 5 10 15

Cys Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr
20 25 30

<210> 156
<211> 30
<212> PRT
<213> homo sapiens

<400> 156

Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys
1 5 10 15

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

<210> 157
<211> 30
<212> PRT
<213> homo sapiens

<400> 157

Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg
1 5 10 15

Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr
20 25 30

<210> 158
<211> 30
<212> PRT
<213> homo sapiens

<400> 158

Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys
1 5 10 15

Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu
20 25 30

<210> 159
<211> 30
<212> PRT
<213> homo sapiens

<400> 159

Cys Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly
1 5 10 15

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

<210> 160
<211> 30
<212> PRT
<213> homo sapiens

<400> 160

Pro Thr Asn Lys Met Thr Val Cys Ser Pro Asp Gly Pro Gly Gly Arg
1 5 10 15

Cys Gln Cys Arg Ala Leu Gly Ser Gly Met Ala Val Asp Cys

20 25 30

<210> 161
<211> 33
<212> PRT
<213> homo sapiens

<400> 161

Cys Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu Arg Arg
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 162
<211> 17
<212> PRT
<213> homo sapiens

<400> 162

Cys Thr His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly
1 5 10 15

Cys

<210> 163
<211> 33
<212> PRT
<213> homo sapiens

<400> 163

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 164
<211> 33
<212> PRT
<213> homo sapiens

<400> 164

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 165
<211> 32
<212> PRT
<213> homo sapiens

<400> 165

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
1 5 10 15

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
20 25 30

<210> 166
<211> 33
<212> PRT
<213> homo sapiens

<400> 166

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg

20 25 30

Cys

<210> 167
<211> 19
<212> PRT
<213> homo sapiens

<400> 167

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
1 5 10 15

Cys Pro Lys

<210> 168
<211> 30
<212> PRT
<213> homo sapiens

<400> 168

Val Cys Ser Pro Asp Gly Pro Gly Gly Arg Cys Gln Cys Arg Ala Leu
1 5 10 15

Gly Ser Gly Met Ala Val Asp Cys Ser Thr Leu Thr Ser Lys
20 25 30

<210> 169
<211> 30
<212> PRT
<213> homo sapiens

<400> 169

Asp Leu Ser Leu Arg Cys Asp Glu Leu Val Arg Thr His His Ile Leu
1 5 10 15

Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn His
20 25 30

<210> 170
<211> 33
<212> PRT
<213> homo sapiens

<400> 170

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
1 5 10 15

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 171
<211> 17
<212> PRT
<213> homo sapiens

<400> 171

Cys Phe Gln Gly Arg Gly Gly Leu Asp Leu Arg Val Arg Gly Glu Pro
1 5 10 15

Cys

<210> 172
<211> 33
<212> PRT
<213> homo sapiens

<400> 172

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 173
<211> 33
<212> PRT
<213> homo sapiens

<400> 173

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

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 174
<211> 33
<212> PRT
<213> homo sapiens

<400> 174

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 175
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<212> PRT
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<400> 175

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1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 176
<211> 33
<212> PRT
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<400> 176

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 177
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<400> 177

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1 5 10 15

Cys Arg Gly Glu Pro Leu Gln Val Glu Arg Thr Leu Ile Tyr Tyr Leu
20 25 30

Cys

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<400> 179

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Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

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Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 181
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1 5 10 15

Asp Leu Arg His Arg Pro Thr Ala Gly Ala Phe Asn His Ser
20 25 30

<210> 182
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<400> 182

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1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 183
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<400> 183

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Cys Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr
20 25 30

Cys

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<400> 184

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<400> 185

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<400> 186

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Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
20 25 30

Cys

<210> 187
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<400> 187

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1 5 10 15

<210> 188
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<400> 188

Cys Tyr Asp Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln
1 5 10 15

Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
20 25 30

Cys

<210> 189
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<212> PRT
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<400> 189

Cys Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr
1 5 10 15

Cys Tyr Asp Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln
20 25 30

Cys

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<400> 190

Asp Cys Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Cys Asn Gln
1 5 10 15

<210> 191

<211> 33
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<400> 191

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1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 192
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<400> 192

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1 5 10 15

Cys Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln
20 25 30

Cys

<210> 193
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<212> PRT
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<400> 193

Cys Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr Ser Val Ala Trp
1 5 10 15

Cys Ala Arg Thr Leu Val Arg Pro Ser Glu His Ala Leu Val Asp Asn
20 25 30

Cys

<210> 194
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<212> PRT
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<400> 194

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Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
20 25 30

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1 5 10 15

<210> 197
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<400> 197

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Cys Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr Ser Val Ala
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Cys

<210> 199
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Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
20 25 30

Cys

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<400> 201

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Cys Asp Glu Leu Val Arg His His Ile Leu Ile Asp Leu Arg His Cys
20 25 30

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<400> 202

Cys Pro Asp Gly Pro Gly Gly Arg Ala Gln Ala Arg Ala Leu Gly Ser
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg

20 25 30

Cys

<210> 203
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<212> PRT
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<400> 203

Cys Thr Leu Val Arg Pro Ser Glu His Ala Leu Val Asp Asn Asp Gly
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Cys Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr Ser Val Ala Trp
20 25 30

Cys

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<400> 204

Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
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Cys Tyr Asp Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln
20 25 30

Cys

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<400> 205

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Cys Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr Ser Val
20 25 30

Cys

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<400> 207

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Cys Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn Gln Thr
20 25 30

Cys

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<400> 208

Cys Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Cys Asn Gln Thr

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<210> 209
<211> 33
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<400> 209

Cys Val Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser
1 5 10 15

Cys Tyr Asp Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln
20 25 30

Cys

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<400> 210

Cys Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg
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Cys Tyr Asp Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln
20 25 30

Cys

<210> 211
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1 5 10 15

His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Cys
20 25 30

<210> 212
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<213> homo sapiens

<400> 212

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Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 213
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Leu Arg Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile
20 25 30

<210> 214
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<400> 214

Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg Cys Asp Glu Leu
1 5 10 15

Val Arg Thr His His Ile Leu Ile Asp Leu Arg His Arg Pro

20 25 30

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<400> 215

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

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 216
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<400> 216

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 217
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<400> 217

Cys Val Asn Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser
1 5 10 15

Cys Pro Asp Ala Asp Pro Glu Gly Arg Phe Lys Ala Arg Gln Ala Asn
20 25 30

Cys

<210> 218
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<400> 218

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 219
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Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

<210> 220
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Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

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<400> 221

Cys Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg
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20 25 30

Cys

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Cys Val Arg Pro Ser Glu His Ala Leu Val Asp Asn Asp Gly Leu Tyr
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Cys Ser Val Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg
20 25 30

Cys

<210> 223
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<400> 223

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
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Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

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20 25 30

Cys

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20 25 30

Cys

<210> 226
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<400> 226

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Cys His Ser Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg
20 25 30

Cys

<210> 227
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<212> PRT
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<400> 227

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
1 5 10 15

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 228
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<400> 228

Cys Ala Gly Ala Phe Asn His Ser Asp Leu Asp Ala Glu Leu Arg Arg
1 5 10 15

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 229
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<212> PRT
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<400> 229

Cys Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg Leu His
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

<210> 230
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<400> 230

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
1 5 10 15

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
20 25 30

Cys

<210> 231
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<212> PRT

<213> homo sapiens

<400> 231

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
1 5 10 15

Cys Val His Tyr Glu Gln Pro Thr Ile Gln Ile Glu Leu Arg Gln Asn
20 25 30

Cys

<210> 232
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<400> 232

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

Cys Gly Val Arg Arg Thr Asp Lys Gly Asp Leu Ser Leu Arg Ala Asp
20 25 30

Cys

<210> 233
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<400> 233

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

Cys His His Ile Leu Ile Asp Leu Arg His Arg Pro Thr Ala Gly Ala
20 25 30

Cys

<210> 234
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<400> 234

Cys Asp Leu Asp Ala Glu Leu Arg Arg Leu Phe Arg Glu Arg Tyr Arg
1 5 10 15

Cys Asp Glu Leu Val Arg Thr His His Ile Leu Ile Asp Leu Arg His
20 25 30

Cys

Claims

1. An antibody against human TROP-2 in which an H chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 92 or 98, and an L chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 93.

2. The antibody according to claim 1, wherein the amino acid sequences of CDR 1 to 3 of the H chain V region of the antibody are shown in SEQ ID NOS: 36 to 38, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region of the antibody are shown in SEQ ID NOS: 41 to 43, respectively.

3. An antibody against human TROP-2 in which an H chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 94 or 95, and an L chain V region of the antibody consists of the amino acid sequence shown in SEQ ID NO: 96.

4. The antibody according to claim 3, wherein the amino acid sequences of CDR 1 to 3 of the H chain V region of the antibody are shown in SEQ ID NOS: 66 to 68, respectively, and/or the amino acid sequences of CDR 1 to 3 of the L chain V region of the antibody are shown in SEQ ID NOS: 71 to 73, respectively.

5. The antibody according to any one of claims 1 to 4, which is a humanized antibody.

6. The antibody according to any one of claims 1 to 5, which has anti-tumor activity *in vivo*.

7. The antibody according to any one of claims 1 to 6, which exhibits 50% or more of tumor growth inhibitory activity at a dosage of 5 to 20 mg/kg body weight.

8. The antibody according to claim 7, wherein the frequency of administration for exhibiting the tumor growth inhibitory activity is at most once a week.

9. The antibody according to any one of claims 1 to 6, which exhibits 50% or more of the tumor growth inhibitory activity by a single administration of the antibody at a dosage of 10 mg/kg body weight.

10. The antibody according to any one of claims 1 to 9, which has anti-tumor activity on two or more types of human tumor cell lines.

11. The antibody according to any one of claims 1 to 10, wherein the dissociation constant (Kd value) is 1.0×10^{-10} M

or less.

12. The antibody according to any one of claims 1 to 11, which is a monoclonal antibody.

13. The antibody according to any one of claims 6 to 12, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

14. The antibody according to any one of claims 6 to 12, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human colorectal cancer, human lung cancer, human breast cancer and human ovarian cancer.

15. The antibody according to any one of claims 6 to 14, wherein the tumor is a recurrent cancer or a metastatic cancer.

16. The antibody according to any one of claims 10 to 15, wherein the tumor cell lines are at least two types selected from the group consisting of a human pancreatic cancer cell line PK-59, a human pancreatic cancer cell line BxPC-3, a human pancreatic cancer cell line KP-3L, a human pancreatic cancer cell line KP-2, a human pancreatic cancer cell line PK-1, a human pancreatic cancer cell line PK-45H, a human pancreatic cancer cell line PK-45P, a human pancreatic cancer cell line TCC-PAN2, a human pancreatic cancer cell line SUIT-2, a human colon cancer cell line CACO-2, a human colon cancer cell line SW480, a human colon cancer cell line DLD-1, a human colon cancer cell line HCT 116, a human breast cancer cell line JIMT-1, a human breast cancer cell line HCC 1143, a human breast cancer cell line MCF-7, a human breast cancer cell line MDA-MB-468, a human prostate cancer cell line DU145, a human prostate cancer cell line PC-3, a human ovarian cancer cell line SK-OV-3, a human lung cancer cell line Calu-3 and a human bile duct cancer cell line TFK-1.

17. The antibody according to any one of claims 10 to 15, wherein the tumor cell lines are at least two types selected from the group consisting of a human pancreatic cancer cell line PK-59, a human pancreatic cancer cell line BxPC-3, a human colon cancer cell line SW480, a human lung cancer cell line Calu-3, a human breast cancer cell line MBA-MD-468 and a human ovarian cancer cell line SK-OV-3.

18. An antibody fragment derived from the antibody according to any one of claims 1 to 17.

19. The antibody fragment according to claim 18, which comprises the amino acid sequence shown in SEQ ID NO: 92 or 98 and/or the amino acid sequence shown in SEQ ID NO: 93.

20. The antibody fragment according to claim 18, which comprises the amino acid sequence shown in SEQ ID NO: 94 or 95 and/or the amino acid sequence shown in SEQ ID NO: 96.

21. An antibody-drug conjugate, which comprises the antibody according to any one of claims 1 to 17 and a substance having anti-tumor activity and/or cell-killing activity.

22. An antibody fragment-drug conjugate, which comprises the antibody fragment according to any one of claims 18 to 20 and a substance having anti-tumor activity and/or cell-killing activity.

23. The conjugate according to claim 21 or 22, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

24. The conjugate according to claim 21 or 22, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human colorectal cancer, human lung cancer, human breast cancer and human ovarian cancer.

25. The conjugate according to any one of claims 21 to 24, wherein the tumor is a recurrent cancer or a metastatic cancer.

26. A pharmaceutical composition, which comprises at least one type selected from the group consisting of the antibody according to any one of claims 1 to 17, the antibody fragment according to any one of claims 18 to 20 and the conjugate according to any one of claims 21 to 25.

27. The composition according to claim 26, which is used in the treatment of tumor.

28. The composition according to claim 27, which does not cause weight reduction as a side effect.

29. The composition according to claim 26, which is used in the diagnosis of tumor.

30. The composition according to any one of claims 26 to 29, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

31. The composition according to any one of claims 26 to 30, wherein the tumor is a recurrent cancer or a metastatic cancer.

32. A tumor therapeutic agent, which comprises at least one type selected from the group consisting of the antibody according to any one of claims 1 to 17, the antibody fragment according to any one of claims 18 to 20 and the conjugate according to any one of claims 21 to 25.

33. The therapeutic agent according to claim 32, which does not cause weight reduction as a side effect.

34. The therapeutic agent according to claim 32 or 33, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

35. A tumor diagnostic agent, which comprises at least one type selected from the group consisting of the antibody according to any one of claims 1 to 17, the antibody fragment according to any one of claims 18 to 20 and the conjugate according to any one of claims 21 to 25.

36. The diagnostic agent according to claim 35, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

37. A method for detecting a tumor, which comprises: allowing at least one type selected from the group consisting of the antibody according to any one of claims 1 to 17, the antibody fragment according to any one of claims 18 to 20 and the conjugate according to any one of claims 21 to 25, to react with a sample collected from a living body; and then detecting a signal(s) of the reacted antibody and/or antibody fragment.

38. The method according to claim 37, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

39. A kit for treating, diagnosing or detecting a tumor, which comprises at least one type selected from the group consisting of the antibody according to any one of claims 1 to 17, the antibody fragment according to any one of claims 18 to 20 and the conjugate according to any one of claims 21 to 25.

40. The kit according to claim 39, wherein the tumor is at least one type selected from the group consisting of human pancreatic cancer, human prostate cancer, human colorectal cancer, human breast cancer, human ovarian cancer, human lung cancer and human bile duct cancer.

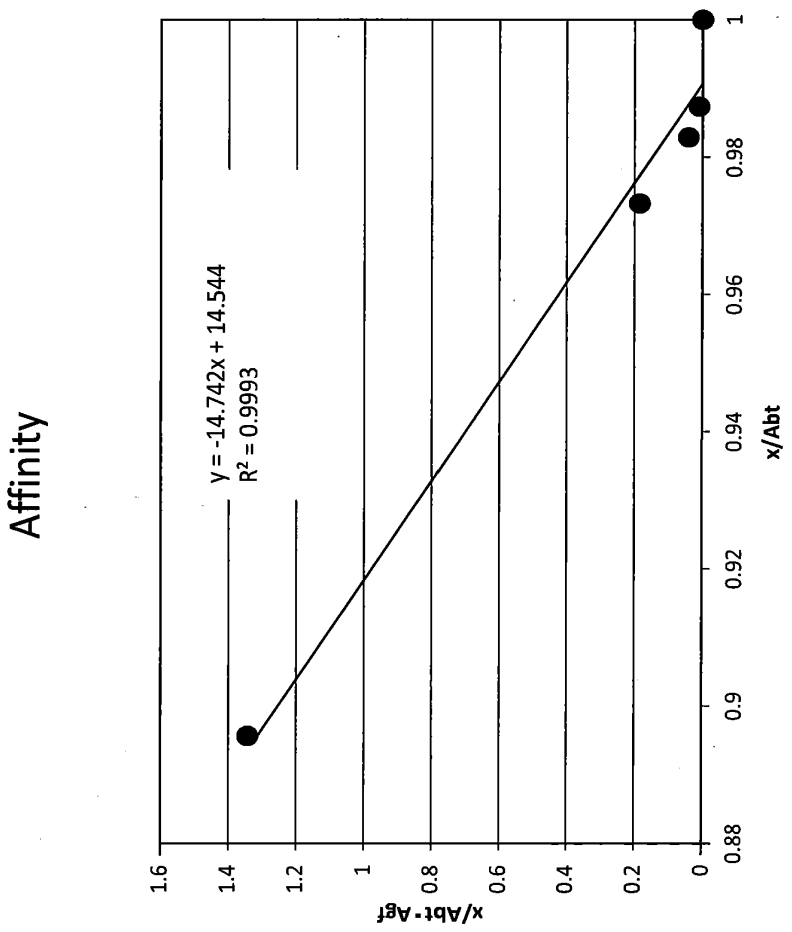
41. A polynucleotide encoding the antibody according to any one of claims 1 to 17.

42. A polynucleotide encoding the antibody fragment according to any one of claims 18 to 20.

43. A recombinant vector comprising the polynucleotide according to claim 41 or 42.

44. A transformant comprising the recombinant vector according to claim 43.

Fig.1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R² = 0.9993
x/Abt
x/Abt-Agf
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0.98
0.96
0.94
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1.2
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0.2
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Fig.2

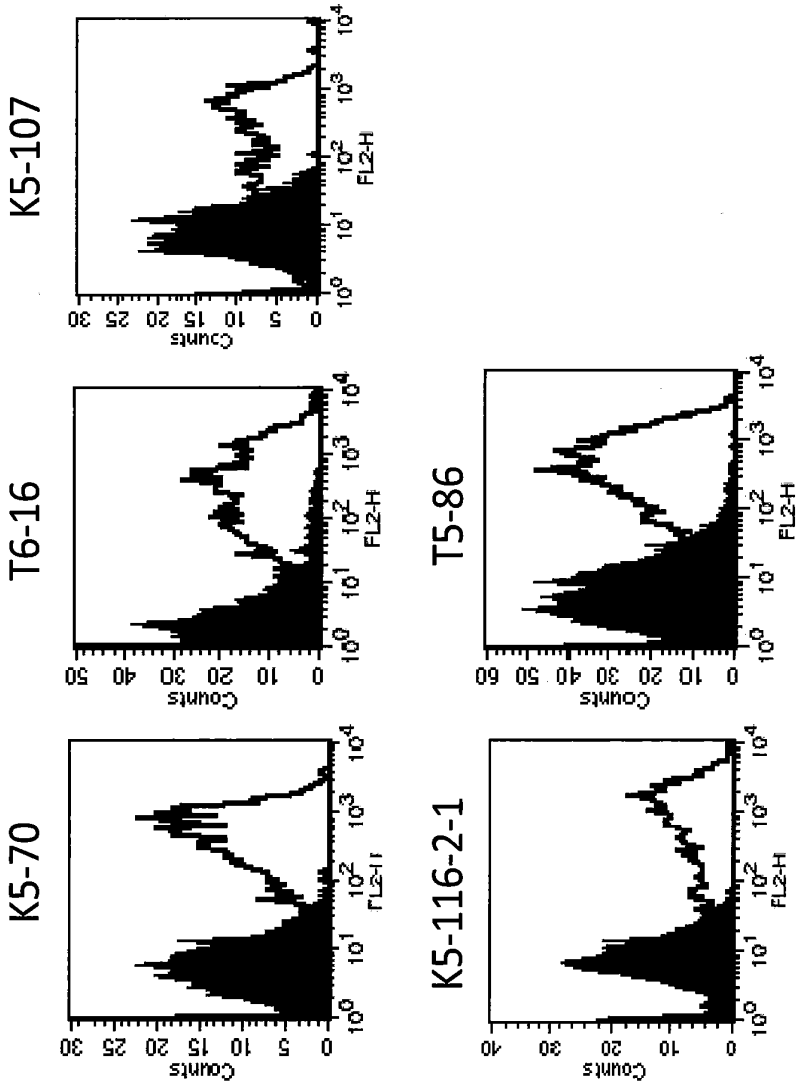

Fig.3

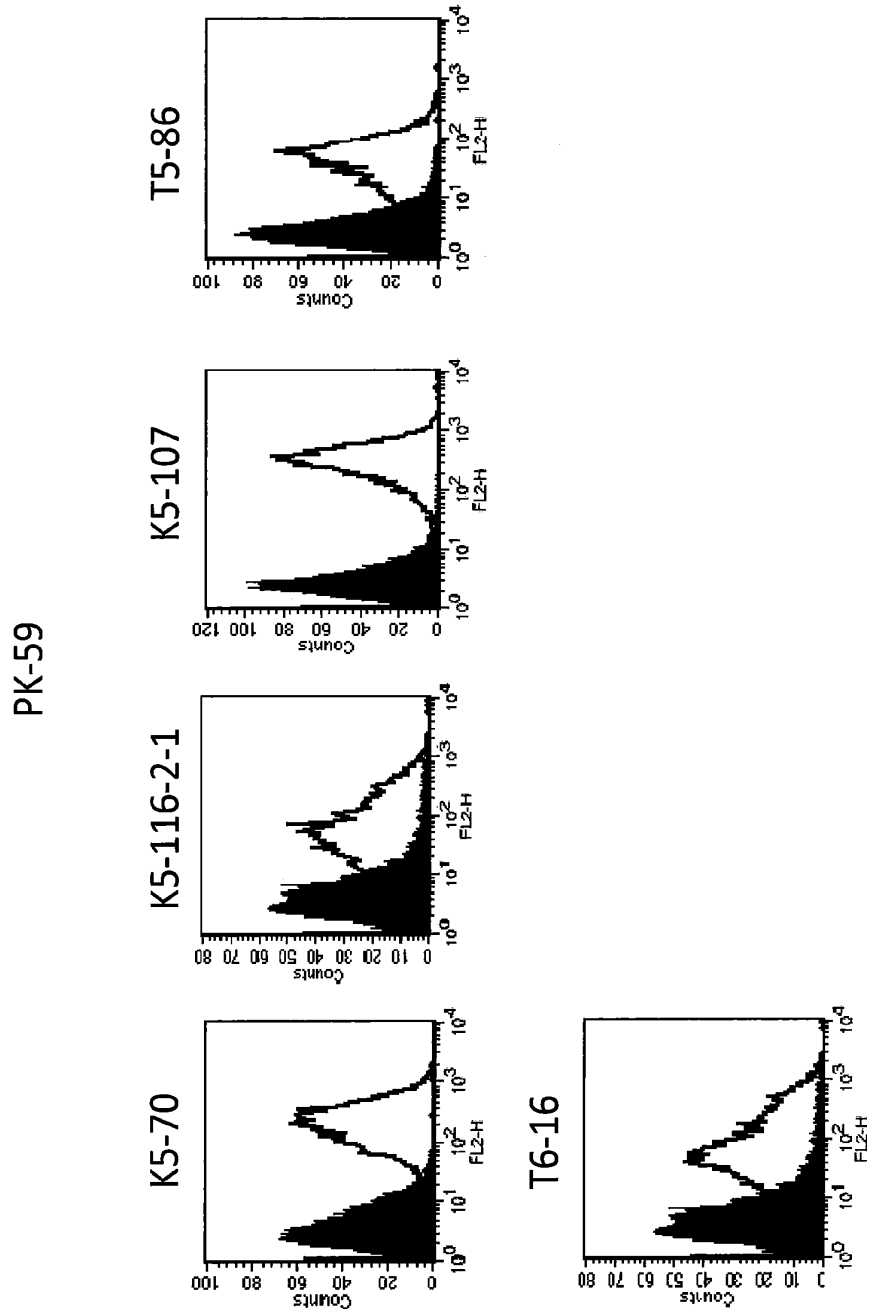

Fig.4

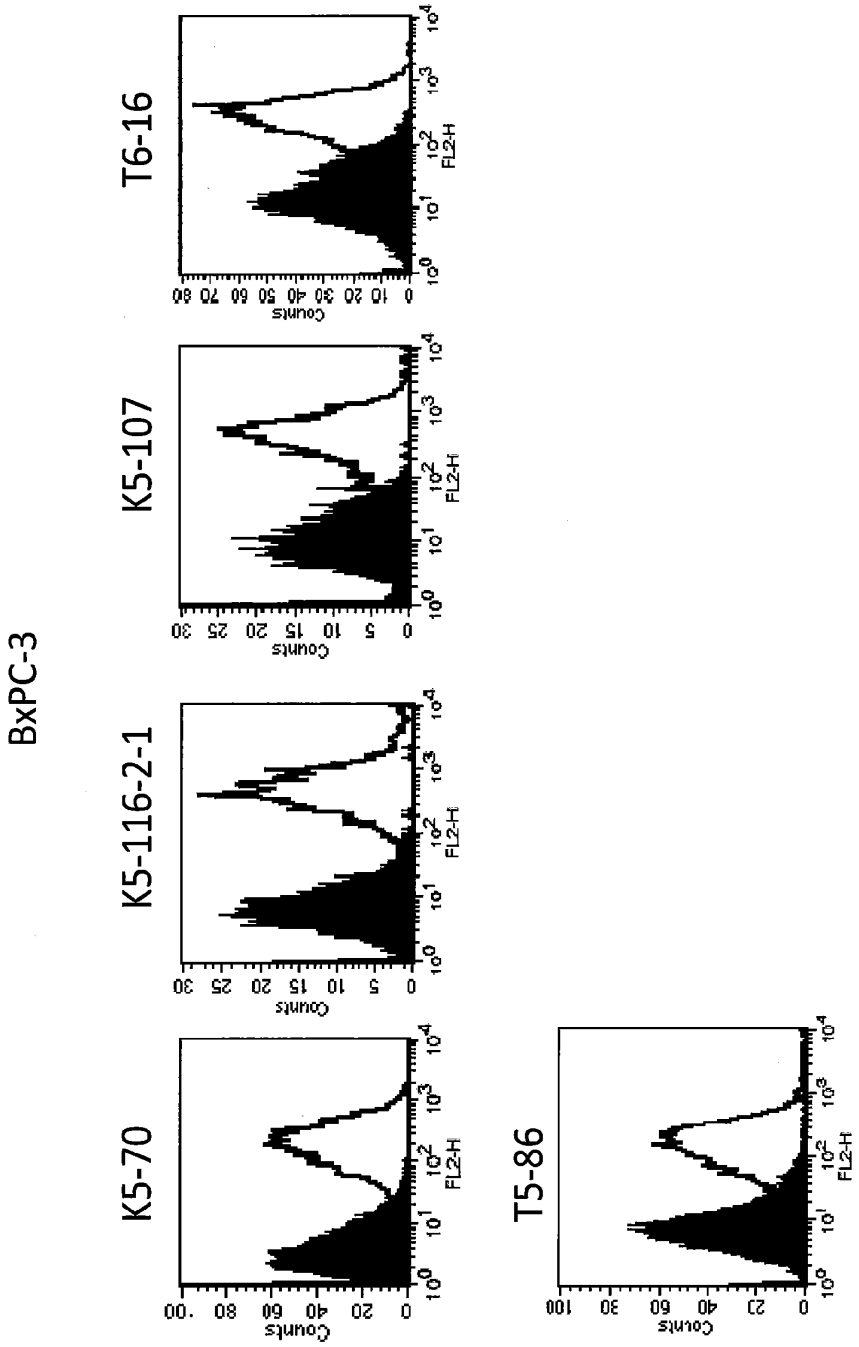

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Fig.5

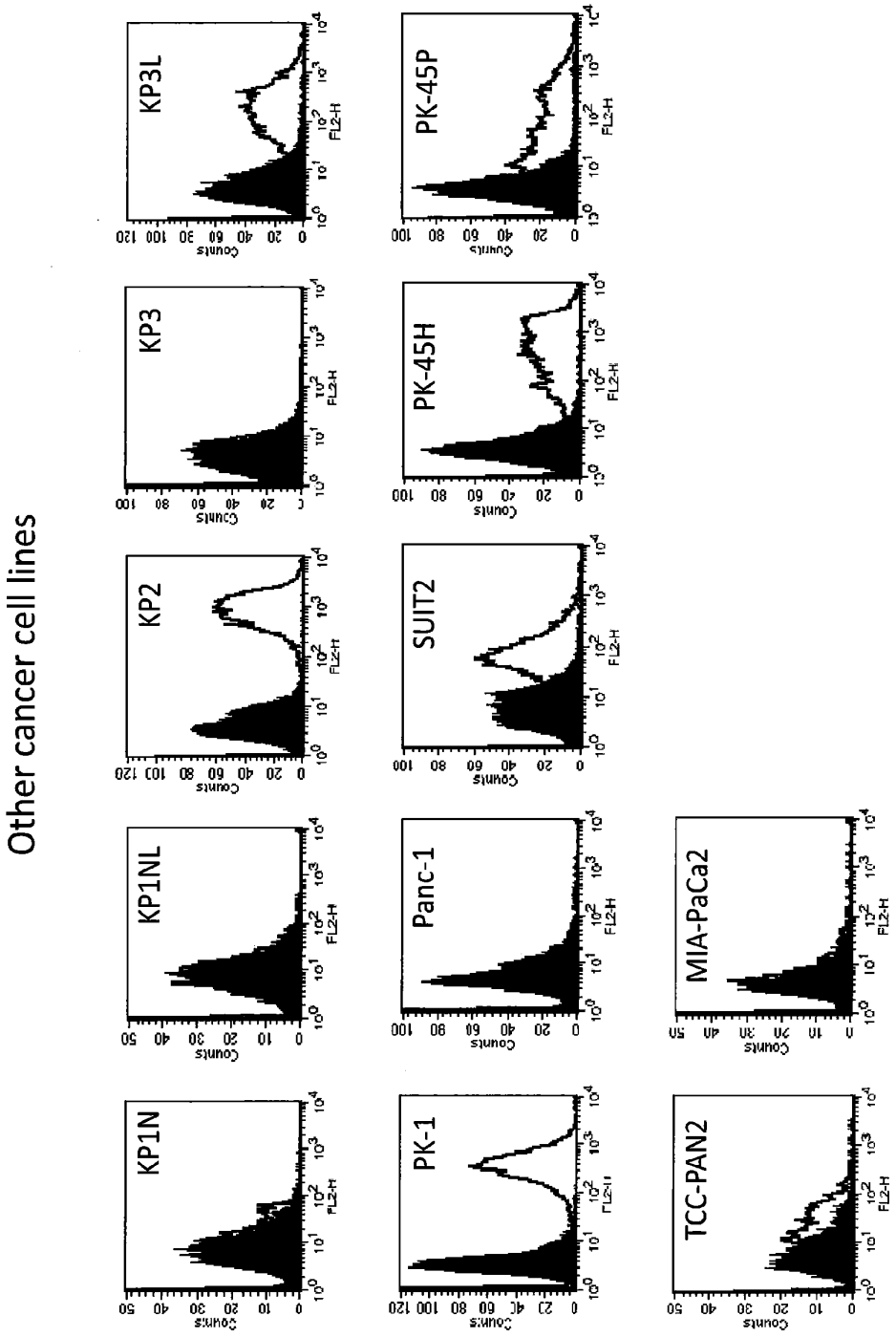

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Fig.6

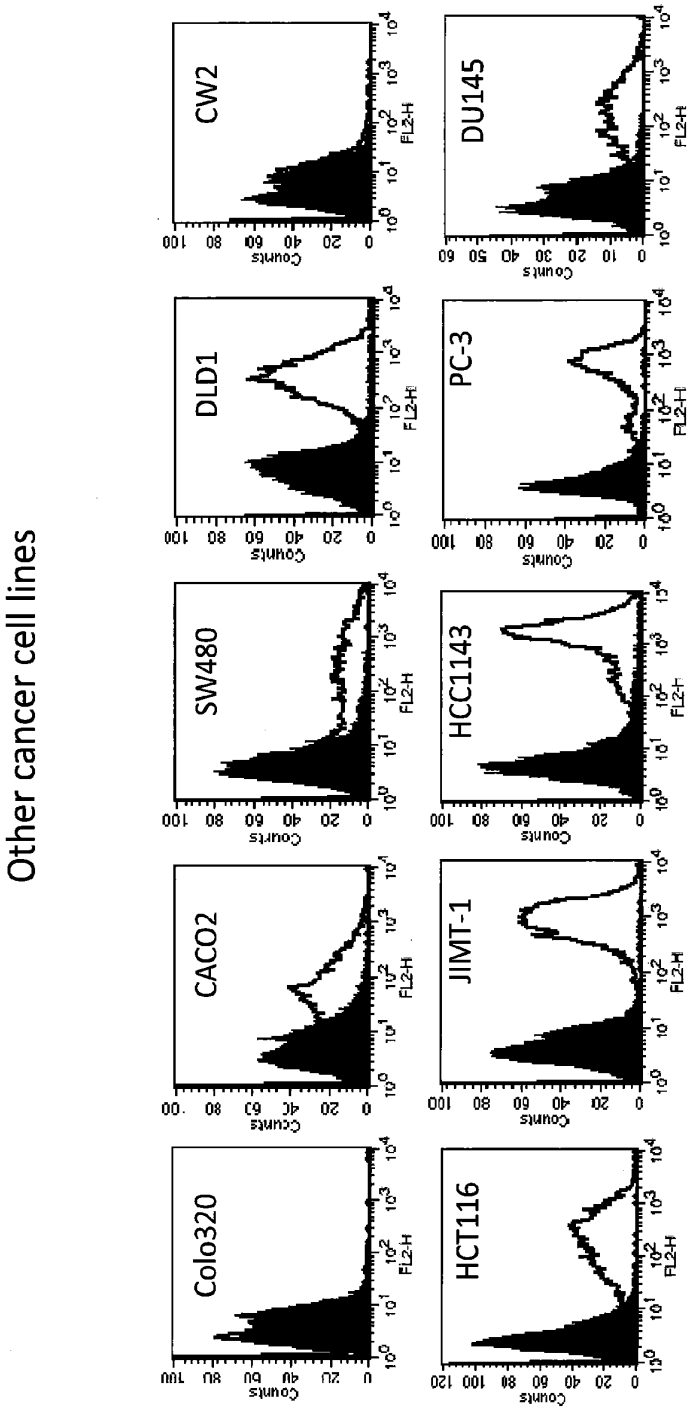

Fig.7

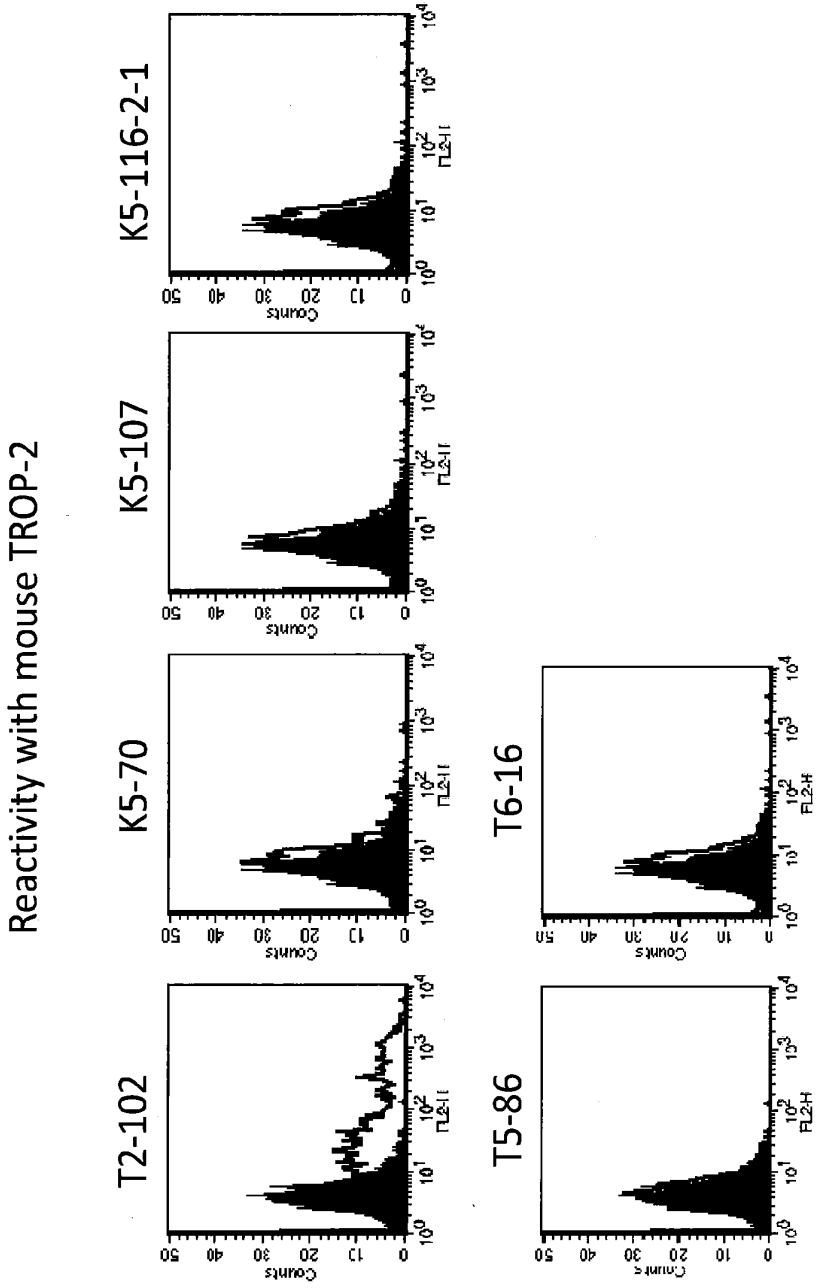
Reactivity with mouse TROP-2
K5-116-2-1
K5-107
K5-70
T2-102
T6-16
T5-86
Counts
FL2-H

Fig.8

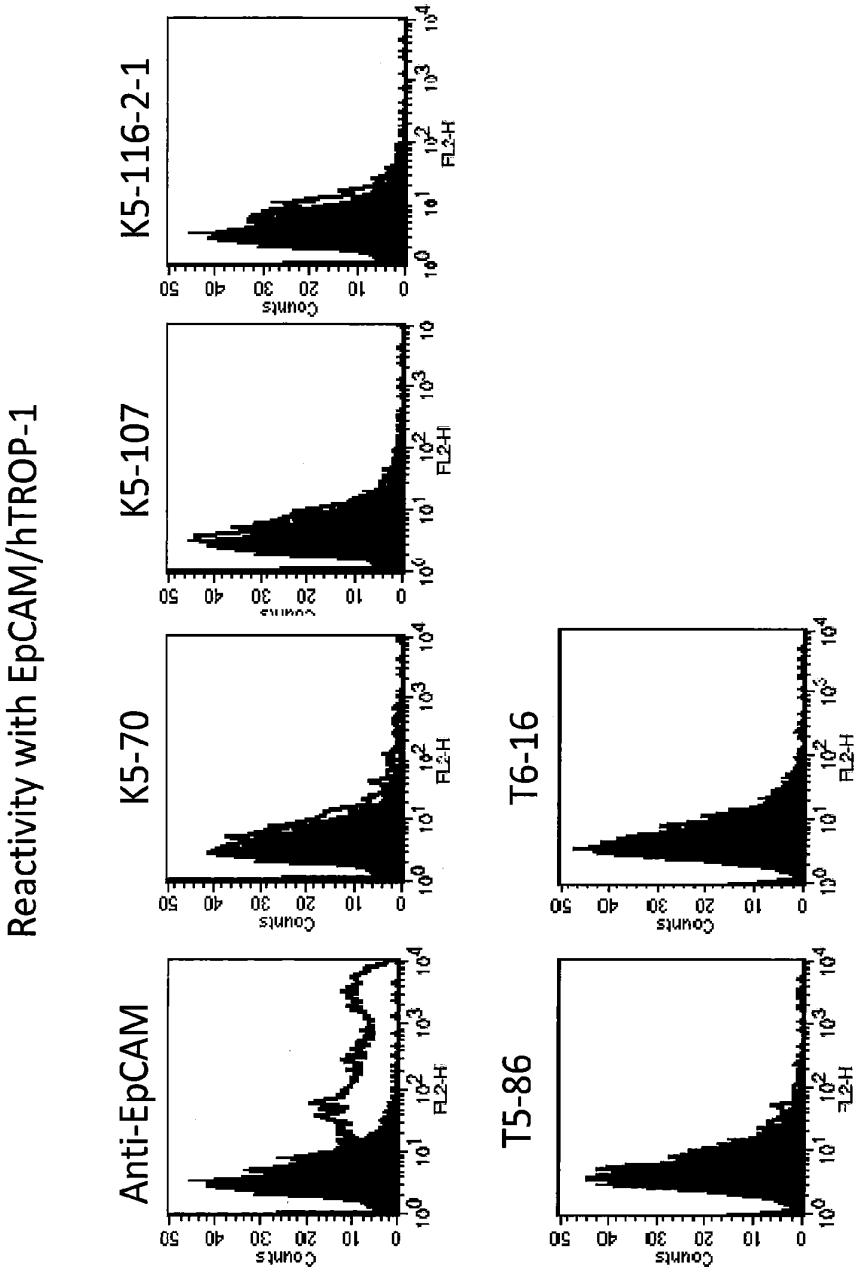
Reactivity with EpCAM/hTROP-1
Anti-EpCAM
K5-70
K5-107
K5-116-2-1
T5-86
T6-16
Counts
FL2-H

Fig.9

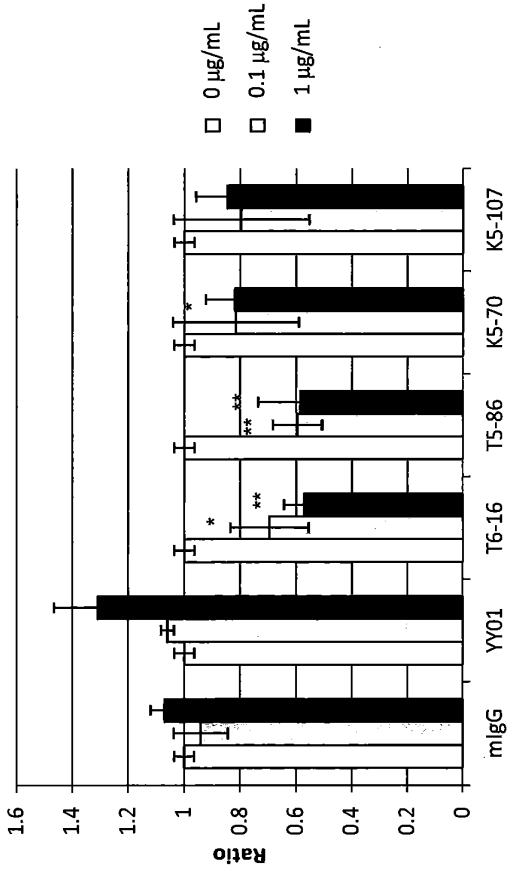
0 µg/mL
0.1 µg/mL
1 µg/mL
Ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mIgG
YY01
T6-16
T5-86
K5-70
K5-107
*
**
**
*
**

Fig.10

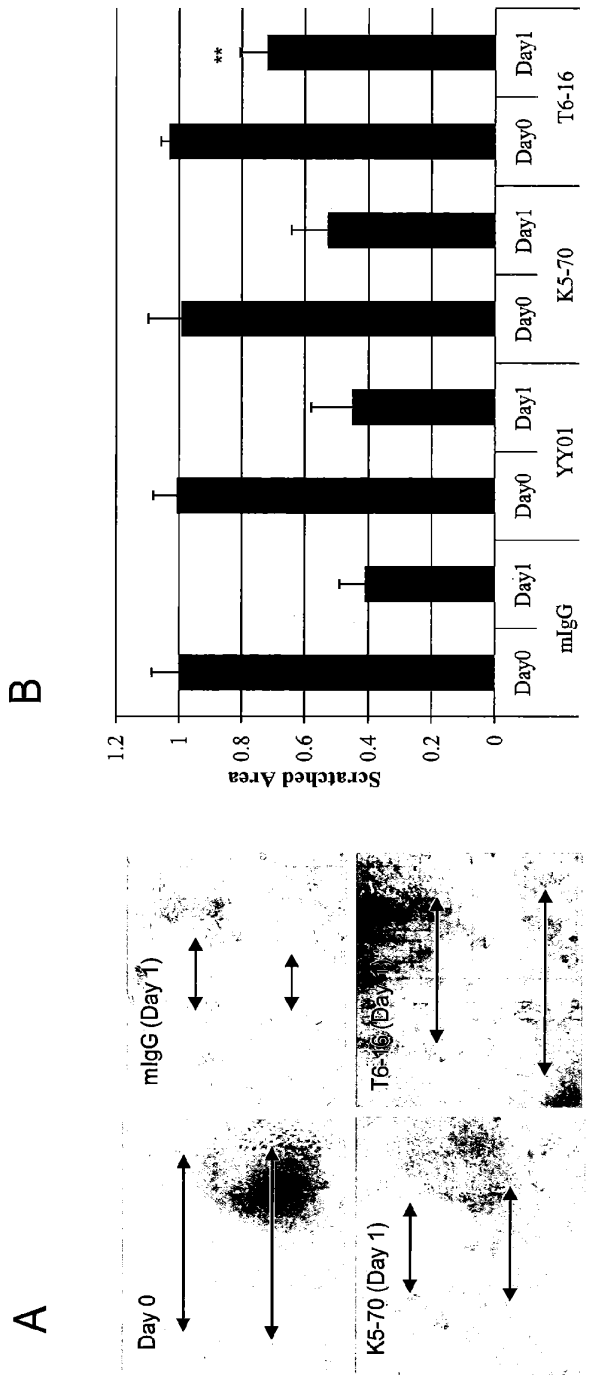
A
Day 0
mIgG (Day 1)
K5-70 (Day 1)
T6-16 (Day 1)
B
Scratched Area
1.2
1
0.8
0.6
0.4
0.2
0
**
mIgG
YY01
K5-70
T6-16
Day0
Day1

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Fig.11

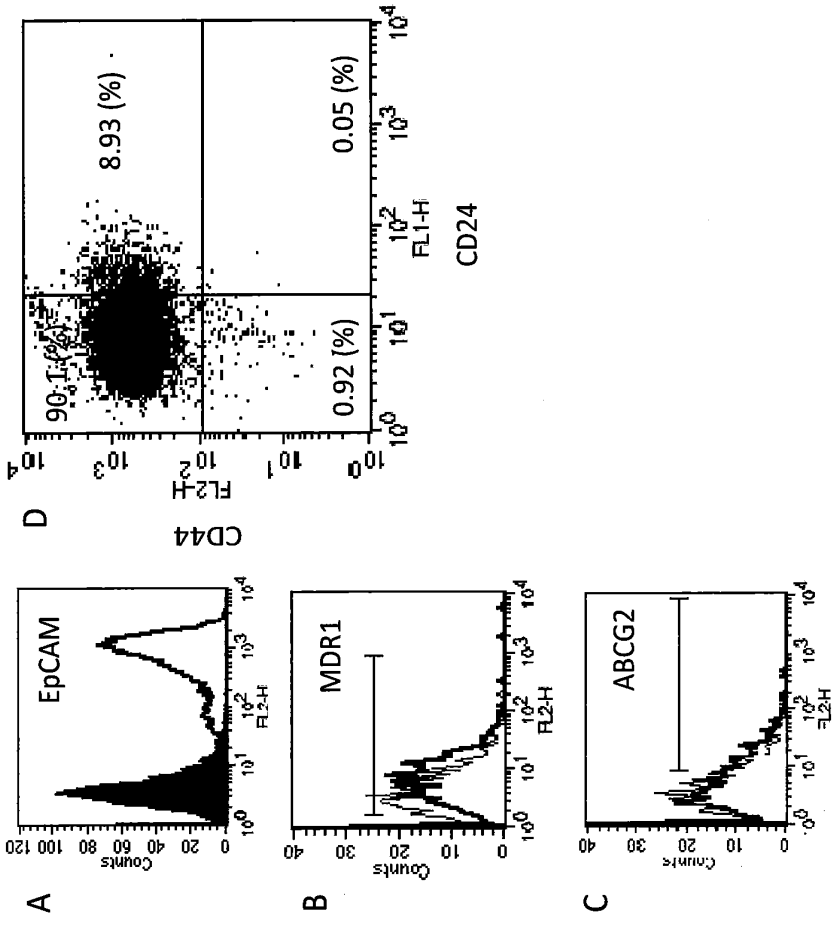

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Fig.12

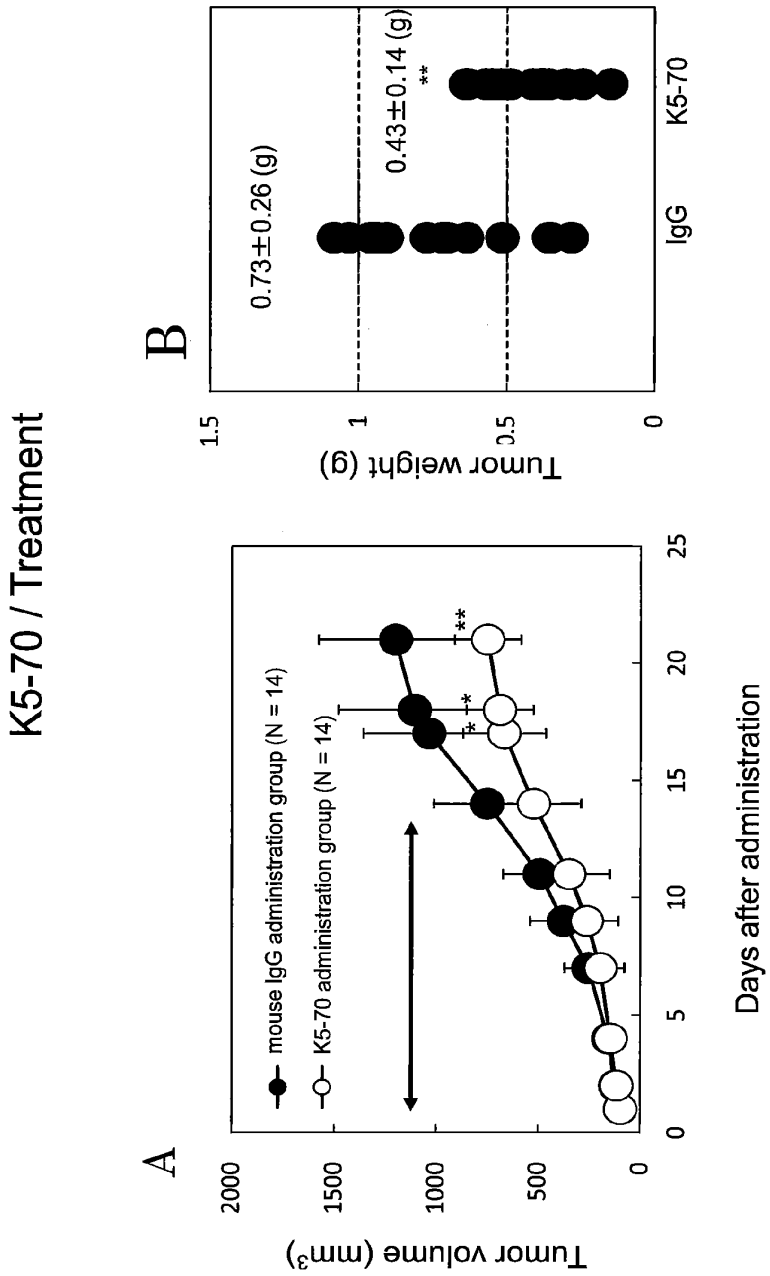

Fig.13

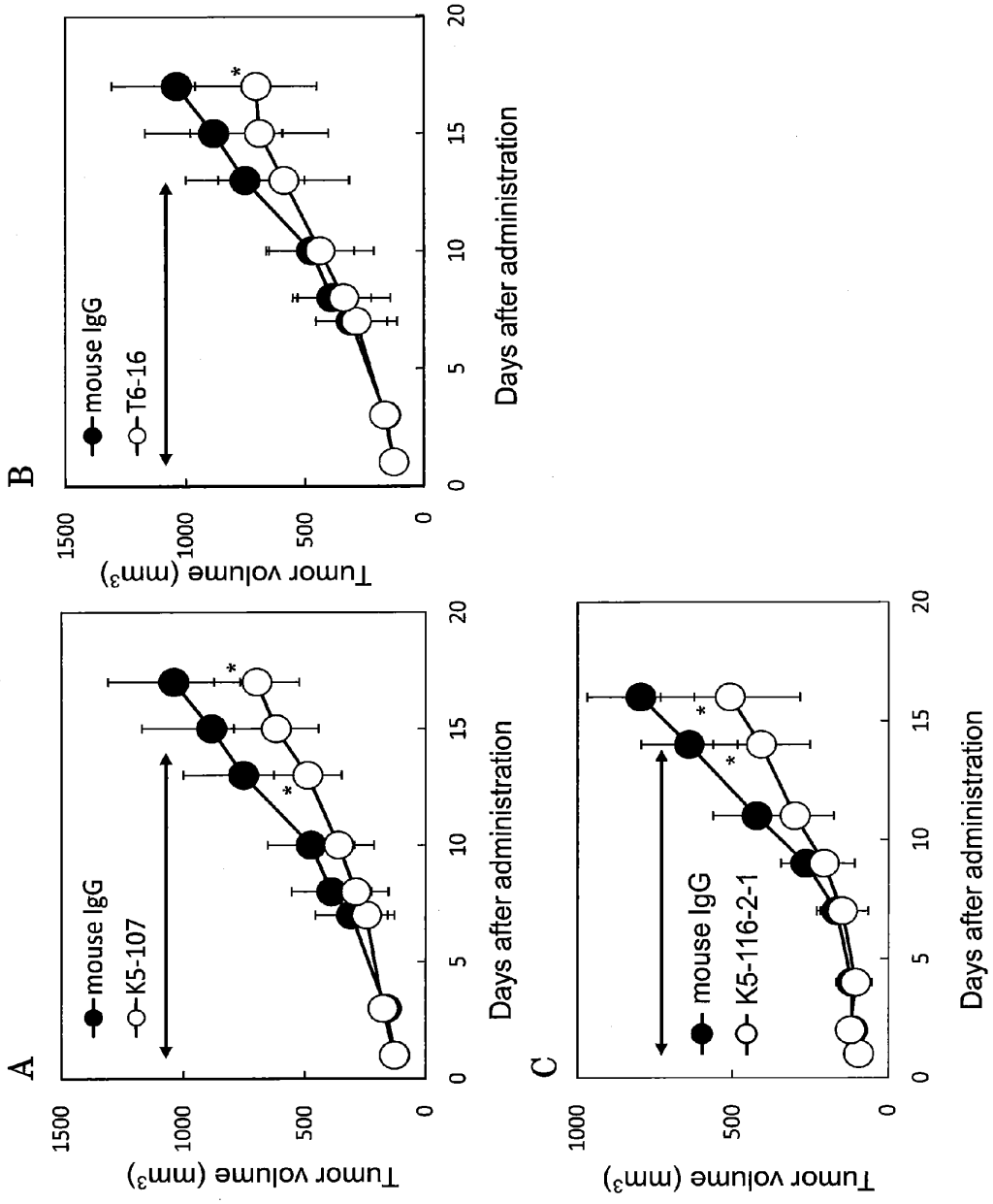
A
B
C
mouse IgG
K5-107
T6-16
K5-116-2-1
Tumor volume (mm3)
Days after administration
0
500
1000
1500
5
10
15
20
*

Fig.14

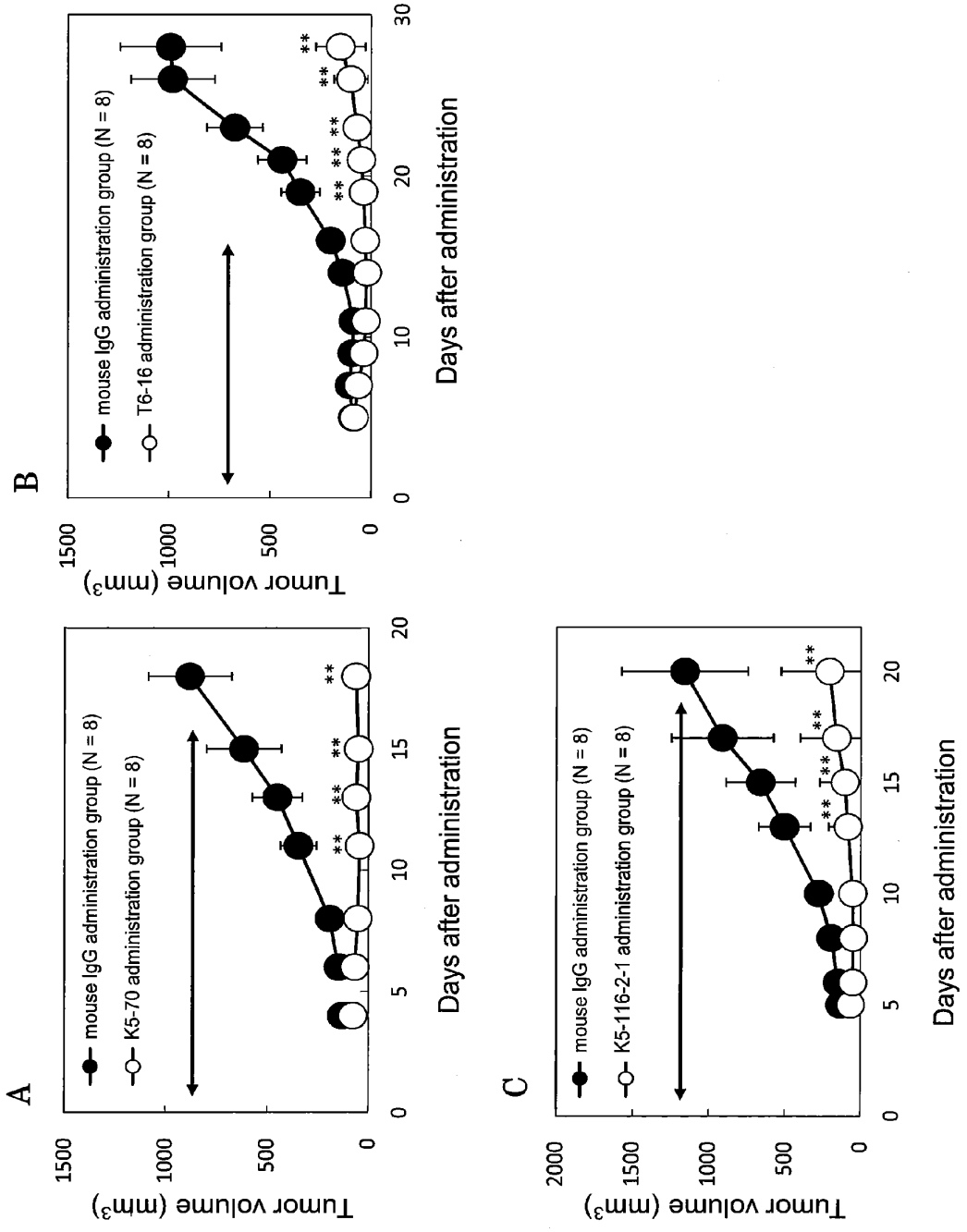
A
B
C
Tumor volume (mm3)
Days after administration
mouse IgG administration group (N = 8)
K5-70 administration group (N = 8)
T6-16 administration group (N = 8)
K5-116-2-1 administration group (N = 8)
**

Fig.15

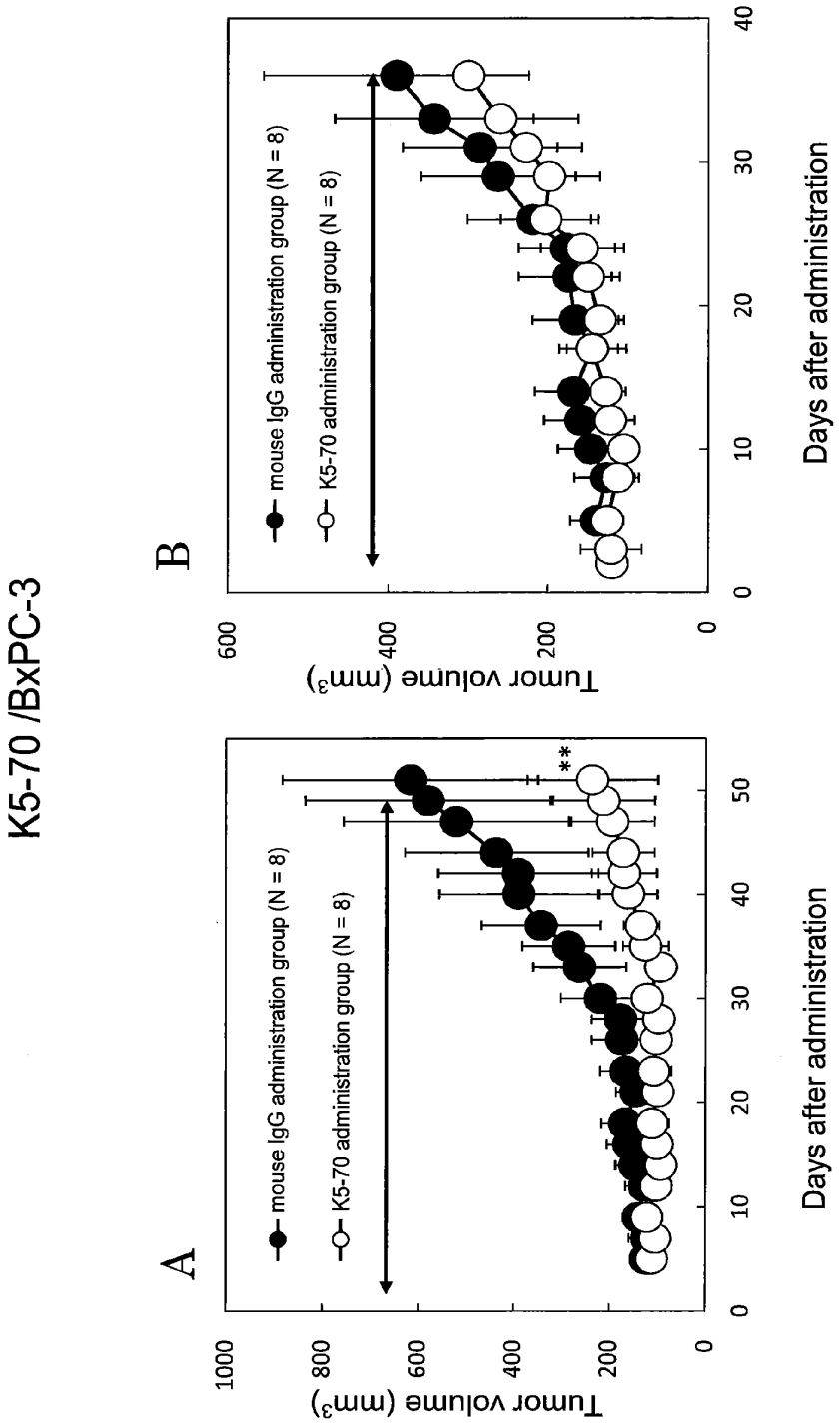
K5-70 /BxPC-3
A
B
mouse IgG administration group (N = 8)
K5-70 administration group (N = 8)
Tumor volume (mm3)
Days after administration
**
0
200
400
600
800
1000
10
20
30
40
50

Fig.16

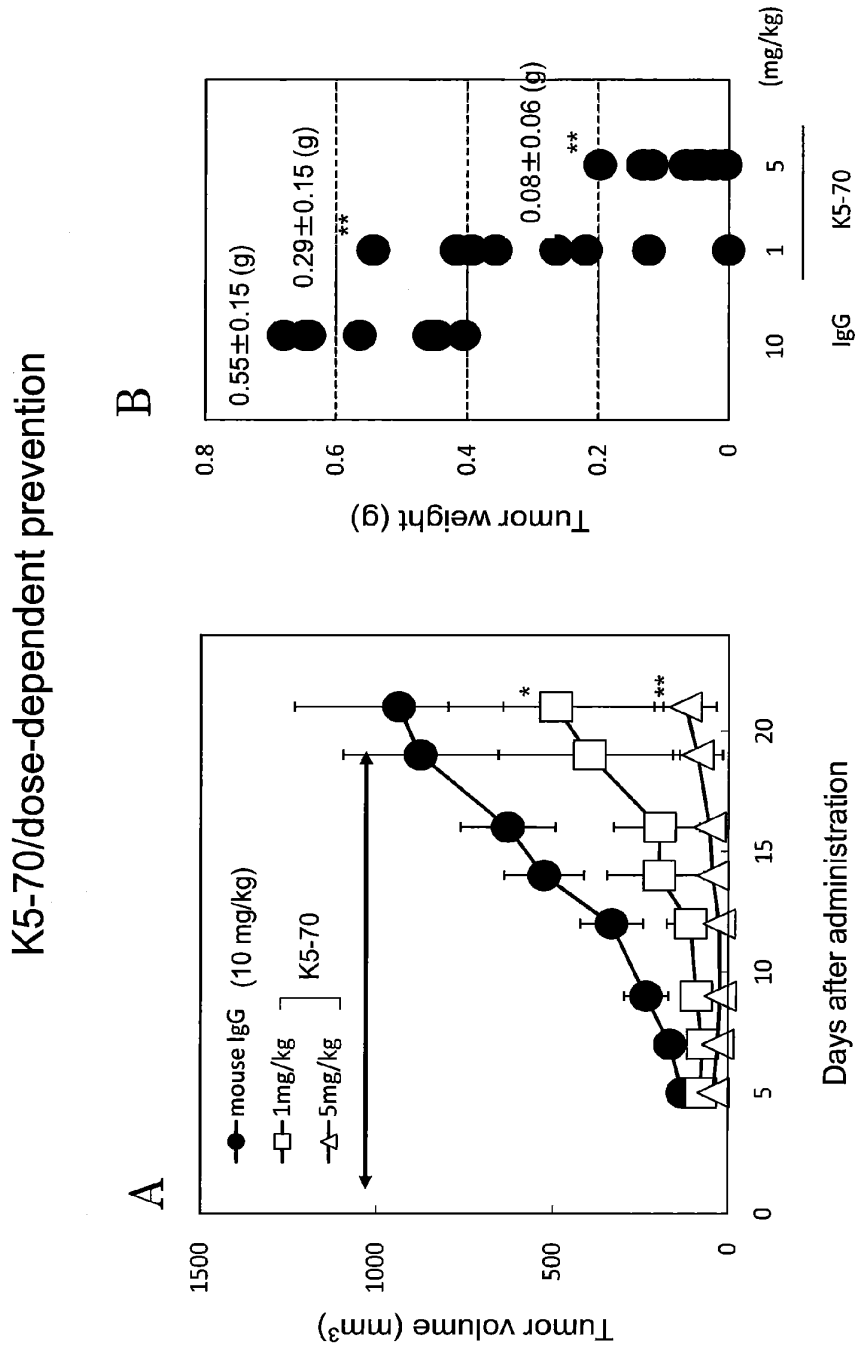
K5-70/dose-dependent prevention
A
mouse IgG (10 mg/kg)
1mg/kg
5mg/kg
K5-70
Tumor volume (mm3)
Days after administration
B
0.55±0.15 (g)
0.29±0.15 (g)
0.08±0.06 (g)
Tumor weight (g)
IgG
K5-70
(mg/kg)

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Fig.17

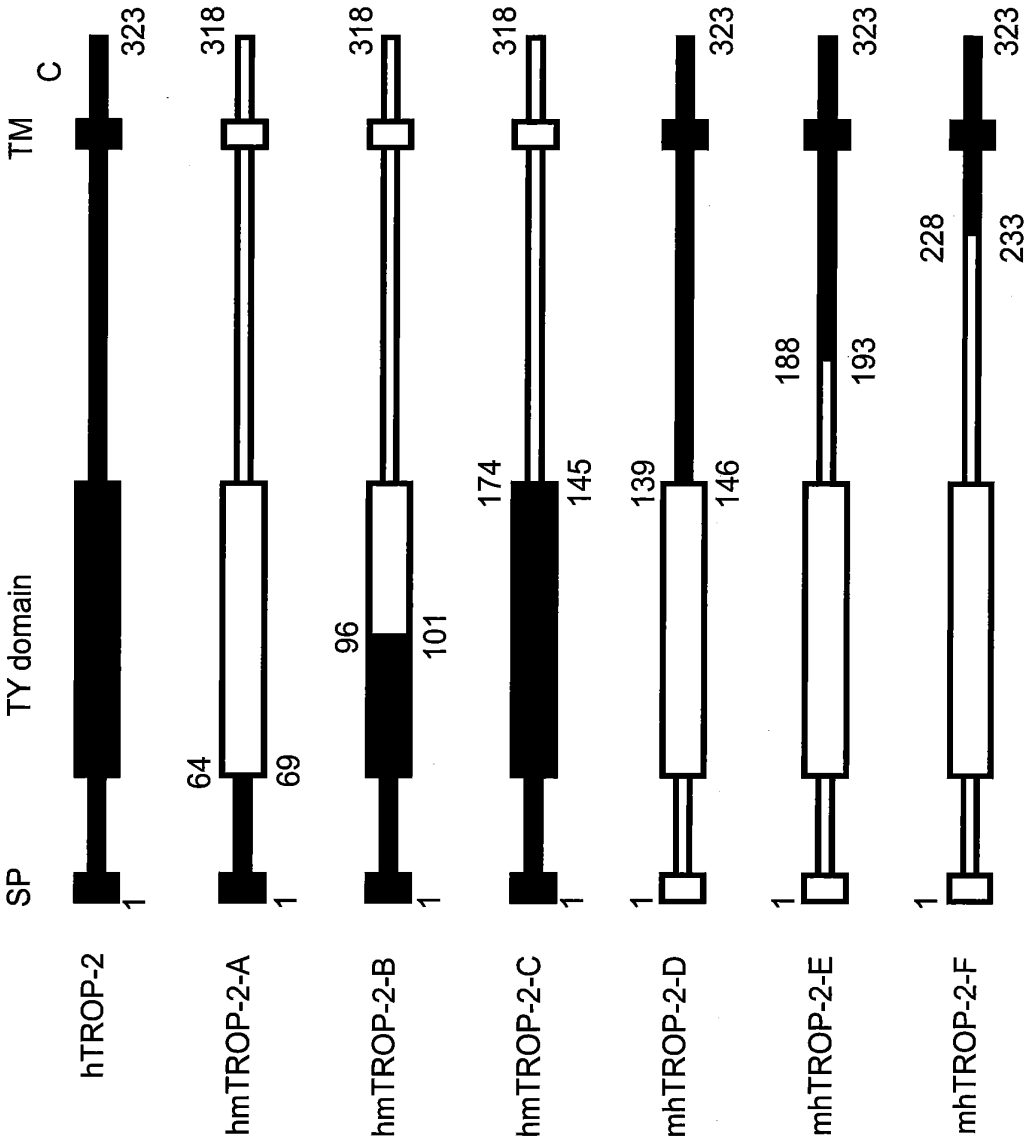

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Fig.18

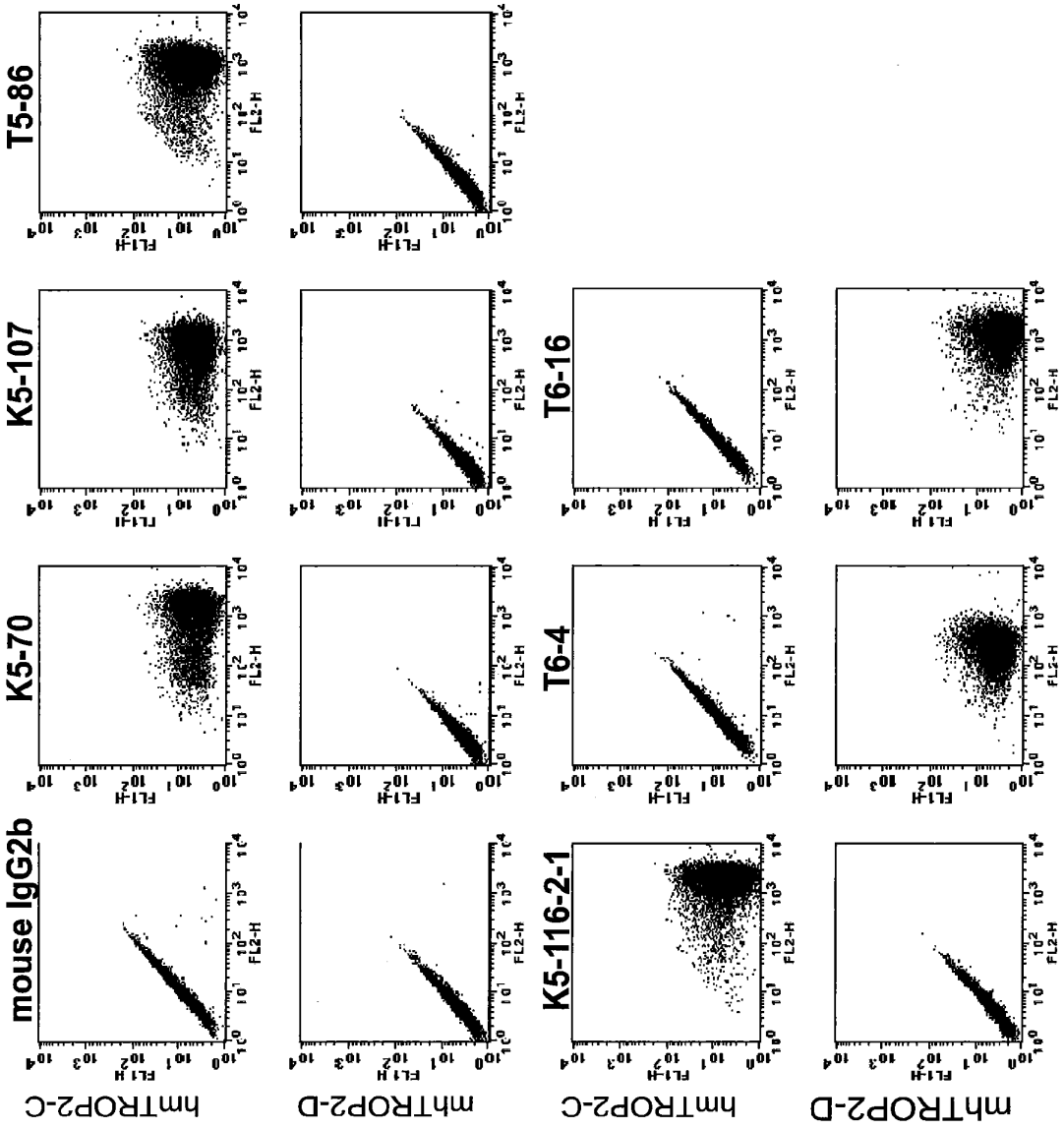

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Fig.19

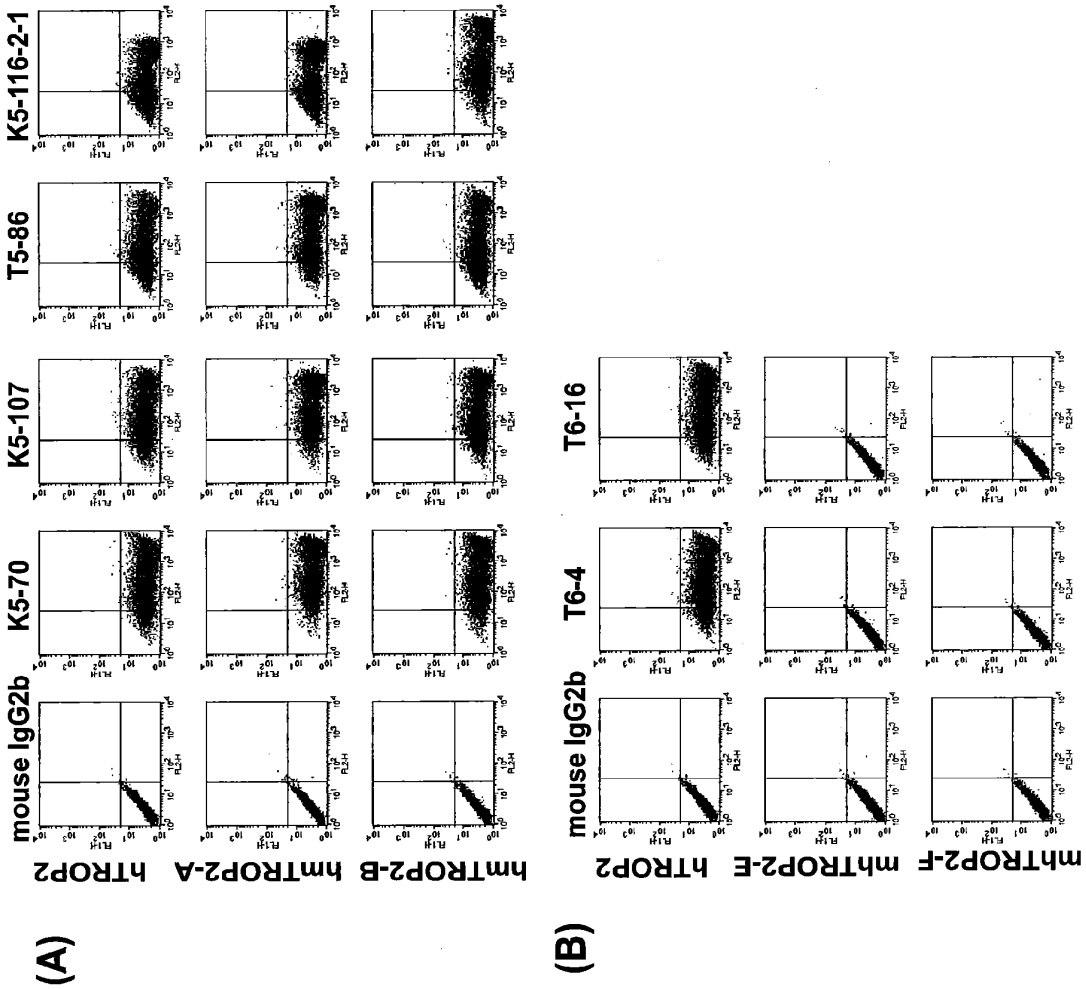

Fig.20

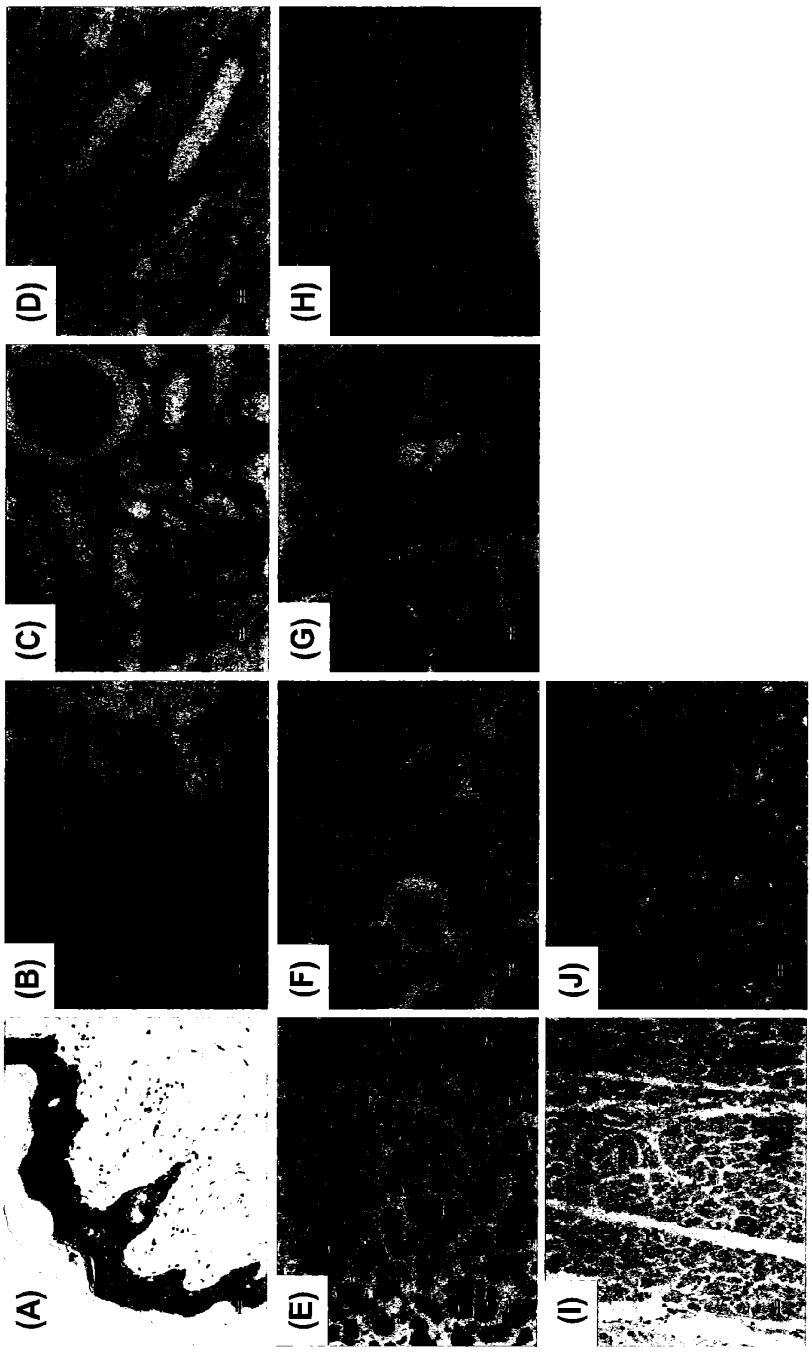

Fig.21

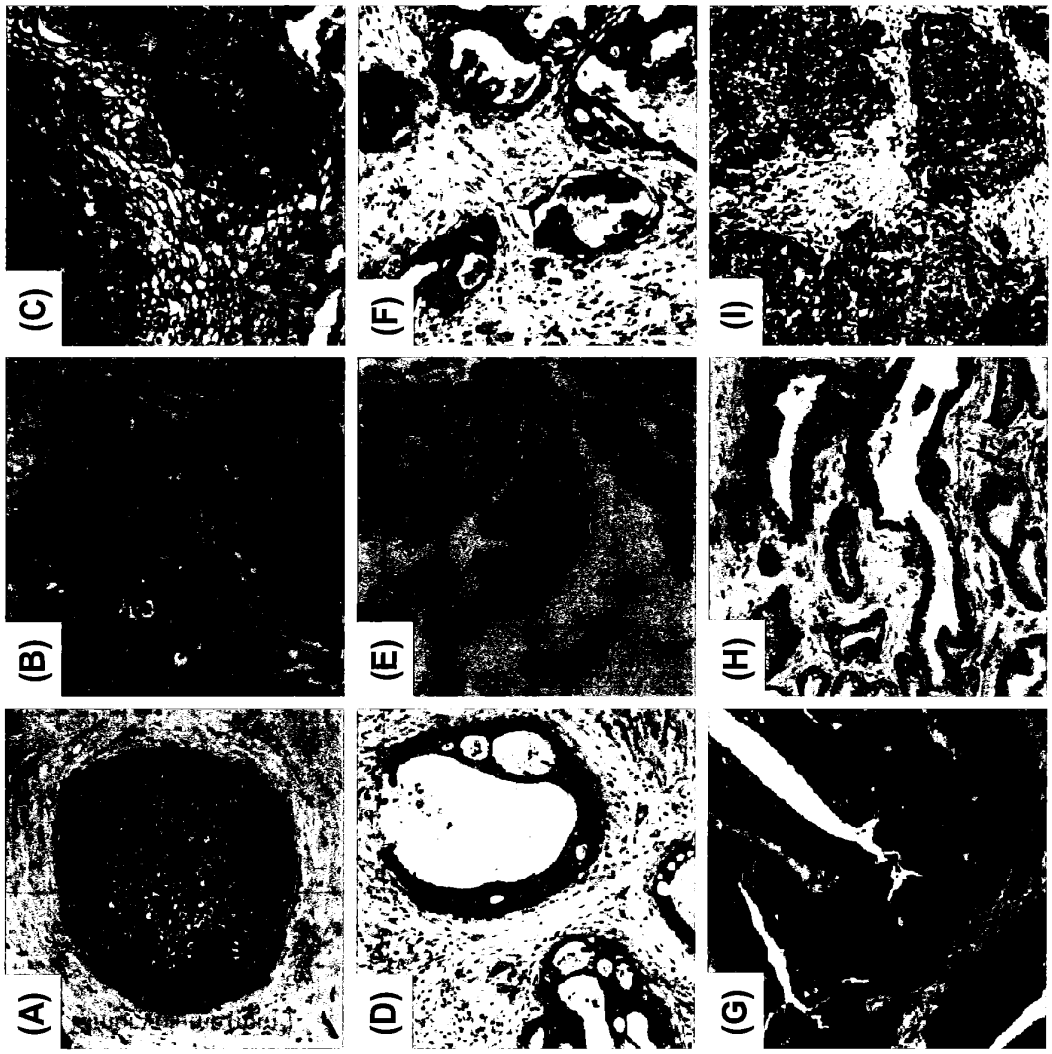

Fig.22

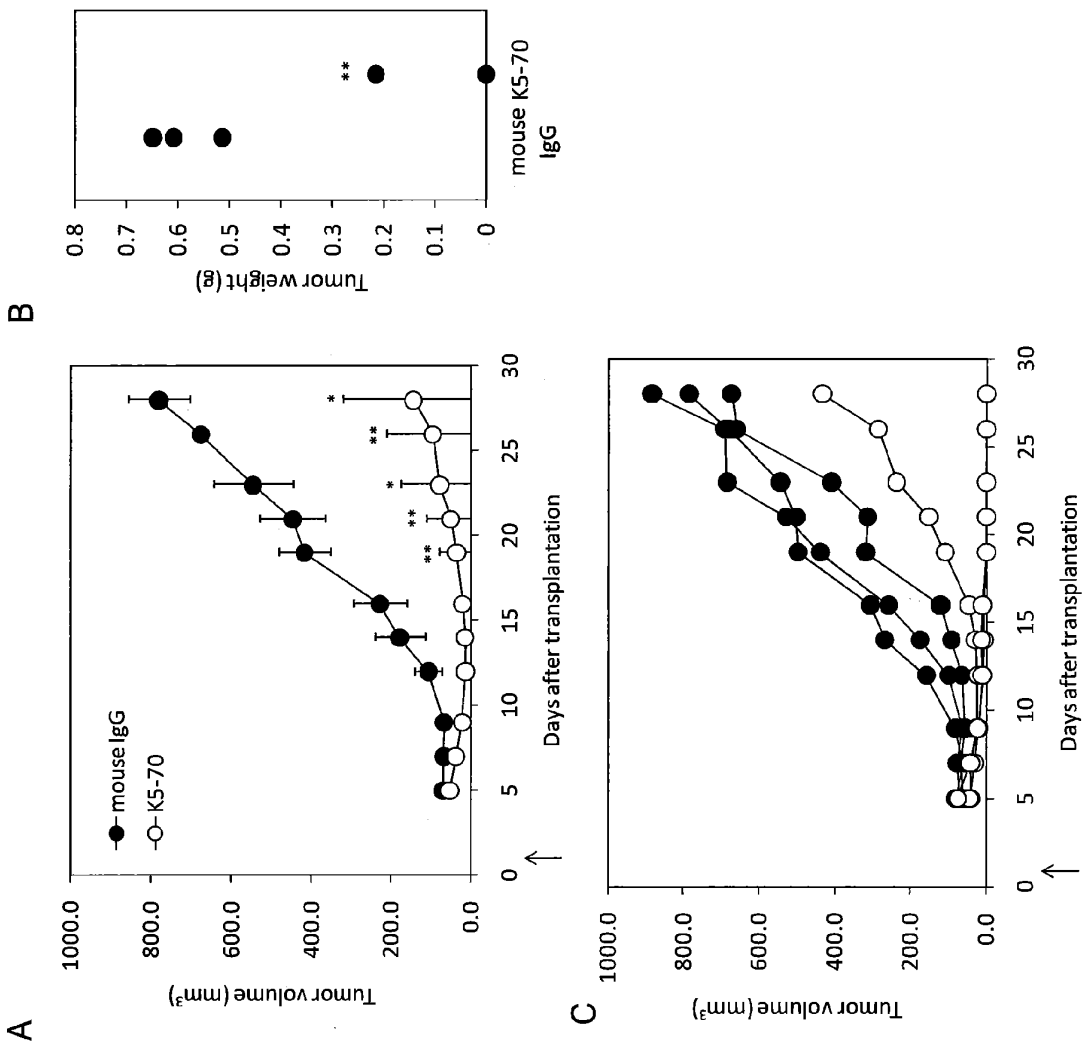
A
B
C
Tumor volume (mm³)
Days after transplantation
mouse IgG
K5-70
Tumor weight (g)
mouse K5-70 IgG

Fig.23

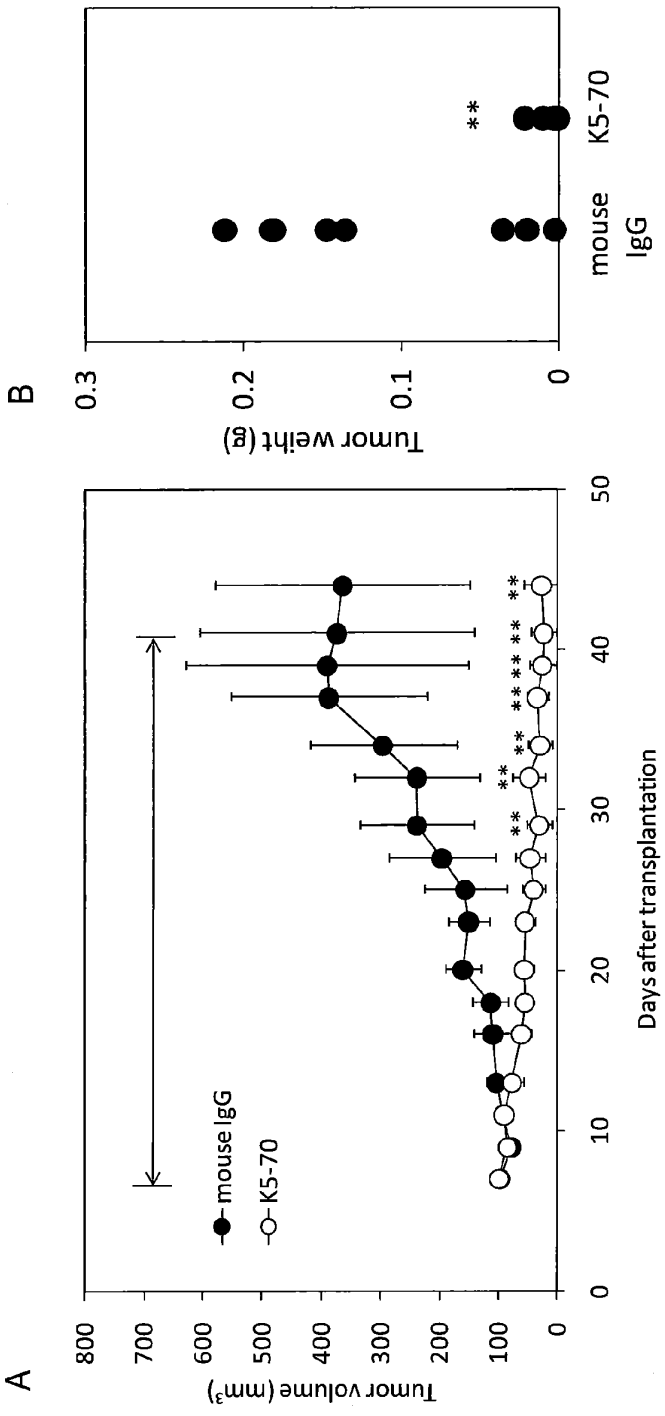
A
Tumor volume (mm3)
800
700
600
500
400
300
200
100
0
mouse IgG
K5-70
0
10
20
30
40
50
Days after transplantation
B
Tumor weight (g)
0.3
0.2
0.1
0
mouse IgG
K5-70
**

Fig.24

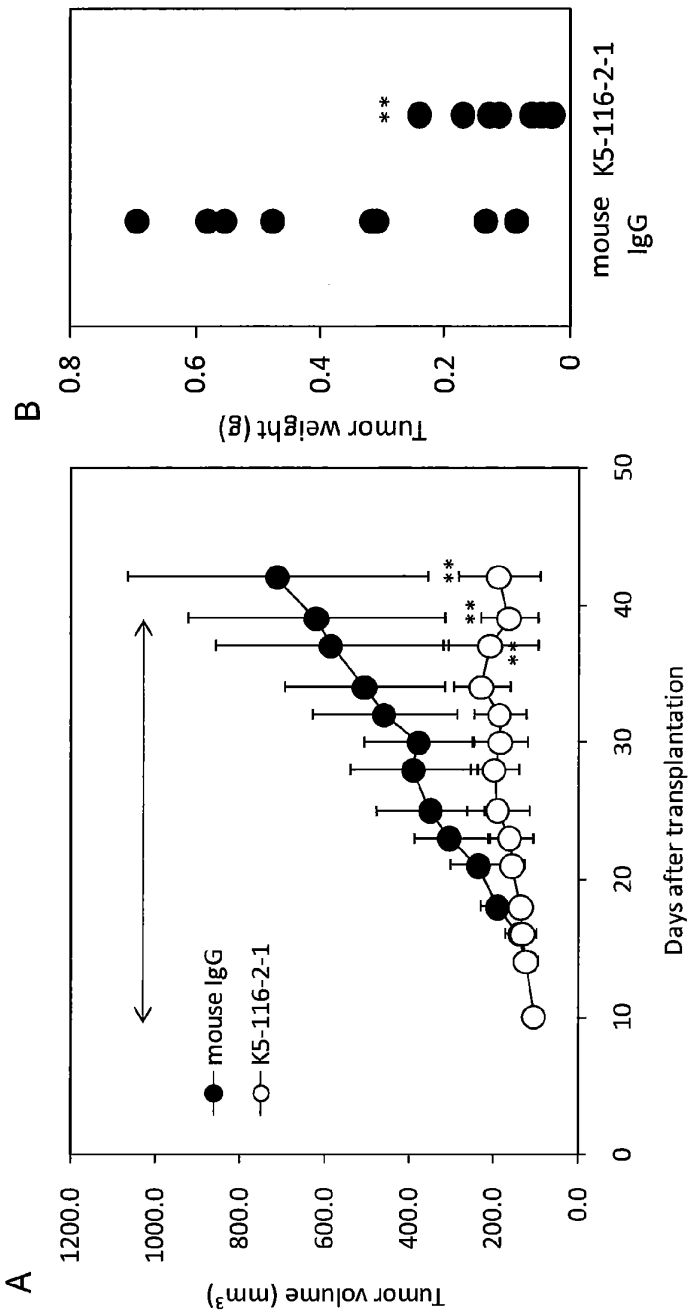
A
Tumor volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
K5-116-2-1
**
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0
mouse IgG
K5-116-2-1

Fig.25

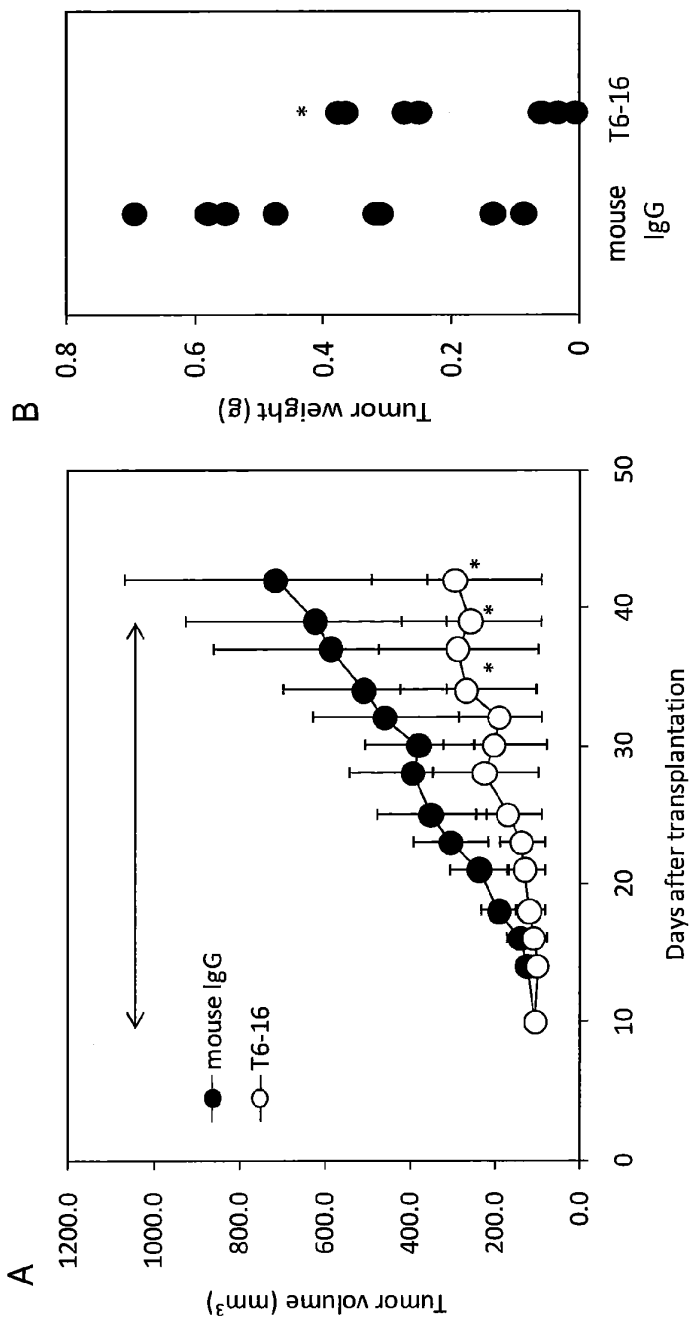
A
Tumor volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
T6-16
*
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0
mouse IgG
T6-16
*

Fig.26

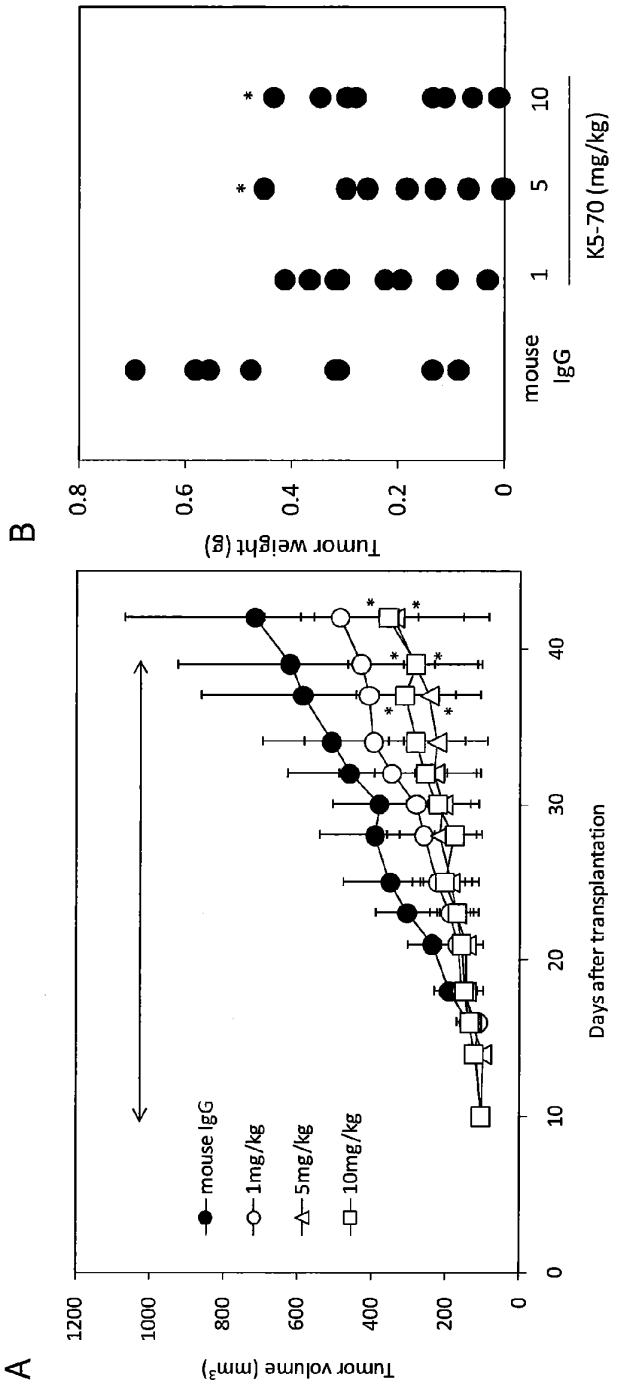
A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
Days after transplantation
mouse IgG
1mg/kg
5mg/kg
10mg/kg
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0
mouse IgG
1
5
10
K5-70 (mg/kg)

Fig.27

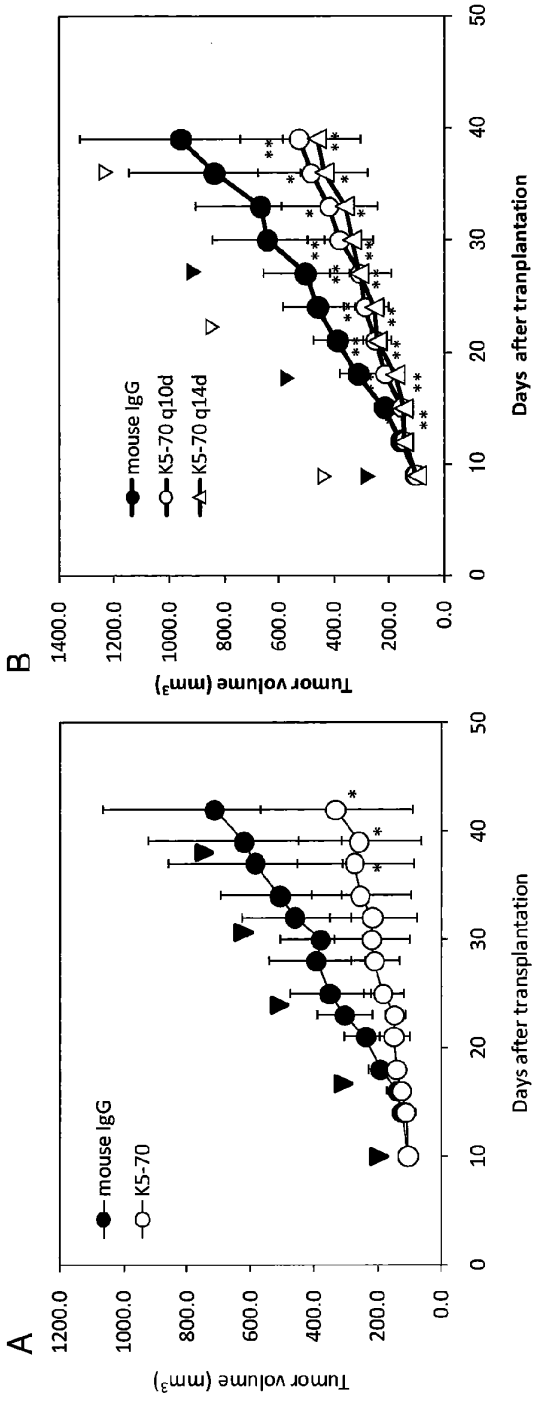
A
Tumor volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
K5-70
B
Tumor volume (mm3)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after tranplantation
mouse IgG
K5-70 q10d
K5-70 q14d

Fig.28

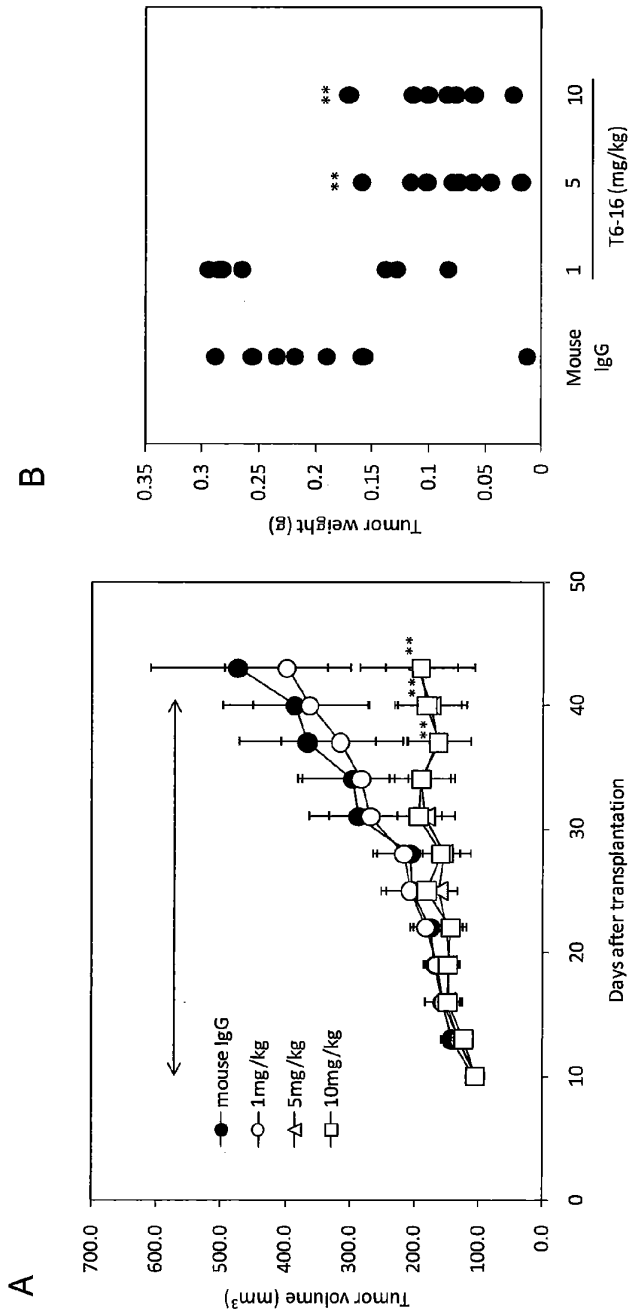
A
Tumor volume (mm3)
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
1mg/kg
5mg/kg
10mg/kg
**
B
Tumor weight (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Mouse IgG
1
5
10
T6-16 (mg/kg)

Fig.29

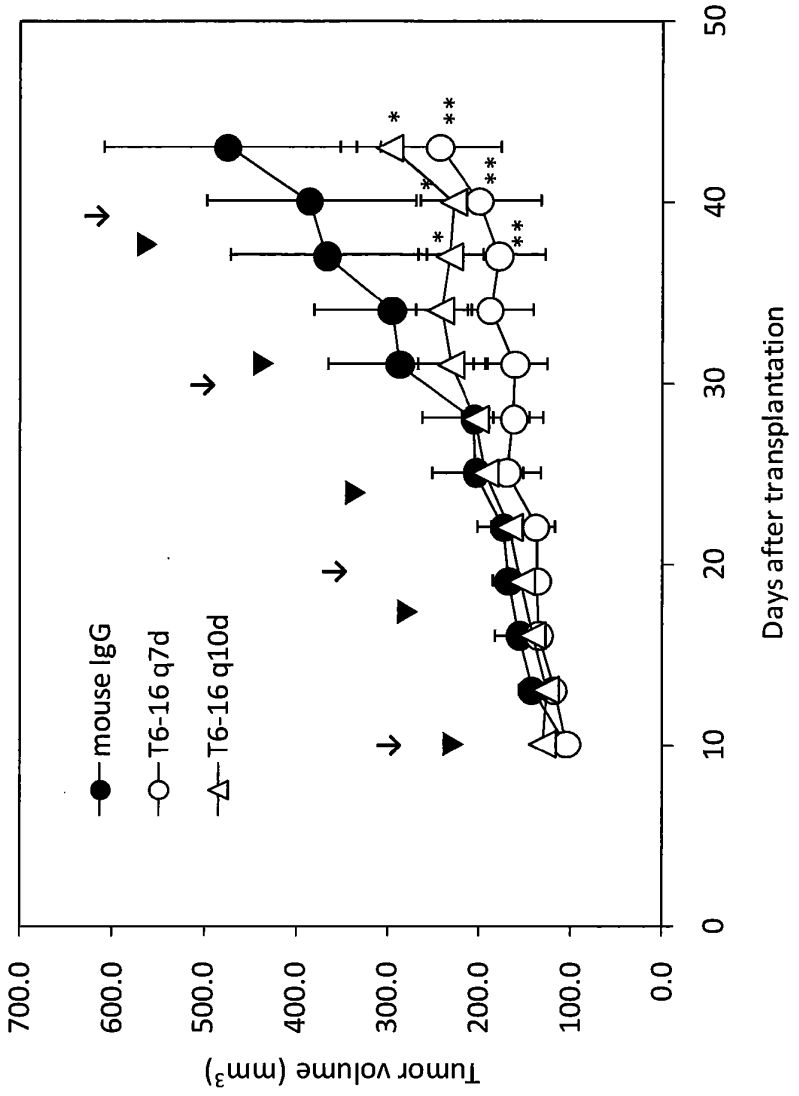
Tumor volume (mm3)
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
T6-16 q7d
T6-16 q10d
*
**

Fig.30

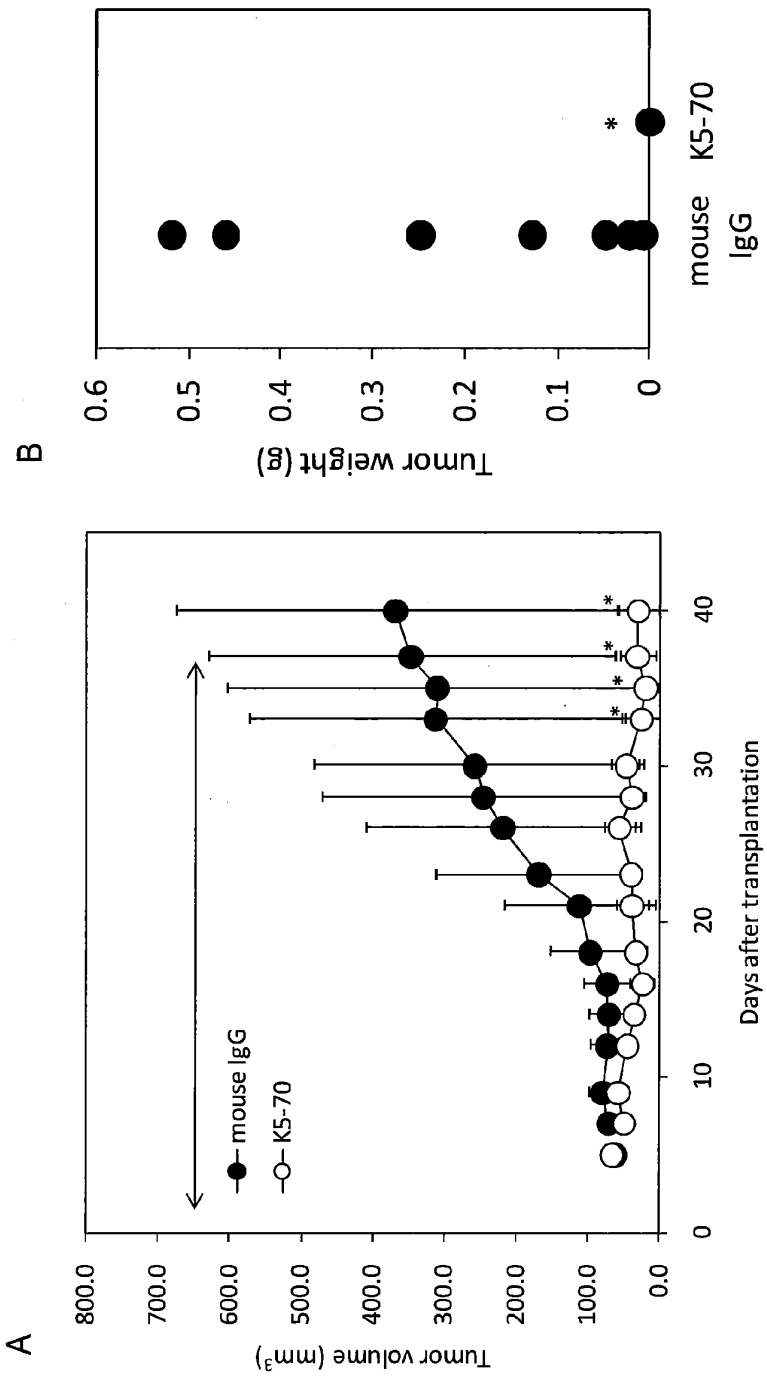
A
Tumor volume (mm3)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0
10
20
30
40
Days after transplantation
mouse IgG
K5-70
*
B
Tumor weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
mouse IgG
K5-70
*

Fig.31

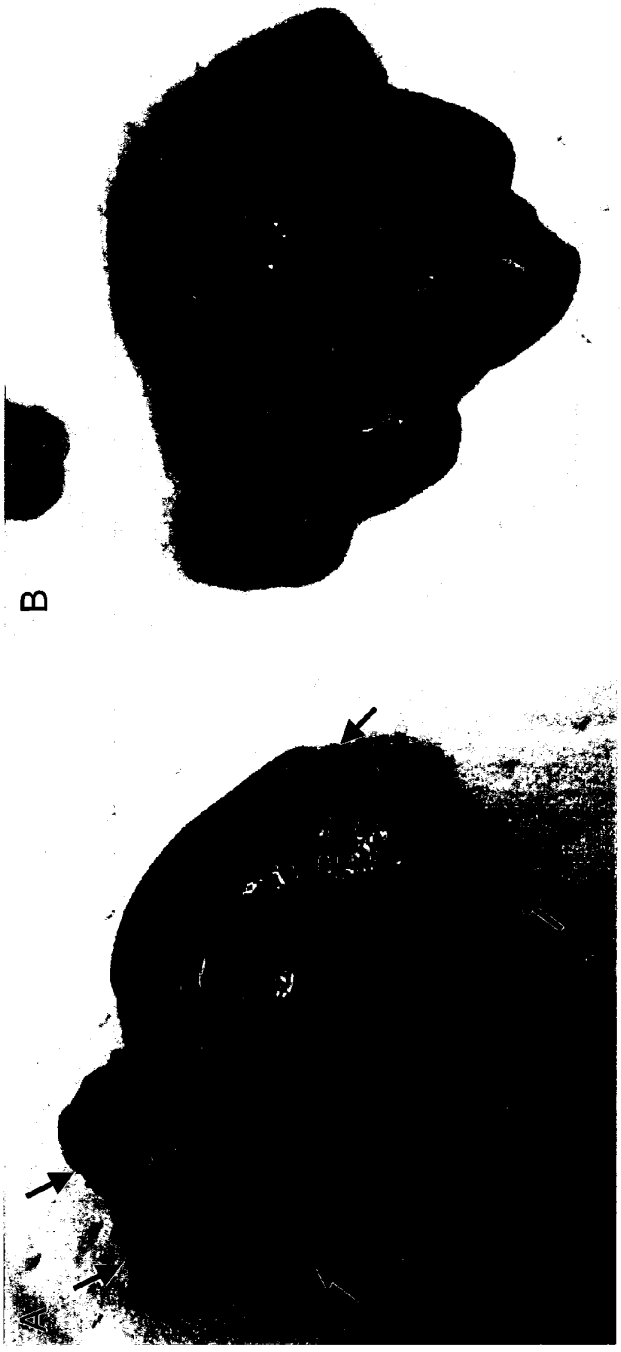

Fig.32

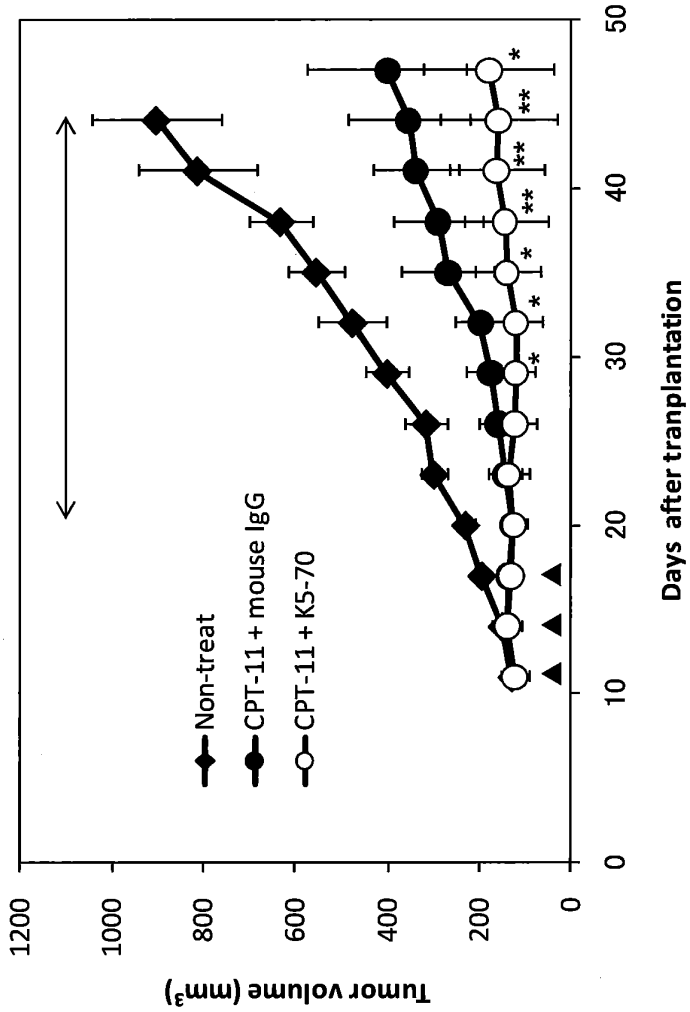
Tumor volume (mm³)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Days after tranplantation
Non-treat
CPT-11 + mouse IgG
CPT-11 + K5-70
*
**
**
**
*
*
*
*

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Fig.33

K5-70
VH

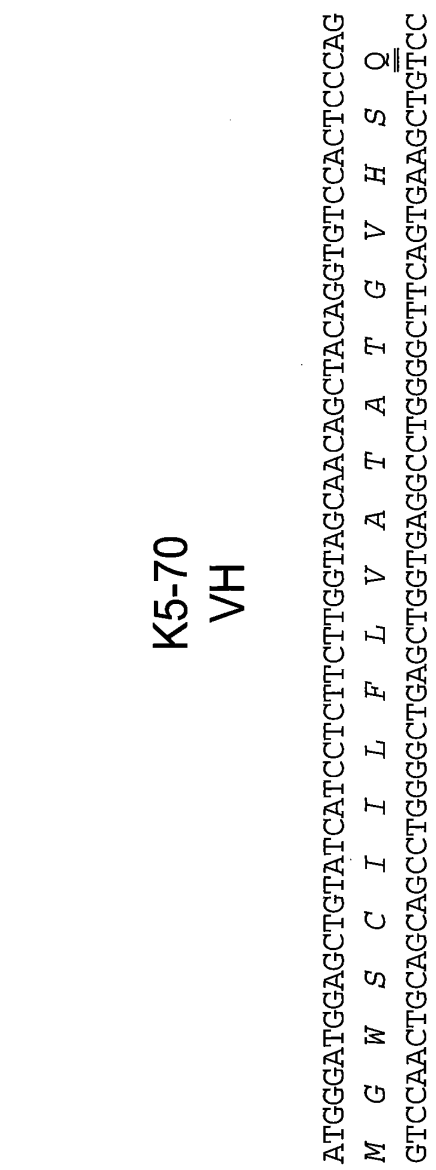

Fig.34

K5-70
VL

ATGGTATCCACACCTCAGTTCCTTGTATTTTTTGCTTTTTCTGGATTCCAGCCTCCAGAGGT
M V S T P Q F L V F L L F W I P A S R G
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
D I L L T Q S P A I L S V S P G E R V S
TTCTCCTGCAGGGCCAGTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACA
F S C R A S Q S I G T S I H W Y Q Q R T
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
N G S P R L L I K Y A S E S I S G I P S
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCT
R F S G S G S G T D F T L S I N S V E S
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGGCCATTCACGTTCGGCTCG
E D I A D Y Y C Q Q S N S W P F T F G S
GGGACAAAGTTGGAAATAAAA
G T K L E I K

Fig.35

K5-107
VH

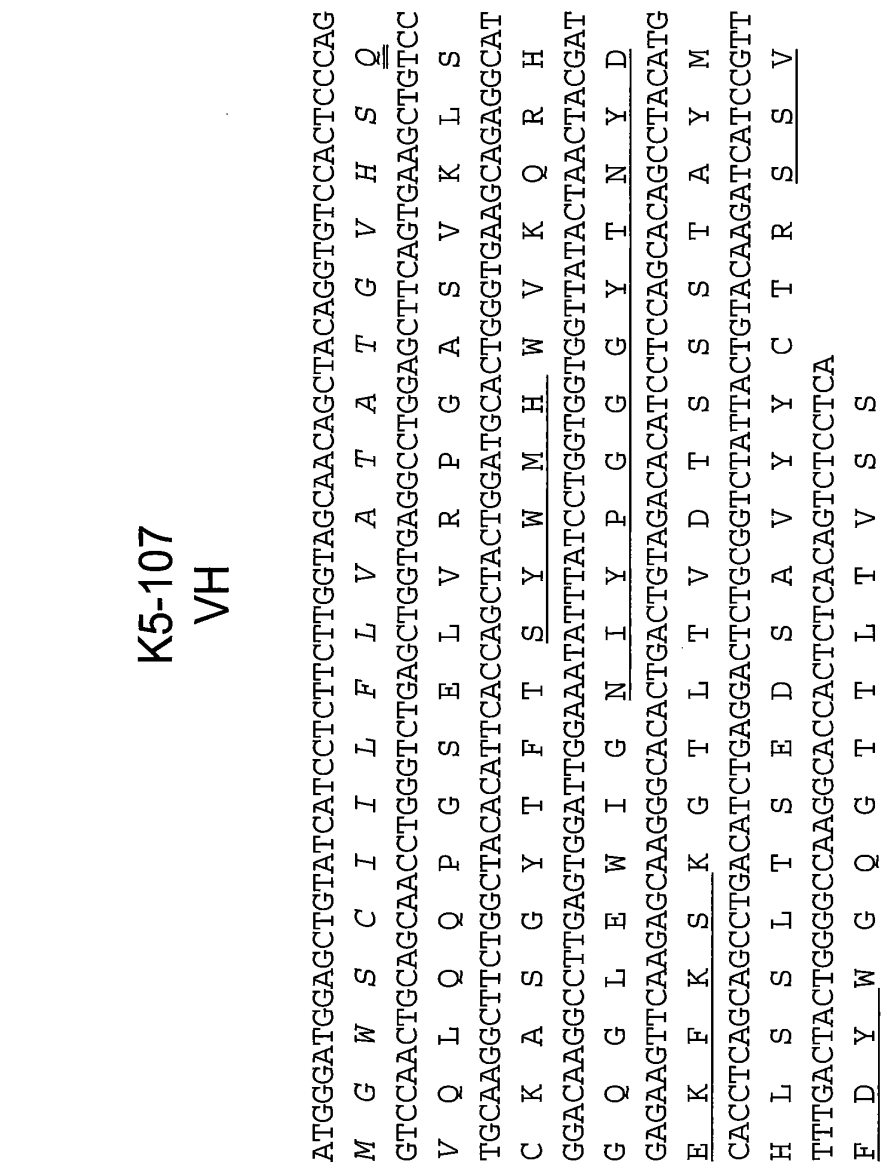

Fig.36

K5-107
VL

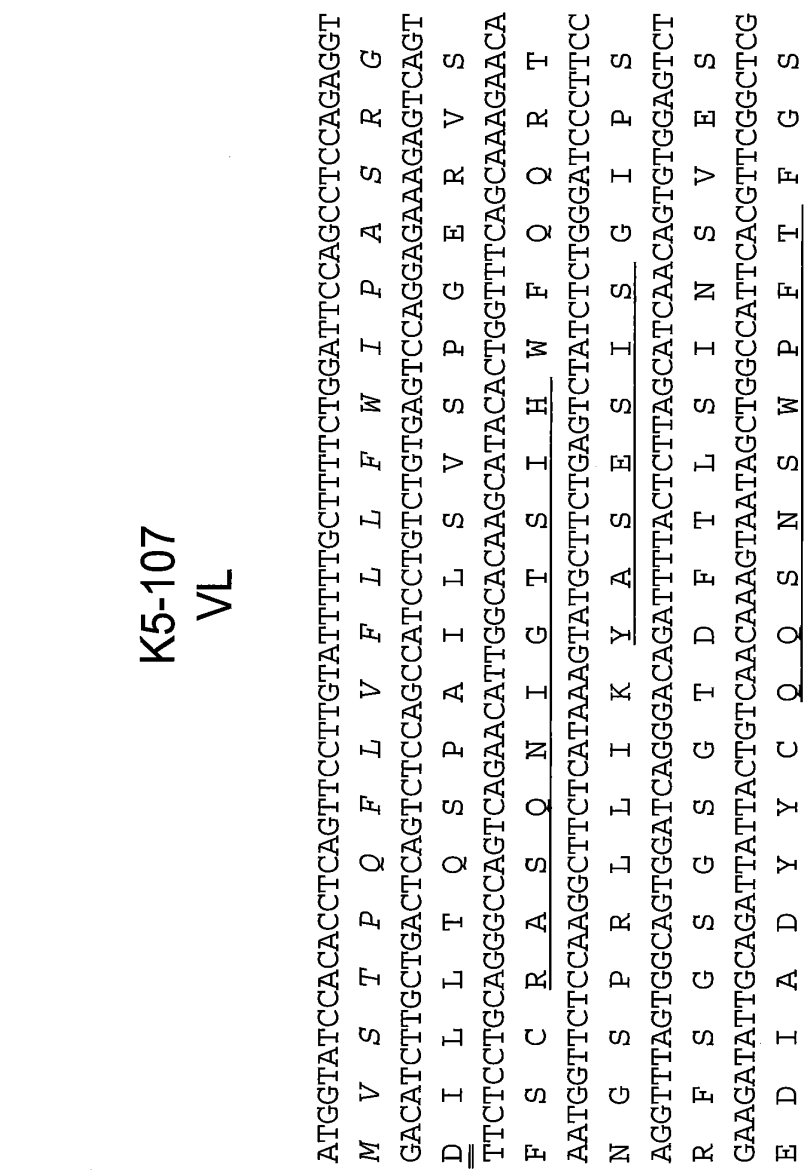

Fig.37

K5-116-2-1
VH

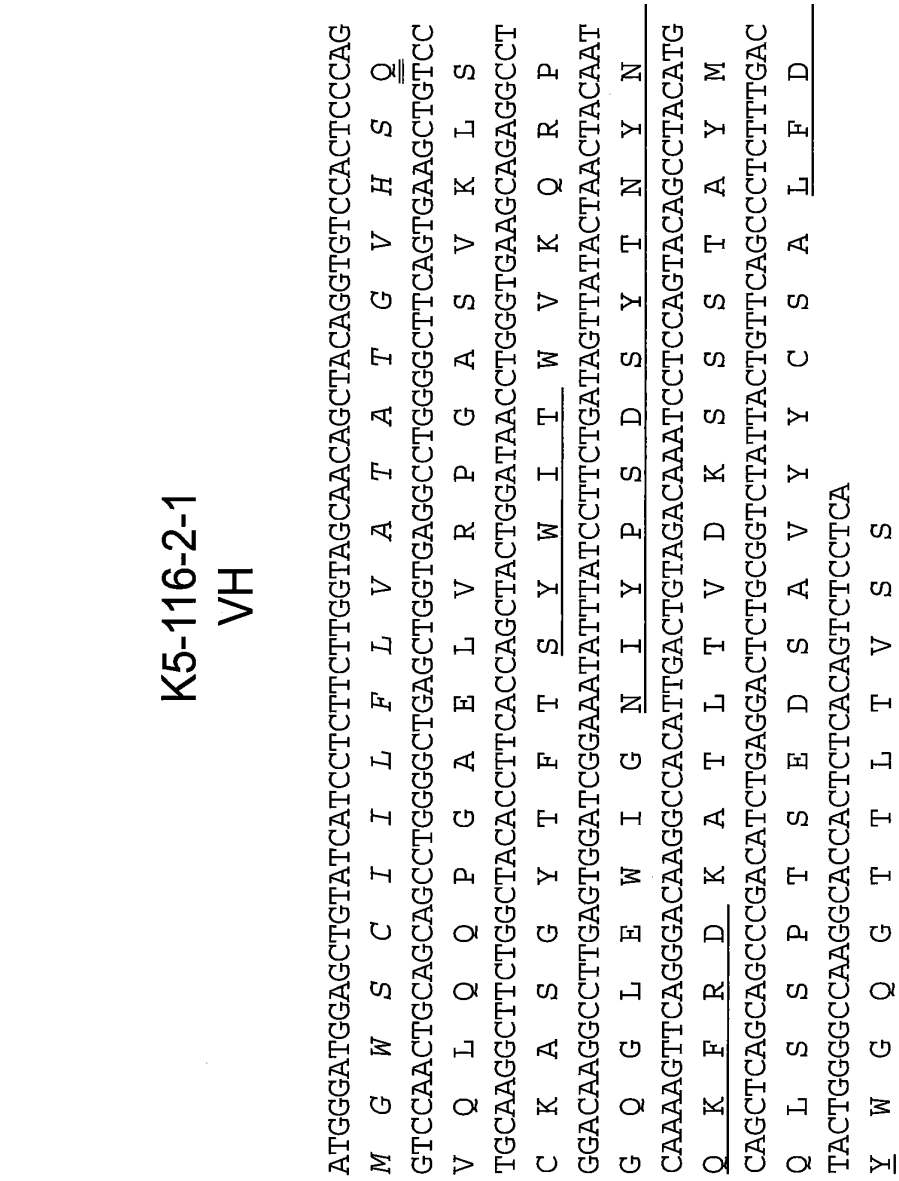

Fig.38

K5-116-2-1
VL

ATGGTATCCACACCTCAGTTCCTTGTATTTTTGCTTTTCTGGATTCCAGCCTCCAGAGGT
M V S T P Q F L V F L L F W I P A S R G
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
D I L L T Q S P A I L S V S P G E R V S
TTCTCCTGCAGGGCCAGTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACA
F S C R A S Q S I G T S I H W Y Q Q R T
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
N G S P R L L I K Y A S E S I S G I P S
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTATTCTTAGCATCAACAGTGTGGAGTCT
R F S G S G S G T D F I L S I N S V E S
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGGCCATTCACGTTCGGCTCG
E D I A D Y Y C Q Q S N S W P F T F G S
GGGACAAAGTTGGAAATAAAA
G T K L E I K

Fig.39

T6-16
VH

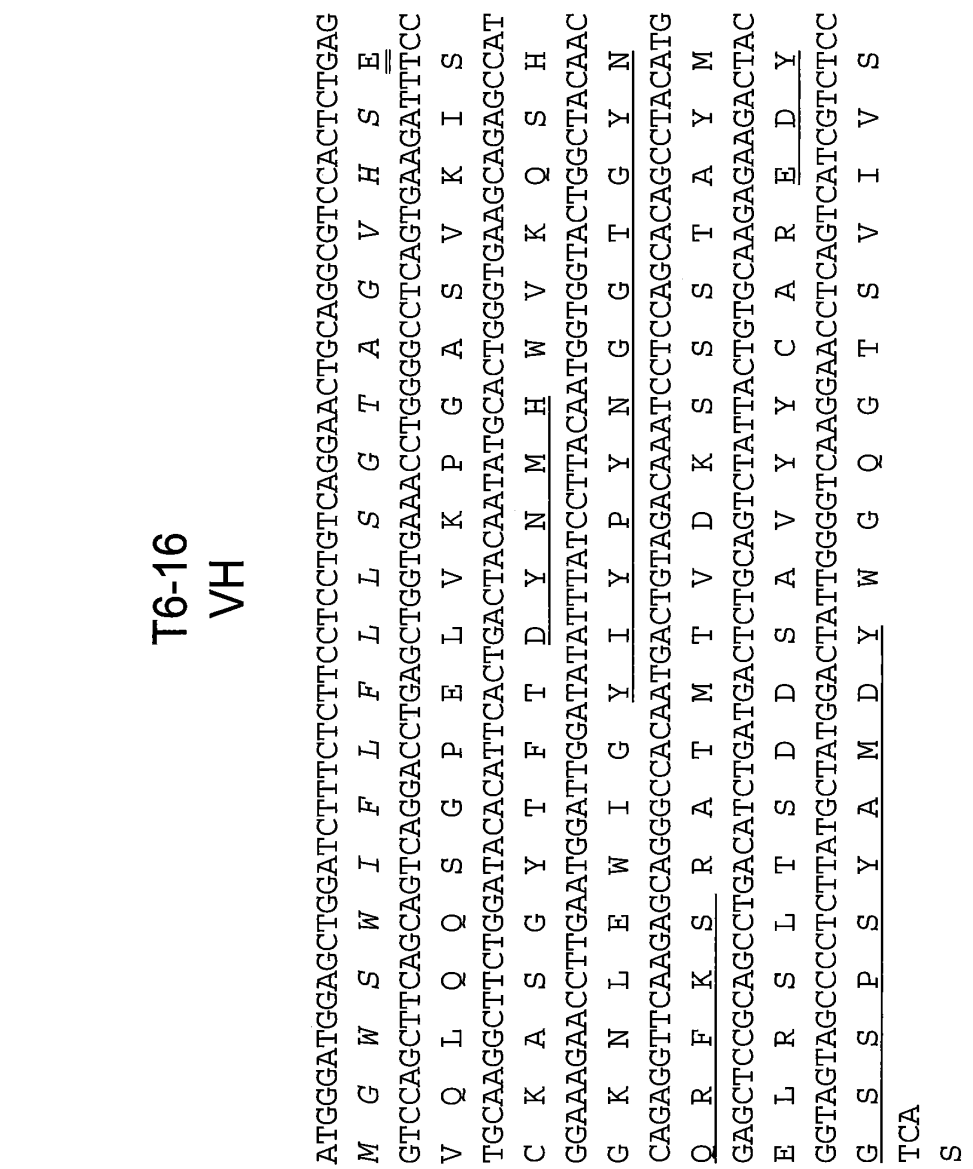

Fig.40

T6-16
VL

ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGAT
M K L P V R L L V L M F W I P A S S S D
GTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAGGCCTCCATC
V V M T Q T P L S L P V S L G D Q A S I
TCTTGCAGATCTAGTCAGAGCCTTGTACACGGTAATGGAAACACCTATTTACATTGGTAC
S C R S S Q S L V H G N G N T Y L H W Y
CTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCT
L Q K P G Q S P K L L I Y K V S N R F S
GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACGGATTTCACACTCAAGATCAGC
G V P D R F S G S G S G T D F T L K I S
AGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAACTACACATGTTCCCACG
R V E A E D L G V Y F C S Q T T H V P T
TTCGGCTCGGGGACAAAGTTGGAAATAAAA
F G S G T K L E I K

Fig.41

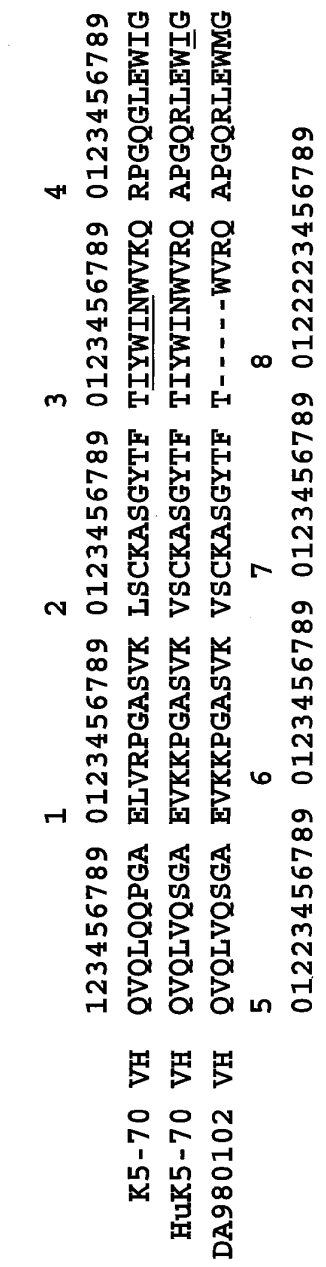
1 2 3 4
123456789 0123456789 0123456789 0123456789
K5-70 VH QVQLQQPGA ELVRPGASVK LSCKASGYTF TIYWINWVKQ RPGQGLEWIG
HuK5-70 VH QVQLVQSGA EVKKPGASVK VSCKASGYTF TIYWINWVRQ APGQRLEWIG
DA980102 VH QVQLVQSGA EVKKPGASVK VSCKASGYTF T----WVRQ APGQRLEWMG
5 6 7 8
01223456789 0123456789 0123456789 0122223456789
a abc
K5-70 VH NIYPSDSYTNY NQKFKDKATL TVDKSSSTAY MQLSSPTSEDSAV
HuK5-70 VH NIYPSDSYTNY NQKFKDKATL TVDTSASTAY MELSSLRSEDTAV
DA980102 VH ----------- ------RVTL TSDTSASTAY MEMSSLRSEDTAV
1 1
9 0 1
0123456789 0123456789 0123
K5-70 VH YYCTRTSMA- -DYWGQGTTL TVSS
HuK5-70 VH YYCTRTSMA- -DYWGQGTLV TVSS
DA980102 VH VHYYCAR----- -WGQGTLV TVSS

Fig.42

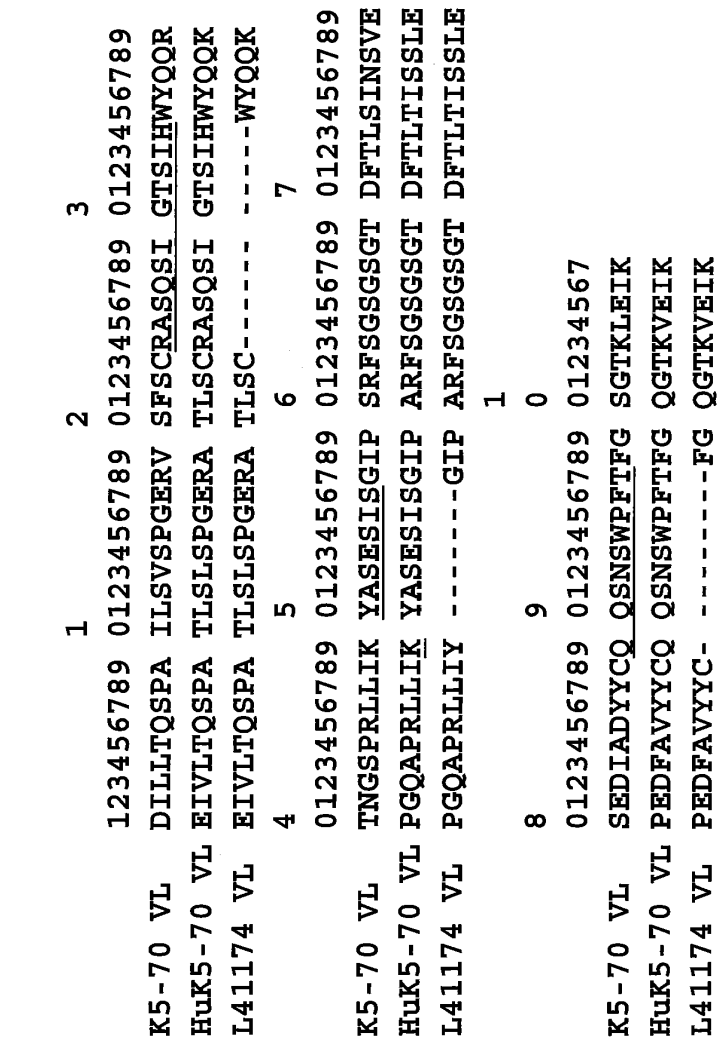
1 2 3
123456789 0123456789 0123456789 0123456789
K5-70 VL DILLTQSPA ILSVSPGERV SFSCRASQSI GTSIHWYQQR
HuK5-70 VL EIVLTQSPA TLSLSPGERA TLSCRASQSI GTSIHWYQQK
L41174 VL EIVLTQSPA TLSLSPGERA TLSC----- -----WYQQK
4 5 6 7
0123456789 0123456789 0123456789 0123456789
K5-70 VL TNGSPRLLIK YASESISGIP SRFSGSGSGT DFTLSINSVE
HuK5-70 VL PGQAPRLLIK YASESISGIP ARFSGSGSGT DFTLTISSLE
L41174 VL PGQAPRLLIY -------GIP ARFSGSGSGT DFTLTISSLE
1
8 9 0
0123456789 0123456789 01234567
K5-70 VL SEDIADYYCQ QSNSWPFTFG SGTKLEIK
HuK5-70 VL PEDFAVYYCQ QSNSWPFTFG QGTKVEIK
L41174 VL PEDFAVYYC- --------FG QGTKVEIK

Fig.43

```
                    1         2         3         4
          123456789 0123456789 0123456789 0123456789
T6-16 VH  EVQLQQSGP ELVKPGASVK ISCKASGYTF TDYNMHWVKQ SHGKNLEWIG
HuT6-16 VH1 QVQLVQSGA EVKKPGASVK VSCKASGYTF TDYNMHWVRQ APGQGLEWIG
HuT6-16 VH2 QVQLVQSGA EVKKPGASVK VSCKASGYTF TDYNMHWVRQ APGQGLEWIG
DA935238 VH QVQLVQSGA EVKKPGASVK VSCKASGYTF T----WVRQ APGQGLEWMG
          5         6         7         8
          0122456789 0123456789 0123456789 0122223456789
             a                      abc
T6-16 VH  YIYPYNGGTGY NQRFKSRATM TVDKSSSTAY MELRSLTSDDSAV
HuT6-16 VH1 YIYPYNGGTGY NQRFKSRATM TVDKSTSTAY MELRSLRSDDTAV
HuT6-16 VH2 YIYPYNGGTGY NQRFKSRATM TVDTSTSTAY MELRSLRSDDTAV
DA935238 VH ---------- ------RVTM TVDTSTSTAY MELRSLRSDDTAV
                    1         1
          9         0         1
          0123456789 0000012345678 0123
                        abcde
T6-16 VH  YYCAREDYGS SPSYAMDYWGQGTSV IVSS
HuT6-16 VH1 YYCAREDYGS SPSYAMDYWGQGTMV TVSS
HuT6-16 VH2 YYCAREDYGS SPSYAMDYWGQGTMV TVSS
DA935238 VH YYCAR----- ---------WGQGTMV TVSS
```

Fig.44

```
                        1          2                3
             123456789 0123456789 012345677777789 0123456789
                                            abcde
T6-16 VL     DVVMTQTPL SLPVSLGDQA SISCRSSQSLVHGNG NTYLHWYLQK
HuT6-16 VL   DIVMTQSPL SLPVTPGEPA SISCRSSQSLVHGNG NTYLHWYLQK
M99608 VL    DIVMTQSPL SLPVTPGEPA SISC----------- -----WYLQK
             4          5          6          7
             0123456789 0123456789 0123456789 0123456789
T6-16 VL     PGQSPKLLIY KVSNRFSGVP DRFSGSGSGT DFTLKISRVE
HuT6-16 VL   PGQSPQLLIY KVSNRFSGVP DRFSGSGSGT DFTLKISRVE
M99608 VL    PGQSPQLLIY -------GVP DRFSGSGSGT DFTLKISRVE
                                   1
             8          9          0
             0123456789 0123456789 01234567
T6-16 VL     AEDLGVYFCS QTTHVP-TFG SGTKLEIK
HuT6-16 VL   AEDVGVYYCS QTTHVP-TFG GGTKVEIK
M99608 VL    AEDVGVYYC----------FG GGTKVEIK
```

Fig.45

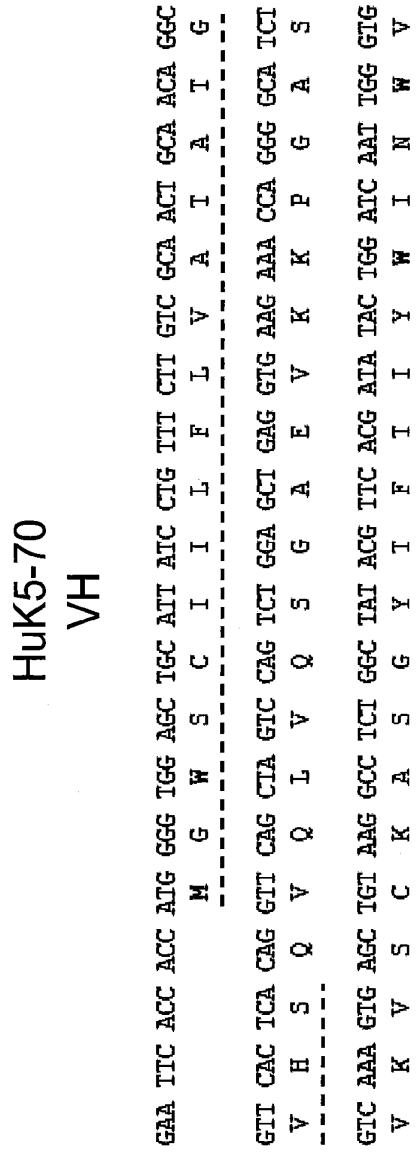
HuK5-70
VH
GAA TTC ACC ACC ATG GGG TGG AGC TGC ATT ATC CTG TTT CTT GTC GCA ACT GCA ACA GGC
M G W S C I I L F L V A T A T G
GTT CAC TCA CAG GTT CAG CTA GTC CAG TCT GGA GCT GAG GTG AAG AAA CCA GGG GCA TCT
V H S Q V Q L V Q S G A E V K K P G A S
GTC AAA GTG AGC TGT AAG GCC TCT GGC TAT ACG TTC ACG ATA TAC TGG ATC AAT TGG GTG
V K V S C K A S G Y T F T I Y W I N W V
AGG CAA GCT CCT GGA CAA CGG TTG GAA TGG ATT GGC AAC ATC TAT CCC TCA GAC TCC TAC
R Q A P G Q R L E W I G N I Y P S D S Y
ACC AAC TAC AAT CAG AAG TTC AAG GAC AAA GCC ACT CTC ACC GTA GAT ACC AGT GCC TCC
T N Y N Q K F K D K A T L T V D T S A S
ACA GCC TAT ATG GAG CTG AGC AGT TTA CGC AGC GAA GAT ACA GCG GTG TAC TAC TGC ACC
T A Y M E L S S L R S E D T A V Y Y C T
AGA ACC TCC ATG GCT GAC TAT TGG GGT CAG GGT ACA CTG GTG ACT GTG AGC TCC GCT AGC
R T S M A D Y W G Q G T L V T V S S

Fig.46

HuK5-70
VL

```
ACC GGT ACC ACC ATG GTA AGC ACA CCC CAG TTC CTC GTC TTC CTC CTG TTT TGG ATT CCC
      M   V   S   T   P   Q   F   L   V   F   L   L   F   W   I   P
      ---------------------------------------------------------------

GCA AGT AGA GGG GAG ATC GTC TTG ACT CAG AGT CCT GCC ACA CTG TCT CTT TCA CCA GGA
  A   S   R   G   E   I   V   L   T   Q   S   P   A   T   L   S   L   S   P   G
  -----------

GAA AGG GCG ACA CTT AGC TGT CGA GCT TCT CAG AGC ATT GGC ACG TCC ATA CAC TGG TAT
  E   R   A   T   L   S   C   R   A   S   Q   S   I   G   T   S   I   H   W   Y
                              ===========================

CAG CAG AAA CCG GGA CAA GCT CCA CGG TTA CTG ATC AAG TAT GCC TCC GAA AGC ATC TCT
  Q   Q   K   P   G   Q   A   P   R   L   L   I   K   Y   A   S   E   S   I   S
                              ===========================

GGG ATT CCT GCA CGC TTT AGC GGA AGC GGT AGT GGT ACC GAC TTC ACT CTG ACC ATA TCC
  G   I   P   A   R   F   S   G   S   G   S   G   T   D   F   T   L   T   I   S

TCA CTA GAA CCC GAG GAT TTT GCC GTG TAC TAC TGC CAG CAG TCC AAC TCA TGG CCT TTC
  S   L   E   P   E   D   F   A   V   Y   Y   C   Q   Q   S   N   S   W   P   F
                                  ===============================

ACC TTT GGC CAA GGC ACC AAA GTG GAG ATC AAG CGT ACG
  T   F   G   Q   G   T   K   V   E   I   K
  ===
```

Fig.47

HuT6-16
VH1

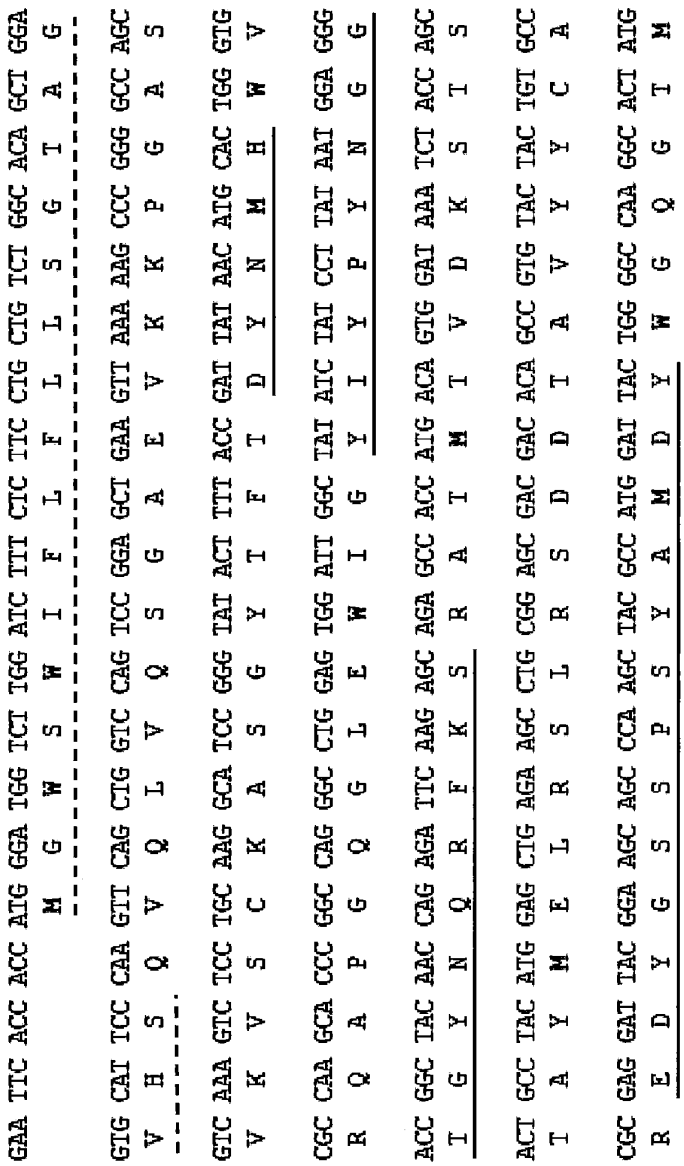

Fig.48

HuT6-16
VH2

```
GAA TTC ACC ACC ATG GGC TGG TCT TGG ATC TTC CTC TTC CTG CTG AGC GGG ACC GCT GGA
          M   G   W   S   W   I   F   L   F   L   L   S   G   T   A   G
          ---------------------------------------------------------------

GTC CAT TCT CAA GTC CAA CTG GTC CAG TCC GGG GCT GAA GTG AAA AAA CCA GGA GCA TCC
 V   H   S   Q   V   Q   L   V   Q   S   G   A   E   V   K   K   P   G   A   S
 ---------.

GTT AAG GTG TCC TGT AAG GCA AGC GGA TAC ACC TTT ACC GAC TAT AAC ATG CAC TGG GTG
 V   K   V   S   C   K   A   S   G   Y   T   F   T   D   Y   N   M   H   W   V

AGG CAG GCC CCC GGA CAG GGG CTG GAG TGG ATC GGC TAT ATT TAT CCT TAC AAC GGG GGC
 R   Q   A   P   G   Q   G   L   E   W   I   G   Y   I   Y   P   Y   N   G   G

ACT GGC TAT AAT CAG AGA TTT AAG AGC CGC GCT ACC ATG ACC GTG GAC ACC TCC ACT TCT
 T   G   Y   N   Q   R   F   K   S   R   A   T   M   T   V   D   T   S   T   S

ACA GCC TAT ATG GAG CTG AGA AGC CTG CGG AGC GAT GAT ACA GCC GTG TAC TAC TGC GCC
 T   A   Y   M   E   L   R   S   L   R   S   D   D   T   A   V   Y   Y   C   A

AGA GAA GAT TAC GGC AGC AGC CCC AGC TAC GCC ATG GAC TAC TGG GGC CAG GGC ACA ATG
 R   E   D   Y   G   S   S   P   S   Y   A   M   D   Y   W   G   Q   G   T   M

GTT ACT GTG AGC AGC GCT AGC
 V   T   V   S   S
```

Fig.49

HuT6-16
VL

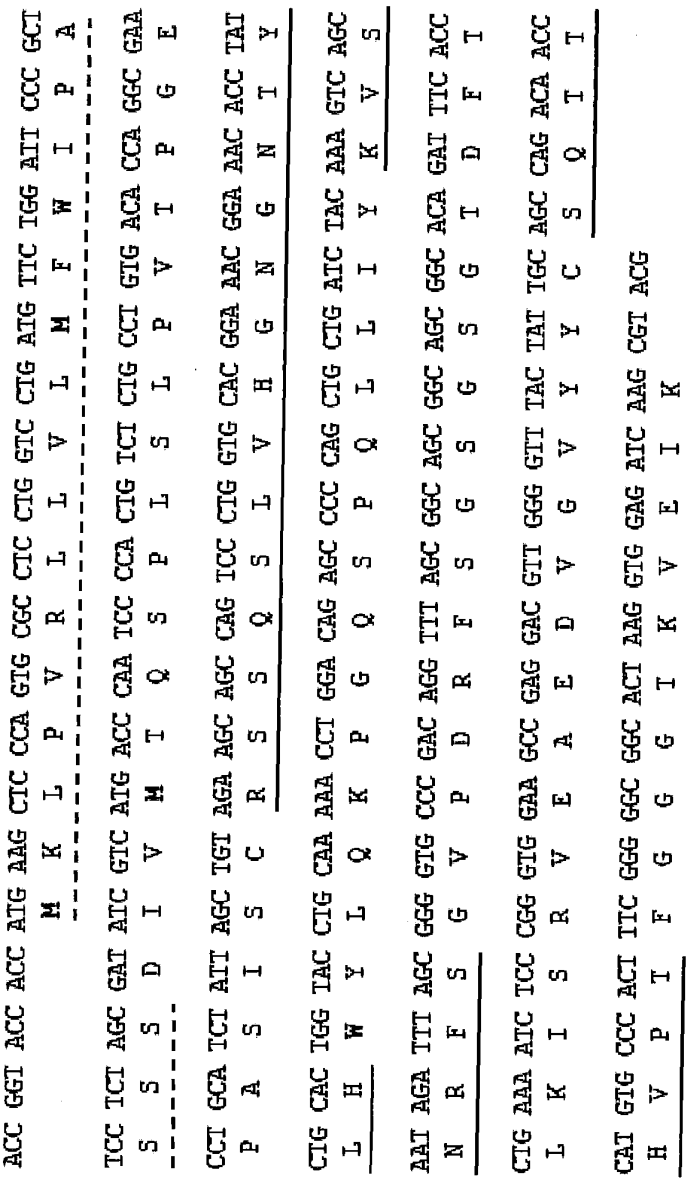

Fig.50

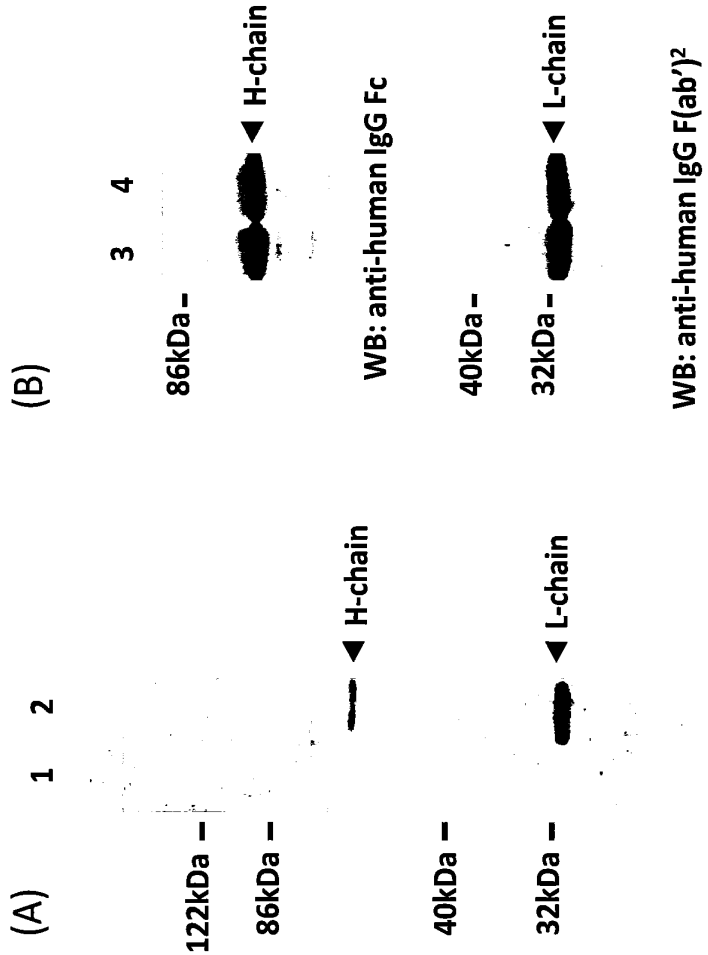
(A)
1
2
122kDa
86kDa
H-chain
40kDa
32kDa
L-chain
WB: anti-human Ig-HRP
(B)
3
4
86kDa
H-chain
WB: anti-human IgG Fc
40kDa
32kDa
L-chain
WB: anti-human IgG F(ab')2

Fig.51

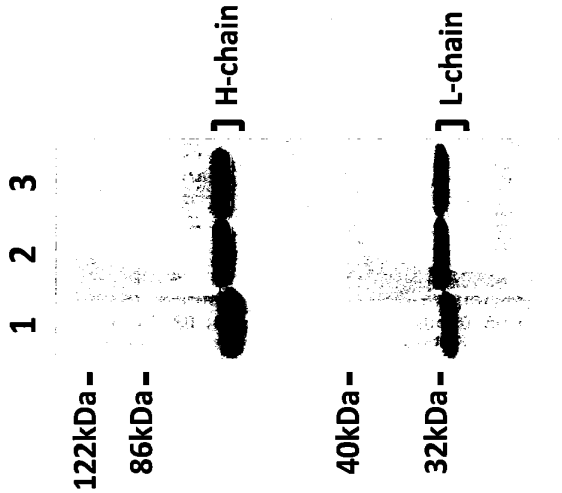

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Fig.52

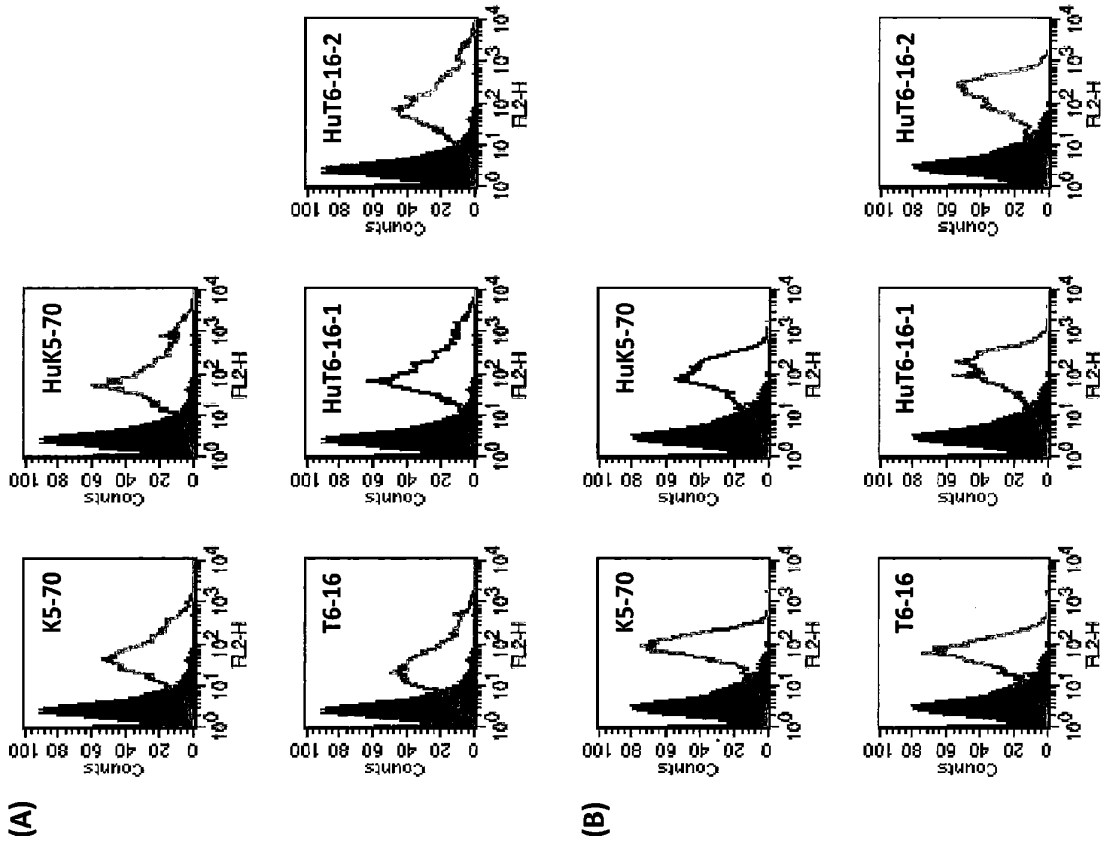

Fig.53

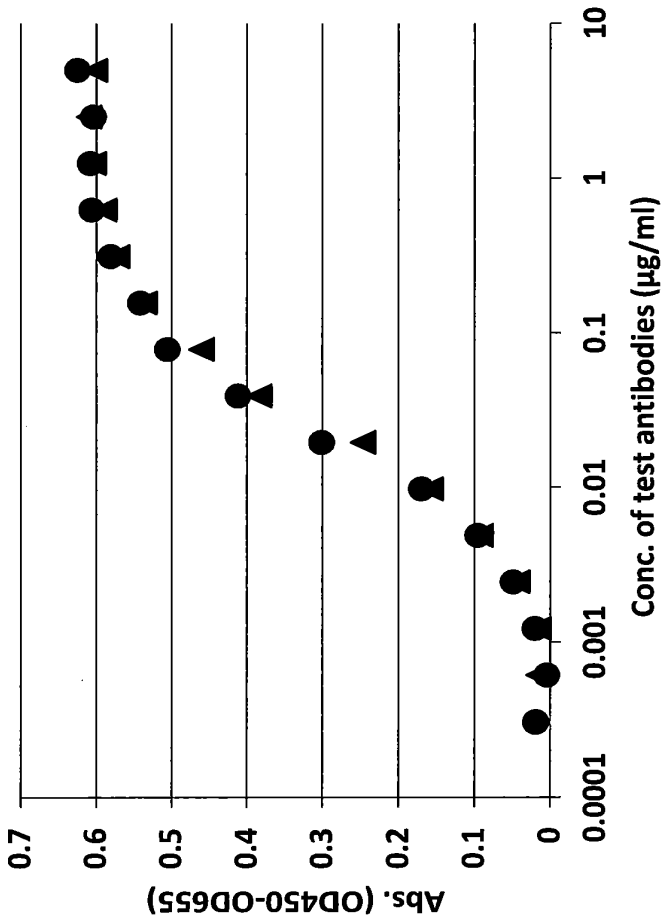
Abs. (OD450-OD655)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0001
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/ml)

Fig.54

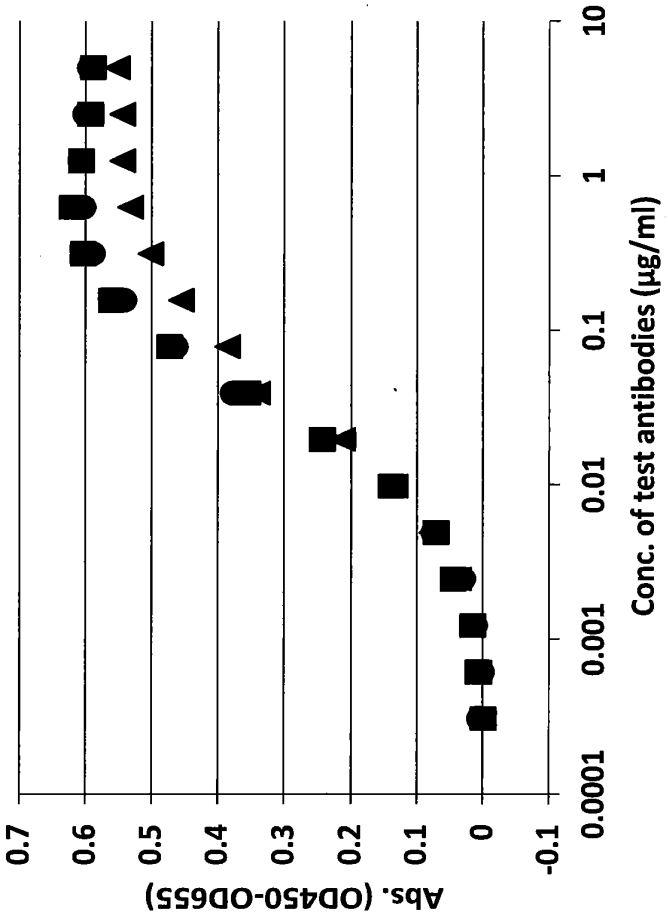
Abs. (OD450-OD655)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0.0001
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/ml)

Fig.55

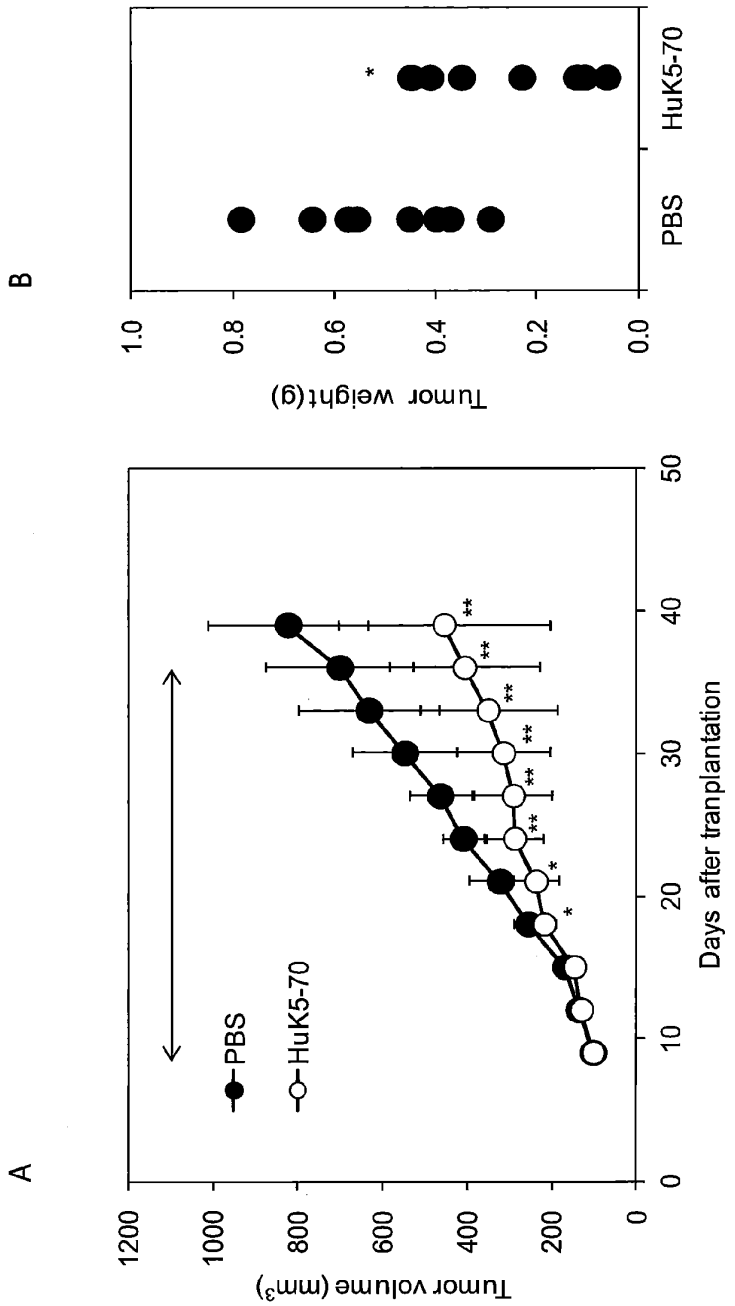
A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Days after tranplantation
PBS
HuK5-70
B
Tumor weight(g)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
HuK5-70

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Fig.56

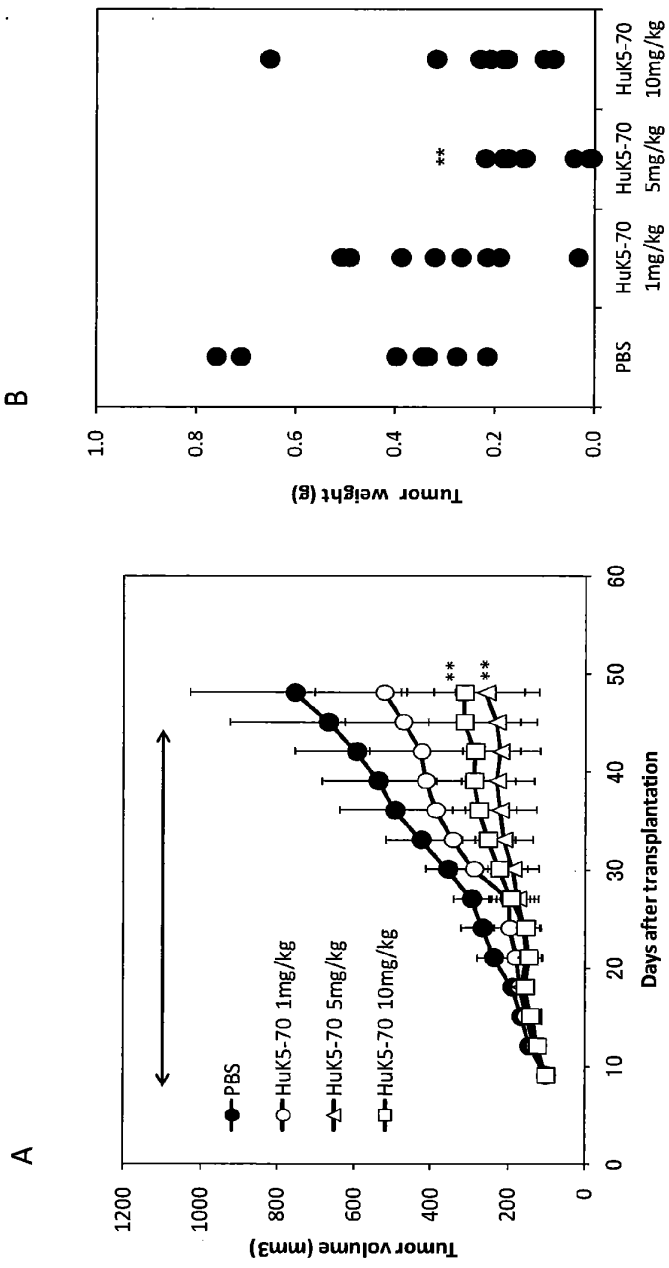

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Fig.57

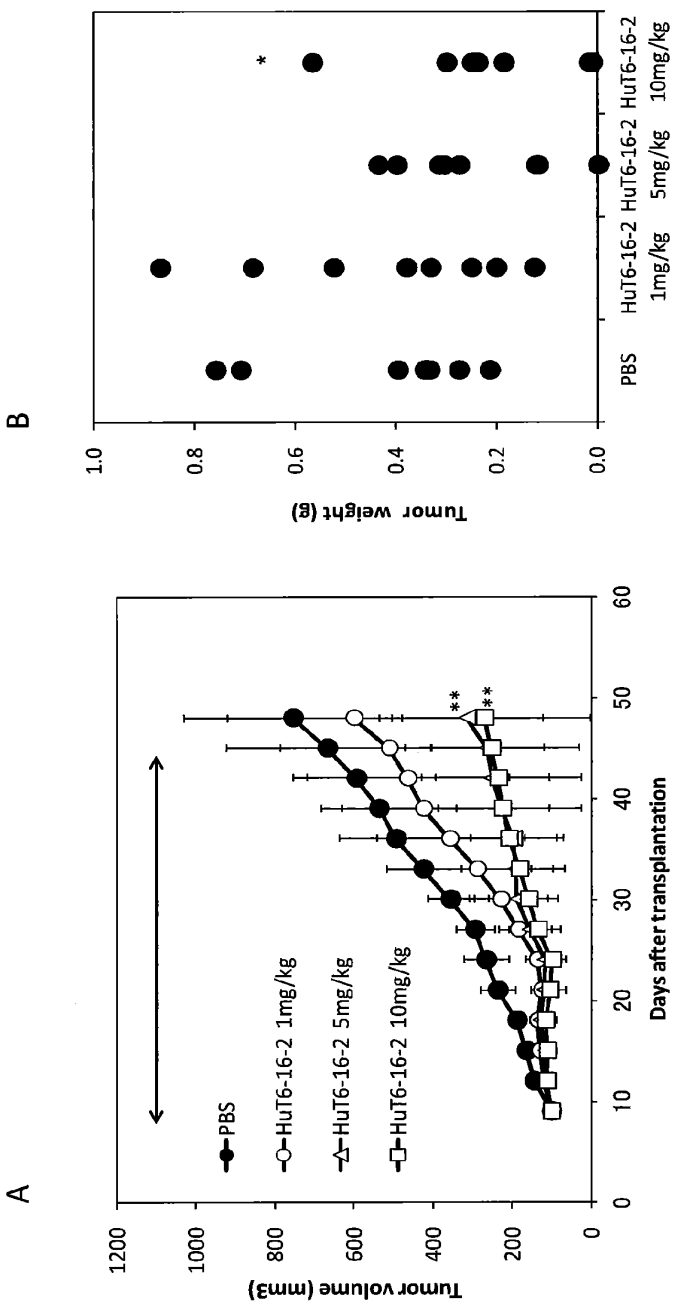


Fig.58

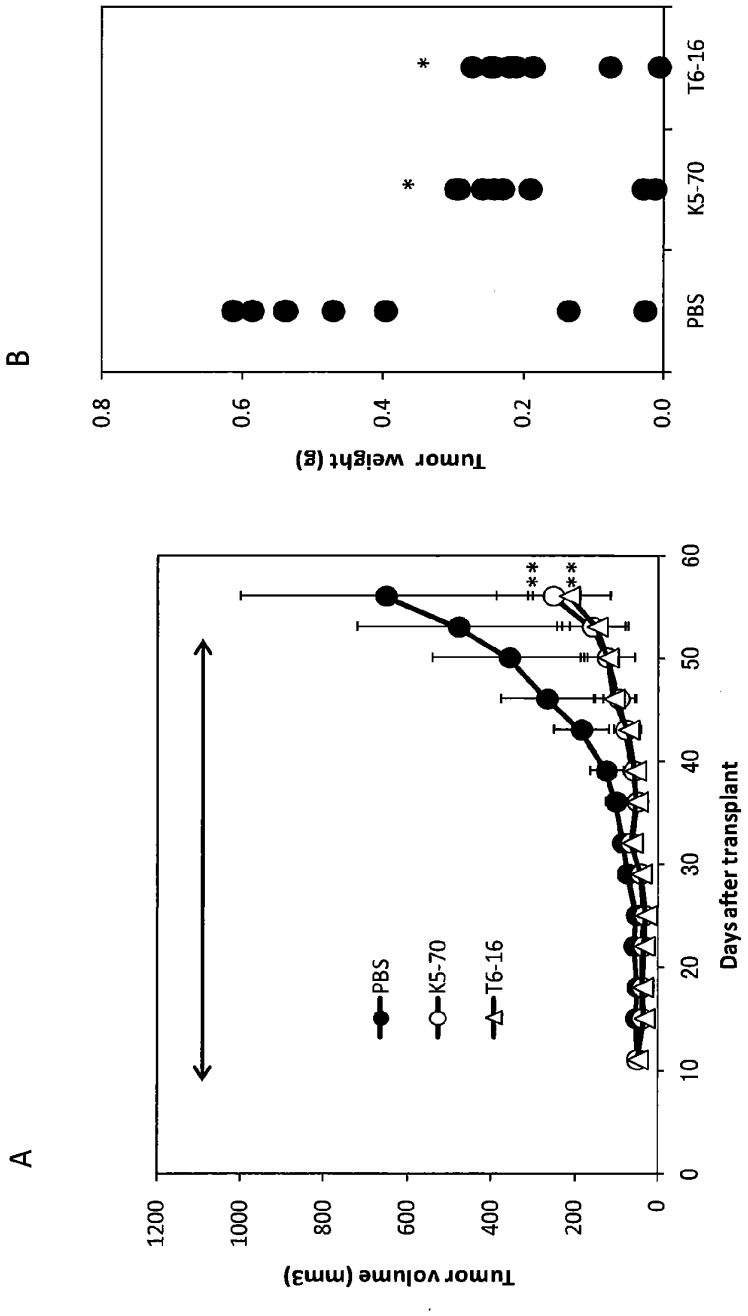

A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Days after transplant
PBS
K5-70
T6-16
**
**
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
*
*
PBS
K5-70
T6-16

Fig.59

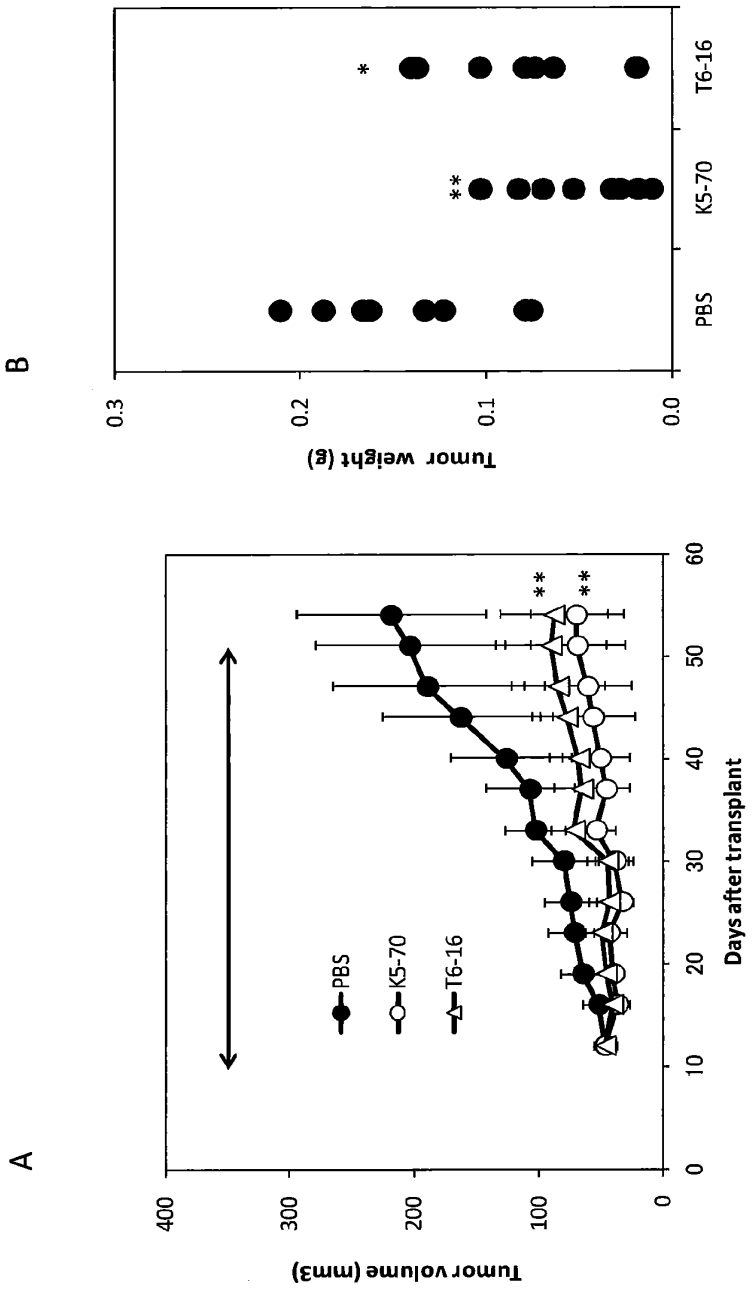
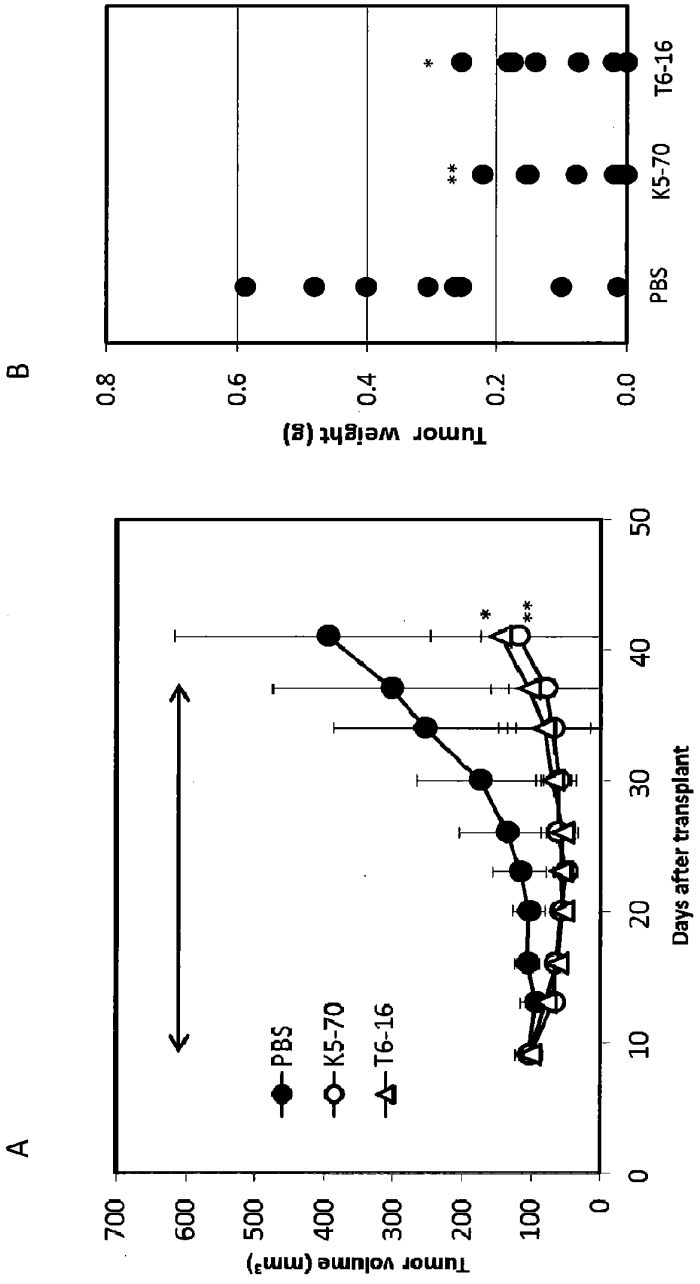

A
Tumor volume (mm3)
400
300
200
100
0
0
10
20
30
40
50
60
Days after transplant
PBS
K5-70
T6-16
**
**
B
Tumor weight (g)
0.3
0.2
0.1
0.0
PBS
K5-70
T6-16
*
**

Fig.60


A
Tumor volume (mm3)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Days after transplant
PBS
K5-70
T6-16
*
**
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
PBS
K5-70
T6-16
*
**

EP 2 799 452 A1

Fig.61

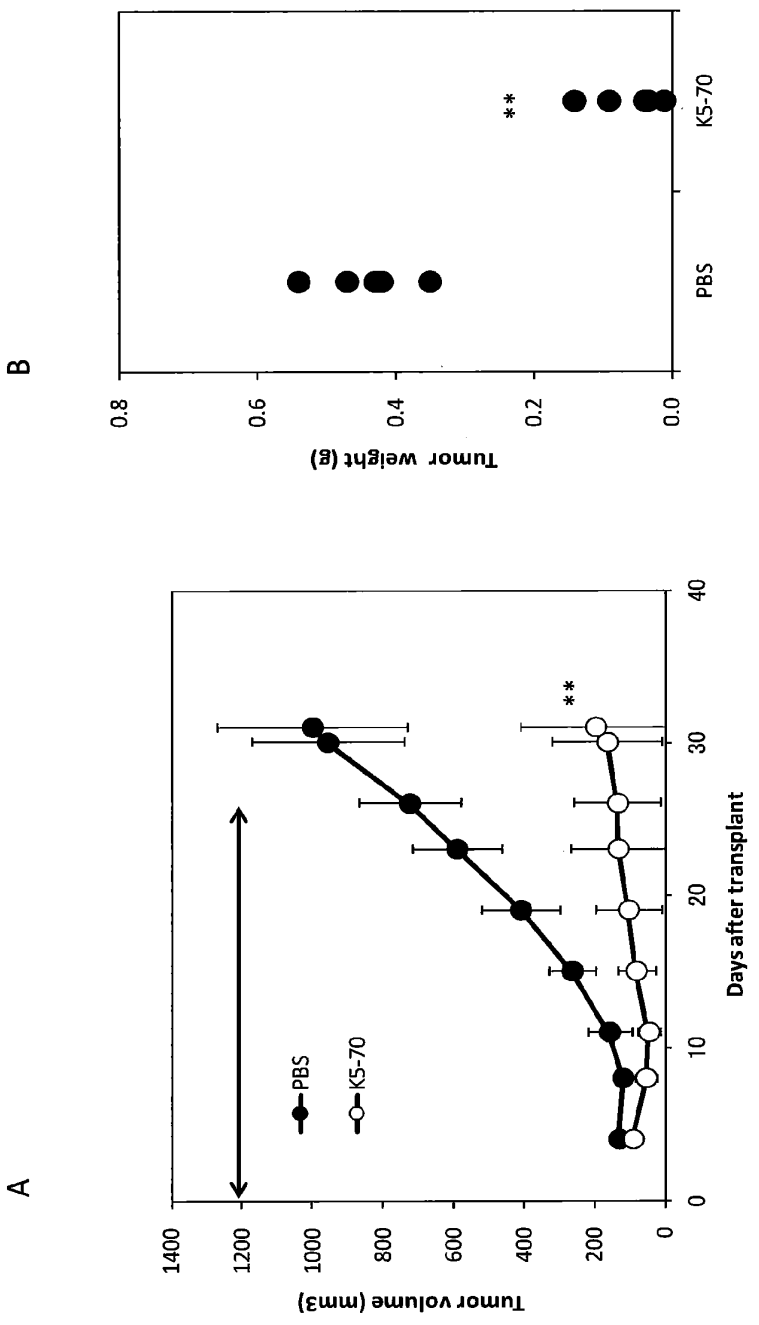


Fig.62

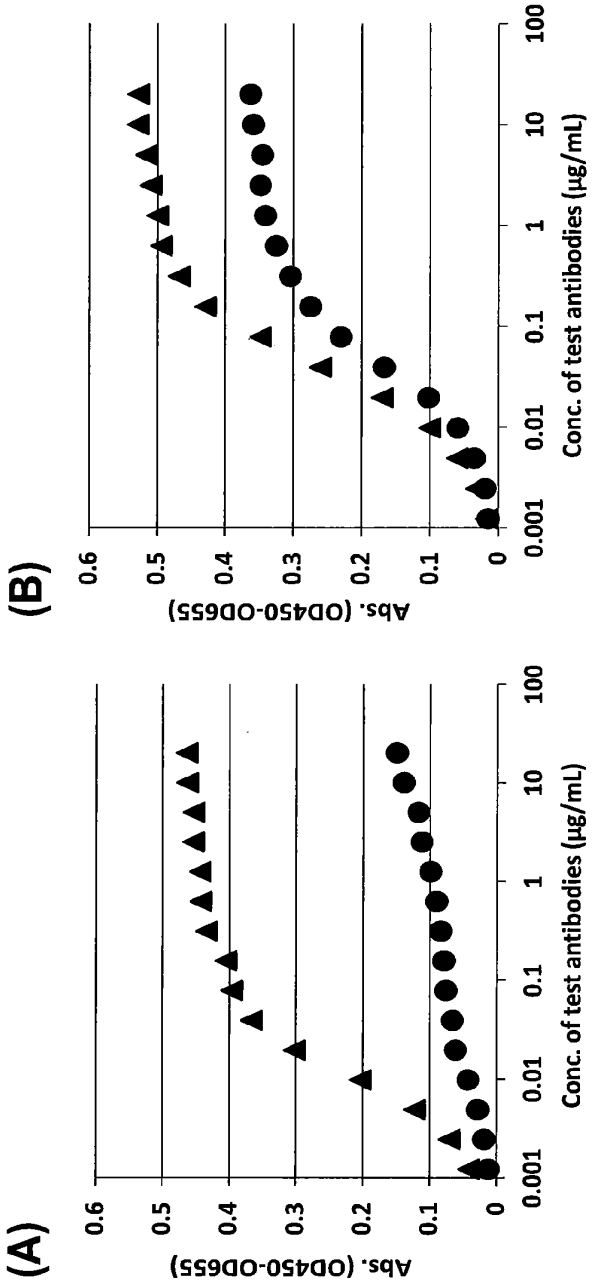

(A)
Abs. (OD450-OD655)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
Conc. of test antibodies (μg/mL)
(B)
Abs. (OD450-OD655)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
Conc. of test antibodies (μg/mL)

Fig.63

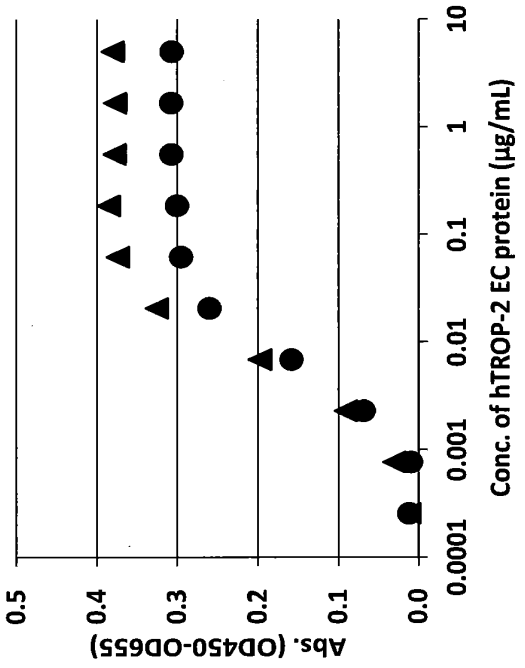

Abs. (OD450-OD655)
0.0
0.1
0.2
0.3
0.4
0.5
0.0001
0.001
0.01
0.1
1
10
Conc. of hTROP-2 EC protein (μg/mL)

Fig.64

```
  EcoRI    Kozak
GAA TTC ACC ACC ATG GGA TGG TCC TGC ATT ATT CTC TTT CTC GTC GCC ACC GCC ACA GGC
                M   G   W   S   C   I   I   L   F   L   V   A   T   A   T   G

GTG CAC AGC CAG GTT CAA CTG CAG CAA CCT GGG GCA GAG CTG GTT CGG CCA GGG GCC TCC
 V   H   S   Q   V   Q   L   Q   Q   P   G   A   E   L   V   R   P   G   A   S

GTC AAA CTG TCC TGC AAA GCT TCT GGC TAC ACT TTC ACC ATC TAC TGG ATC AAC TGG GTG
 V   K   L   S   C   K   A   S   G   Y   T   F   T   I   Y   W   I   N   W   V

AAG CAG AGG CCC GGC CAG GGC CTG GAA TGG ATC GGA AAT ATC TAT CCT AGC GAT TCT TAC
 K   Q   R   P   G   Q   G   L   E   W   I   G   N   I   Y   P   S   D   S   Y

ACA AAT TAC AAC CAG AAG TTC AAG GAC AAG GCT ACC CTG ACC GTG GAC AAA TCT AGC TCC
 T   N   Y   N   Q   K   F   K   D   K   A   T   L   T   V   D   K   S   S   S

ACA GCC TAC ATG CAG CTG AGC AGC CCC ACT AGC GAG GAT AGC GCA GTG TAT TAT TGT ACC
 T   A   Y   M   Q   L   S   S   P   T   S   E   D   S   A   V   Y   Y   C   T

                                                                  NheI
AGA ACC AGC ATG GCC GAC TAT TGG GGA CAG GGC ACA ACT CTG ACC GTG AGC AGC GCT AGC
 R   T   S   M   A   D   Y   W   G   Q   G   T   T   L   T   V   S   S
```

Fig.65

```
 AgeI    Kozak
ACC GGT ACC ACC ATG GTT AGC ACA CCT CAA TTT CTG GTC TTC CTG CTC TTC TGG ATT CCT
                 M   V   S   T   P   Q   F   L   V   F   L   L   F   W   I   P

GCC AGC AGA GGA GAT ATC CTC CTG ACA CAA AGC CCC GCA ATC CTG AGC GTG TCC CCC GGA
 A   S   R   G   D   I   L   L   T   Q   S   P   A   I   L   S   V   S   P   G

GAG CGC GTG AGC TTT AGC TGC CGG GCC AGC CAG AGC ATT GGA ACC AGC ATC CAC TGG TAT
 E   R   V   S   F   S   C   R   A   S   Q   S   I   G   T   S   I   H   W   Y

CAG CAG AGA ACC AAC GGG TCT CCC AGG CTG CTG ATT AAA TAC GCT TCT GAG TCT ATT TCC
 Q   Q   R   T   N   G   S   P   R   L   L   I   K   Y   A   S   E   S   I   S

GGG ATC CCA AGC AGA TTC TCC GGC TCT GGC AGC GGC ACT GAT TTT ACT CTG TCT ATC AAC
 G   I   P   S   R   F   S   G   S   G   S   G   T   D   F   T   L   S   I   N

AGC GTG GAG TCC GAA GAC ATC GCC GAC TAC TAT TGT CAG CAG AGC AAT TCC TGG CCA TTC
 S   V   E   S   E   D   I   A   D   Y   Y   C   Q   Q   S   N   S   W   P   F

                                                 BsiWI
ACC TTT GGC AGC GGC ACC AAG CTG GAA ATC AAG CGT ACG
 T   F   G   S   G   T   K   L   E   I   K
```

Fig.66

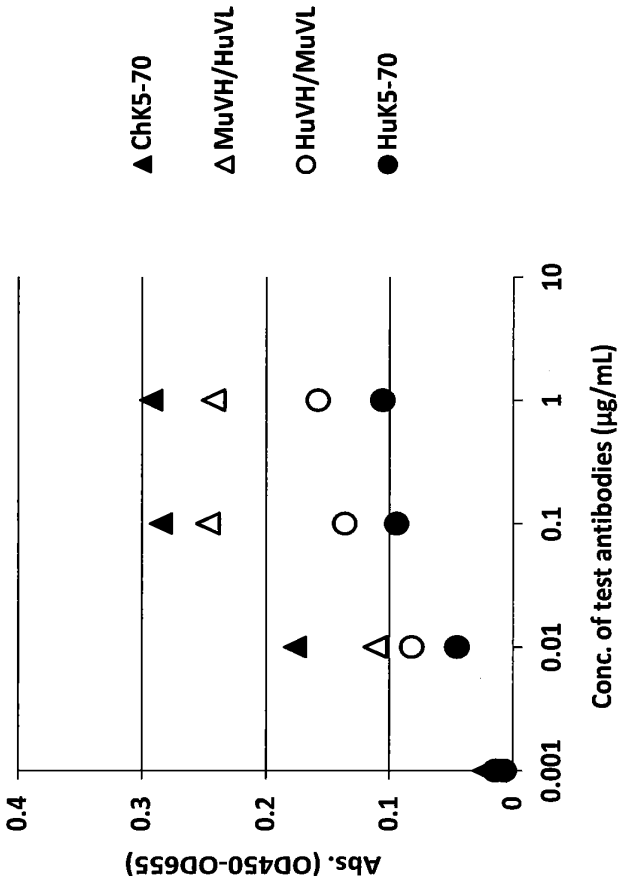

▲ChK5-70
△MuVH/HuVL
○HuVH/MuVL
●HuK5-70
Abs. (OD450-OD655)
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/mL)

Fig.67

	123456789	1 0123456789	2 0123456789	3 0123456789	4 0123456789	5 01223456789
						a
HuK5-70 VH	QVQLVQSGA	EVKKPGASVK	VSCKASGYTF	TIYWINWVRQ	APGQRLEWIG	NIYPSDSYTNY
V5Q	----Q----	----------	----------	----------	----------	----------
S7P	------P--	----------	----------	----------	----------	----------
V11L	---------	-L--------	----------	----------	----------	----------
K12V	---------	--V-------	----------	----------	----------	----------
K13R	---------	---R------	----------	----------	----------	----------
V20L	---------	----------	L---------	----------	----------	----------
R38K	---------	----------	----------	--------K-	----------	----------
A40R	---------	----------	----------	----------	R---------	----------
R44G	---------	----------	----------	----------	----G-----	----------
T73K	---------	----------	----------	----------	----------	----------
A75S	---------	----------	----------	----------	----------	----------
E81Q	---------	----------	----------	----------	----------	----------
L82cP	---------	----------	----------	----------	----------	----------
R83T	---------	----------	----------	----------	----------	----------
T87S	---------	----------	----------	----------	----------	----------
L108T	---------	----------	----------	----------	----------	----------
V109L	---------	----------	----------	----------	----------	----------
V11L/R38K	---------	-L--------	----------	--------K-	----------	----------

	6 0123456789	7 0123456789 abc	8 0122223456789	9 0123456789	1 0 0123456789	1 1 0 0123
HuK5-70 VH	NQKFKDKATL	TVDTSASTAY	MELSSLRSEDTAV	YYCTRTSMA-	-DYWGQGTLV	TVSS
V5Q	----------	----------	----------	----------	----------	----
S7P	----------	----------	----------	----------	----------	----
V11L	----------	----------	----------	----------	----------	----
K12V	----------	----------	----------	----------	----------	----
K13R	----------	----------	----------	----------	----------	----
V20L	----------	----------	----------	----------	----------	----
R38K	----------	----------	----------	----------	----------	----
A40R	----------	----------	----------	----------	----------	----
R44G	----------	----------	----------	----------	----------	----
T73K	----------	---K------	----------	----------	----------	----
A75S	----------	-----S----	----------	----------	----------	----
E81Q	----------	----------	-Q----------	----------	----------	----
L82cP	----------	----------	-----P-------	----------	----------	----
R83T	----------	----------	------T------	----------	----------	----
T87S	----------	----------	----------S--	----------	----------	----
L108T	----------	----------	----------	----------	--------T-	----
V109L	----------	----------	----------	----------	---------L	----
V11L/R38K	----------	----------	----------	----------	----------	----

Fig.68

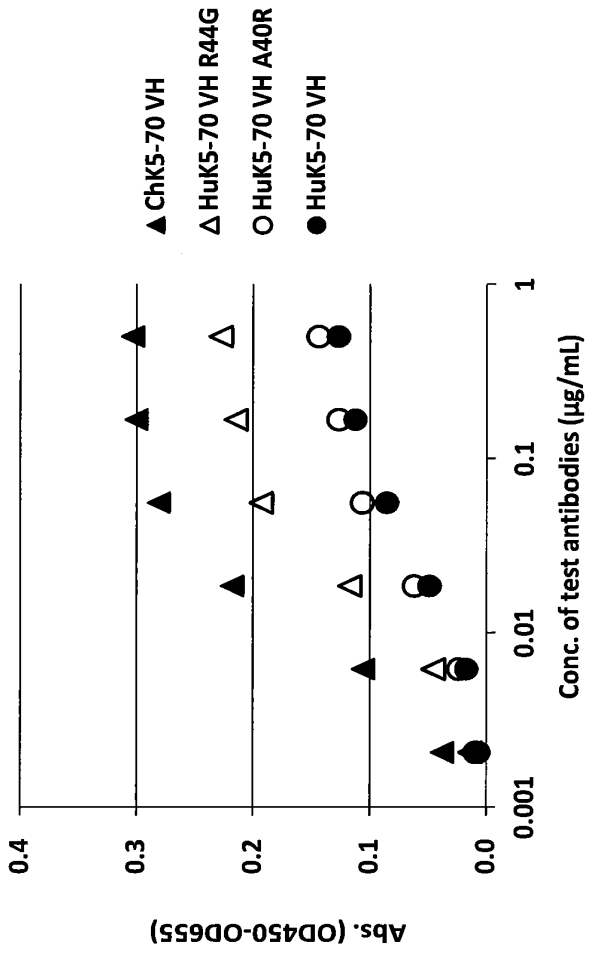

▲ ChK5-70 VH
△ HuK5-70 VH R44G
○ HuK5-70 VH A40R
● HuK5-70 VH
Abs. (OD450-OD655)
0.4
0.3
0.2
0.1
0.0
0.001
0.01
0.1
1
Conc. of test antibodies (μg/mL)

Fig.69

```
  EcoRI    Kozak
GAA TTC ACC ACC ATG GGC TGG AGC TGT ATT ATT CTC TTC CTC GTC GCC ACT GCA ACC GGG
                M   G   W   S   C   I   I   L   F   L   V   A   T   A   T   G

GTC CAC TCC CAG GTC CAG CTG GTG CAG AGC GGA GCT GAG GTG AAG AAG CCC GGC GCC AGC
 V   H   S   Q   V   Q   L   V   Q   S   G   A   E   V   K   K   P   G   A   S
         =

GTT AAG GTT TCT TGC AAA GCC TCT GGC TAT ACA TTC ACA ATC TAT TGG ATC AAT TGG GTG
 V   K   V   S   C   K   A   S   G   Y   T   F   T   I   Y   W   I   N   W   V

CGG CAG GCT CCA GGA CAA GGC CTG GAA TGG ATC GGC AAT ATC TAT CCT TCC GAC TCC TAC
 R   Q   A   P   G   Q   G   L   E   W   I   G   N   I   Y   P   S   D   S   Y

ACC AAC TAT AAC CAG AAA TTT AAG GAC AAA GCA ACA CTG ACC GTG GAC ACC TCT GCC AGC
 T   N   Y   N   Q   K   F   K   D   K   A   T   L   T   V   D   T   S   A   S

ACT GCT TAC ATG GAG CTG AGC AGC CTG AGA AGC GAA GAT ACT GCC GTG TAC TAC TGC ACC
 T   A   Y   M   E   L   S   S   L   R   S   E   D   T   A   V   Y   Y   C   T

                                                                  NheI
AGG ACA AGC ATG GCC GAT TAC TGG GGA CAA GGG ACC CTG GTG ACC GTG AGC AGC GCT AGC
 R   T   S   M   A   D   Y   W   G   Q   G   T   L   V   T   V   S   S
```

Fig.70

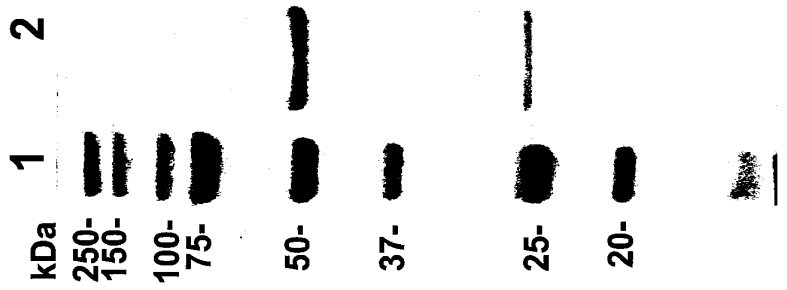


Fig.71

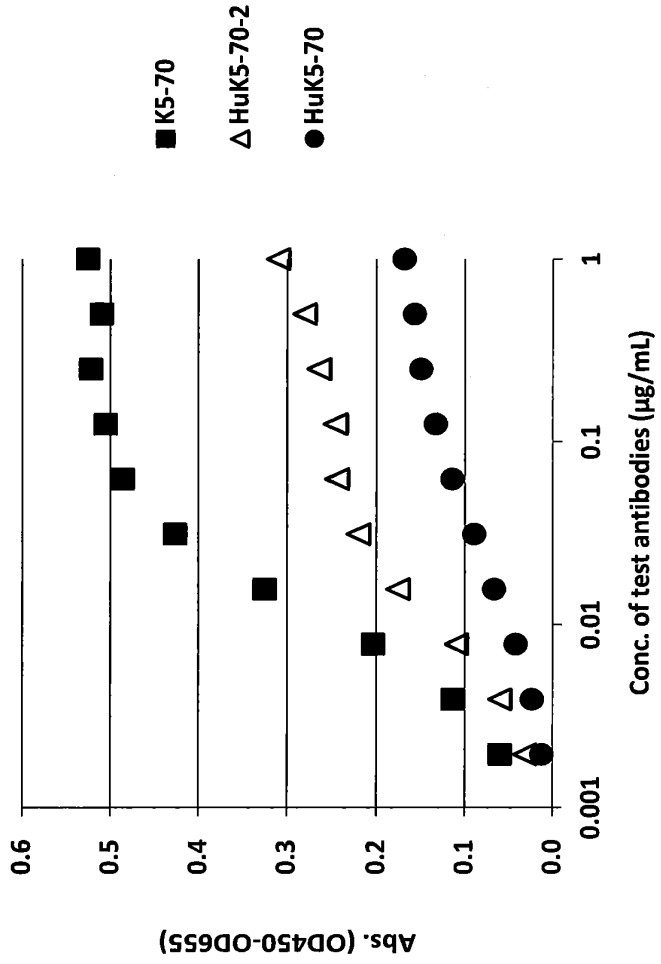

■K5-70
△HuK5-70-2
●HuK5-70
Abs. (OD450-OD655)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.001
0.01
0.1
1
Conc. of test antibodies (μg/mL)

Fig.72A

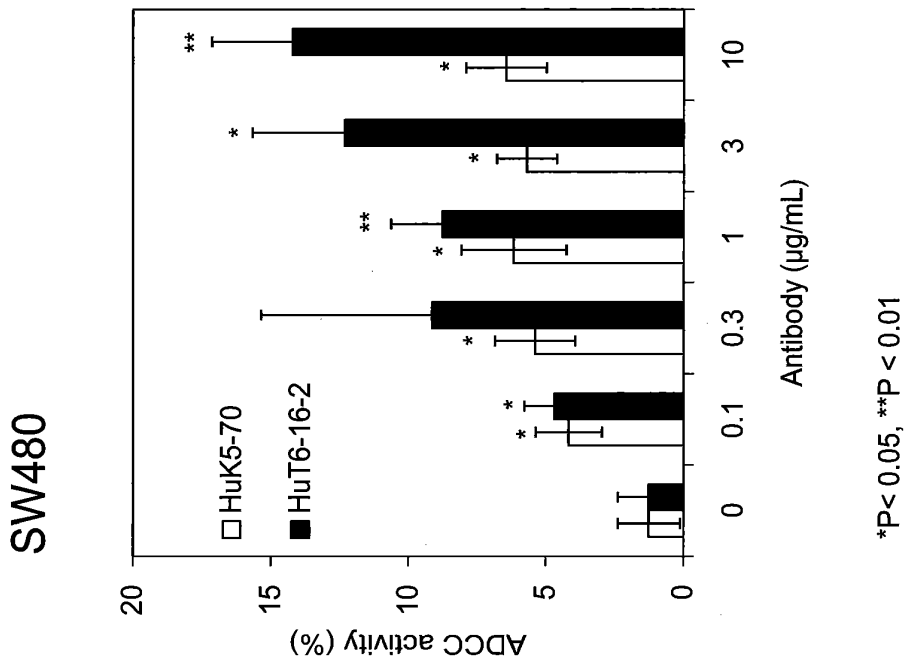

SW480
□HuK5-70
■HuT6-16-2
ADCC activity (%)
0
5
10
15
20
0
0.1
0.3
1
3
10
Antibody (μg/mL)
*P< 0.05, **P < 0.01

Fig.72B

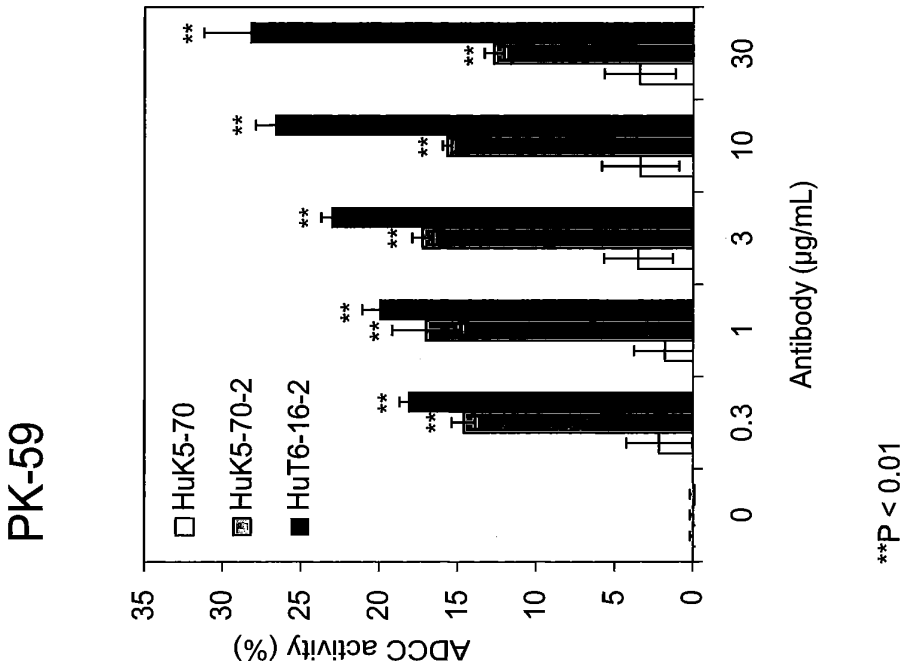
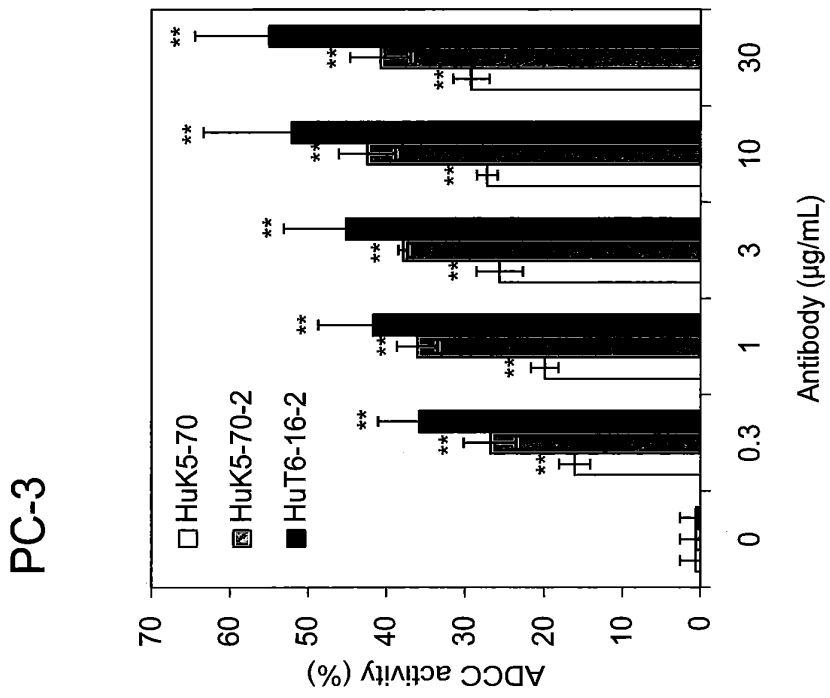

PK-59
HuK5-70
HuK5-70-2
HuT6-16-2
ADCC activity (%)
0
5
10
15
20
25
30
35
0
0.3
1
3
10
30
Antibody (μg/mL)
**
**P < 0.01

Fig.72C


PC-3
HuK5-70
HuK5-70-2
HuT6-16-2
ADCC activity (%)
0
10
20
30
40
50
60
70
0
0.3
1
3
10
30
Antibody (μg/mL)
**P < 0.01

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/30(2006.01)i, *A61K39/395*(2006.01)i, *A61P35/00*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/08*(2006.01)i, *G01N33/53*(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)
C07K16/30, A61K39/395, A61P35/00, C12N15/09, C12P21/08, G01N33/53

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 1996-2013
Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, WPI, BIOSIS(STN), CAplus(STN), MEDLINE(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	US 2007/0202113 A1 (Arius Research, Inc.), 30 August 2007 (30.08.2007), & JP 2009-527230 A & EP 1996700 A & WO 2007/095748 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2009-528995 A (Arius Research, Inc.), 13 August 2009 (13.08.2009), & US 2007/0202113 A1 & EP 1996700 A & WO 2007/095748 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2009-527230 A (Arius Research, Inc.), 30 July 2009 (30.07.2009), & US 2007/0202043 A1 & EP 1996701 A & WO 2007/095749 A1	18-20,22-40, 42-44/1-17, 21,41

☒ 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
26 February, 2013 (26.02.13)	12 March, 2013 (12.03.13)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	JP 2006-502698 A (Immunomedics, Inc.), 26 January 2006 (26.01.2006), & US 2004/0001825 A1 & EP 1483295 A & WO 2003/074566 A2	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2010-528056 A (F. Hoffmann-La Roche AG.), 19 August 2010 (19.08.2010), & US 2008/0131428 A1 & EP 2155791 A & WO 2008/144891 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	WO 2010/089782 A1 (Alberti Saverio), 12 August 2010 (12.08.2010), & JP 2012-516887 A & EP 2393512 A	18-20,22-40, 42-44/1-17, 21,41
P,X	WO 2011/145744 A1 (Livtech, Inc.), 24 November 2011 (24.11.2011), (Family: none)	1-44

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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:
See extra sheet.

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 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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

Continuation of Box No.III of continuation of first sheet(2)

Each of the antibodies described in claims 1 and 3 is specified by an amino acid sequence for an H-chain V-region thereof and an amino acid sequence for an L-chain V-region thereof. Although these amino acid sequences have a certain degree of homology with each other in their framework regions, the sequences for these regions are derived from known sequences, as described in the description of the present application (see the statements in examples 32-33). Therefore, both of the antibodies have no significant homology with each other when compared with those in the prior art techniques, and do not share any common structure, i.e., any common essential structural factor.

Both of the antibodies are common in a matter that the antibodies are anti-human TROP-2 antibodies each having an anti-tumor activity *in vivo* and having a human-originated framework region. However, an mouse anti-human TROP-2 antibody having an anti-tumor activity *in vivo* is disclosed in a document known before the priority date of the present application (US 2007/0202113 A1) and the humanization by a CDR graft method or the like on the basis of an antibody of a mouse or the like is a technique known to persons skilled in the art, as described in the description of the present application (see page 42). When these matters are taken in to consideration, a matter that a humanized antibody having a human-originated framework region can be produced on the basis of a mouse anti-human TROP-2 antibody having an anti-tumor activity *in vivo* can be conceived easily by persons skilled in the art. Therefore, the common matter cannot be regarded as a special technical feature in the meaning within PCT Rule 13.2, second sentence.

Consequently, it is not considered that the inventions of claims 1-44 are relevant to a group of inventions which are so linked as to form a single general inventive concept.

Accordingly, two inventions, which comprise (A) the inventions of claims 1-2, and the parts of the inventions of claims 5-44 which are concerned with claims 1-2 and (B) the inventions of claims 3-4, and the parts of the inventions of claims 5-44 which are concerned with claims 3-4, are set forth in claims 1-44.

Form PCT/ISA/210 (extra sheet) (July 2009)

REFERENCES CITED IN THE DESCRIPTION

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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 907 824 A1

(12) EUROPEAN PATENT APPLICATION
published in accordance with Art. 153(4) EPC

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(51) Int Cl.:
C07K 16/28 *(2006.01)* ***A61K 39/395*** *(2006.01)*
A61P 35/00 *(2006.01)* ***A61K 31/4745*** *(2006.01)*
A61K 31/48 *(2006.01)*

(86) International application number:
PCT/JP2013/006069

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Designated Extension States:
BA ME

(30) Priority: **11.10.2012 JP 2012225887**

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Tokyo 103-8426 (JP)

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- **MIYAZAKI, Hideki**
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- **KASUYA, Yuji**
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- **MORITA, Koji**
  Tokyo 140-8710 (JP)
- **ABE, Yuki**
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- **OGITANI, Yusuke**
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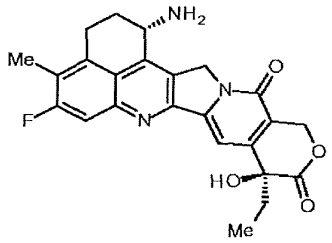
(54) ANTIBODY-DRUG CONJUGATE

(57) As an antitumor drug which is excellent in terms of antitumor effect and safety, there is provided an antibody-drug conjugate in which an antitumor compound represented by the following formula is conjugated to an antibody via a linker having a structure represented by the following formula: $-L^1-L^2-L^P-NH-(CH_2)_{n^1}-L^a-L^b-L^c-$ wherein the antibody is connected to the terminal of L^1, and the antitumor compound is connected to the terminal of L^c with the nitrogen atom of the amino group at position 1 as a connecting position.

[Formula 1]



EP 2 907 824 A1

Description

Technical Field

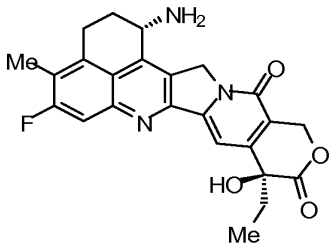
[0001] The present invention relates to an antibody-drug conjugate having an antitumor drug conjugated to an antibody capable of targeting tumor cells via a linker structure moiety, the conjugate being useful as an antitumor drug.

Background Art

[0002] An antibody-drug conjugate (ADC) having a drug with cytotoxicity conjugated to an antibody, whose antigen is expressed on a surface of cancer cells and which also binds to an antigen capable of cellular internalization, and therefore can deliver the drug selectively to cancer cells and is thus expected to cause accumulation of the drug within cancer cells and to kill the cancer cells (see, Non Patent Literatures 1 to 3). As an ADC, Mylotarg (Gemtuzumab ozogamicin) in which calicheamicin is conjugated to an anti-CD33 antibody is approved as a therapeutic agent for acute myeloid leukemia. Further, Adcetris (Brentuximab vedotin), in which auristatin E is conjugated to an anti-CD30 antibody, has recently been approved as a therapeutic agent for Hodgkin's lymphoma and anaplastic large cell lymphoma (see, Non Patent Literature 4). The drugs contained in ADCs which have been approved until now target DNA or tubulin.

[0003] With regard to an antitumor, low-molecular-weight compounds, camptothecin derivatives, compounds that inhibit topoisomerase I to exhibit an antitumor effect, are known. Among them, an antitumor compound represented by the formula below

[Formula 1]



(exatecan, chemical name: (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-10,13(9H,15H)-dione) is a water soluble derivative of camptothecin (Patent Literature 1 and 2). Unlike irinotecan currently used in clinical settings, an activation by an enzyme is unnecessary. Further, the inhibitory activity on topoisomerase I is higher than SN-38 whiich is a main pharmaceutically active substance of irinotecan and topotecan also used in clinical settings, and higher in vitro cytocidal activity is obtained for against various cancer cells. In particular, it exhibits the effect against cancer cells which have resistance to SN-38 or the like due to expression of P-glycoprotein. Further, in a human tumor subcutaneously transplanted mouse model, it exhibited a potent antitumor effect, and thus has undergone the clinical studies, but has not been put on the market yet (see, Non Patent Literatures 5 to 10). It remains unclear whether or not exatecan functions effectively as an ADC.

[0004] DE-310 is a complex in which exatecan is conjugated to a biodegradable carboxymethyldextran polyalcohol polymer via a GGFG peptide spacer (Patent Literature 3). By converting exatecan into a form of a polymer prodrug, so that a high blood retention property can be maintained and also a high targetable property to a tumor area is passively increased by utilizing the increased permeability of newly formed blood vessels within tumor and retention property in tumor tissues. With DE-310, through a cleavage of the peptide spacer by enzyme, exatecan and exatecan with glycine connected to an amino group are continuously released as a main active substance. As a result, the pharmacokinetics are improved and DE-310 was found to have higher effectiveness than exatecan administered alone even though the dosage of exatecan is lower than the case of administration of exatecan alone according to various tumor evaluation models in non-clinical studies. A clinical study was conducted for DE-310, and effective cases were confirmed in humans, in which a report suggesting that the main active substance accumulates in a tumor than in normal tissues was present, however, there is also a report indicating that the accumulation of DE-310 and the main active substance in a tumor is not much different from the accumulation in normal tissues in humans, and thus no passive targeting is observed in humans (see, Non Patent Literatures 11 to 14). As a result, DE-310 was not also commercialized, and it remains unclear whether or not exatecan effectively functions as a drug oriented for such targeting.

[0005] As a compound relating to DE-310, a complex in which a structure moiety represented by $-NH(CH_2)_4C(=O)-$ is

inserted between -GGFG-spacer and exatecan to form - GGFG-NH(CH_2)$_4$C(=O)- used as a spacer structure is also known (Patent Literature 4). However, the antitumor effect of the complex is not known at all.

[Citation List]

[Patent Literature]

[0006]

[Patent Literature 1] Japanese Patent Laid-Open No. 5-59061
[Patent Literature 2] Japanese Patent Laid-Open No. 8-337584
[Patent Literature 3] International Publication No. WO 1997/46260
[Patent Literature 4] International Publication No. WO 2000/25825

[Non Patent Literature]

[0007]

[Non Patent Literature 1] Ducry, L., et al. Bioconjugate Chem. (2010) 21, 5-13.; Antibody-Drug Conjugates: Linking cytotoxic payloads to monoclonal antibodies.
[Non Patent Literature 2] Alley, S. C., et al. Current Opinion in Chemical Biology (2010) 14, 529-537.; Antibody-drug conjugates: targeted drug delivery for cancer.
[Non Patent Literature 3] Damle N.K. Expert Opin. Biol. Ther. (2004) 4, 1445-1452.; Tumour-targeted chemotherapy with immunoconjugates of calicheamicin.
[Non Patent Literature 4] Senter P. D., et al. Nature Biotechnology (2012) 30, 631-637.; The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma.
[Non Patent Literature 5] Kumazawa, E., Tohgo, A., Exp. Opin. Invest. Drugs (1998) 7, 625-632.; Antitumour activity of DX-8951f: a new camptothecin derivative.
[Non Patent Literature 6] Mitsui, I., Kumazawa, E., Hirota, Y., et al. Jpn J. Cancer Res. (1995) 86, 776-786.; A new water-soluble camptothecin derivative, DX-8951f, exhibits potent antitumor activity against human tumors in vitro and in vivo.
[Non Patent Literature 7] Takiguchi, S., Tohgo, A., et al. Jpn J. Cancer Res. (1997) 88, 760-769.; Antitumor effect of DX-8951, a novel camptothecin analog, on human pancreatic tumor cells and their CPT-11-resistant variants cultured in vitro and xenografted into nude mice.
[Non Patent Literature 8] Joto, N. et al. Int J Cancer (1997) 72, 680-686.; DX-8951f, a water-soluble camptothecin analog, exhibits potent antitumor activity against a human lung cancer cell line and its SN-38-resistant variant.
[Non Patent Literature 9] Kumazawa, E. et al. Cancer Chemother. Pharmacol. (1998) 42, 210-220.; Potent and broad antitumor effects of DX-8951f, a water-soluble camptothecin derivative, against various human tumors xenografted in nude mice.
[Non Patent Literature 10] De Jager, R., et al. Ann N Y Acad Sci (2000) 922, 260-273.; DX-8951f: summary of phase I clinical trials.
[Non Patent Literature 11] Inoue, K. et al. Polymer Drugs in the Clinical Stage, Edited by Maeda et al. (2003), 145-153.; CM-dextran-polyalcohol-camptothecin conjugate, DE-310 with a novel carrier system and its preclinical data.
[Non Patent Literature 12] Kumazawa, E. et al. Cancer Sci (2004) 95, 168-175.; DE-310, a novel macromolecular carrier system for the camptothecin analog DX-8951f: Potent antitumor activities in various murine tumor models.
[Non Patent Literature 13] Soepenberg, O. et al. Clinical Cancer Research, (2005) 11, 703-711.; Phase I and pharmacokinetic study of DE-310 in Patients with Advanced Solid Tumors.
[Non Patent Literature 14] Wente M. N. et al. Investigational New Drugs (2005) 23, 339-347.; DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX-8951), in patients with operable solid tumors.

[Summary of Invention]

[Technical Problem]

[0008] With regard to the treatment of tumor by an antibody, an insufficient antitumor effect may be observed even when the antibody recognizes an antigen and binds to tumor cells, and there is a case in which a more effective antitumor antibody is needed. Further, many antitumor low-molecular-weight compounds have a problem in safety like side effect

and toxicity even the compounds have an excellent antitumor effect, it remains as a subject to achieve a superior therapeutic effect by further enhancing the safety. Thus, an object of the present invention is to obtain to provide an antitumor drug having an excellent therapeutic effect, which is excellent in terms of antitumor effect and safety.

[Means to Solve the Problem]

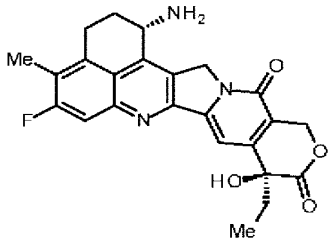
[0009] The inventors thought that, when an antitumor compound exatecan is converted into an antibody-drug conjugate, via a linker structure moiety, by conjugation to the antibody, which is capable of targeting tumor cells, that is having a property of recognizing tumor cells, a property of binding to tumor cells, a property of internalizing within tumor cells, a cytocidal activity against tumor cells, or the like, the antitumor compound can be more surely delivered to tumor cells to specifically exhibit the antitumor effect of the compound in tumor cells, and thus the antitumor effect can be surely exhibited and also an enhanced cytocidal effect of the antibody is expected, and a dose of the antitumor compound can be reduced compared to a case of administering the compound alone, and thus an influence of the antitumor compound on normal cells can be alleviated so that higher safety can be achieved.

[0010] In this connection, the inventors created a linker with a specific structure and succeeded in obtaining an antibody-drug conjugate in which the antibody and exatecan are conjugated to each other via the linker, and confirmed an excellent antitumor effect exhibited by the conjugate to thereby complete the present invention.

[0011] Specifically, the present invention relates to the followings.

[1] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 2]



is conjugated to an antibody via a linker having a structure represented by the following formula: $-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$.

Here, the antibody is connected to the terminal of L^1, the antitumor compound is connected to the terminal of L^c with the nitrogen atom of the amino group at position 1 as connecting position,

wherein

n^1 represents an integer of 0 to 6,

L^1 represents -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, $-CH_2$-C(=O)-NH-$(CH_2)n^3$-C(=O)-, -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-, or -C(=O)-$(CH_2)n^4$-C(=O)-,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8,

L^2 represents -NH-$(CH_2$-CH_2-O$)n^5$-CH_2-CH_2-C(=O)-, -S-$(CH_2)n^6$-C(=O)-, or a single bond,

wherein n^5 represents an integer of 1 to 6, n^6 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 2 to 7 amino acids,

L^a represents -C(=O)-NH-, $-NR^1-(CH_2)n^7$-, -O-, or a single bond,

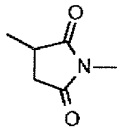
wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, - (CH_2) n^8-COOH, or -$(CH_2)n^9$-OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,

L^b represents $-CR^2(-R^3)$-, -O-, $-NR^4$-, or a single bond,

wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^a-NH_2$, $-(CH_2)n^b$-COOH, or $-(CH_2)n^c$-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other,

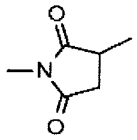
L^c represents $-CH_2$- or -C(=O)-, -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 3]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
-(N-ly-3-diminiccuS)- has a structure represented by the following formula:

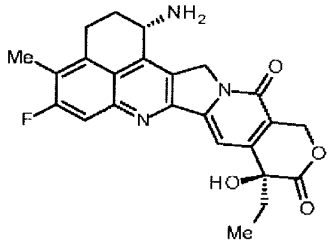
[Formula 4]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and when L^2 is -S-$(CH_2)n^6$-C(=O)-, L^1 is -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-.

The present invention further relates to each of the followings.
[2] The antibody-drug conjugate according to [1], wherein L^c is -C(=O)-.
[3] The antibody-drug conjugate according to [1] or [2], wherein the bond between the antibody and L^1 is a thioether bond which is formed at a disulfide bond site present in a hinge part of the antibody,
a disulfide bond which is formed at a disulfide bond site present in a hinge part of the antibody, or an amide bond which is formed at an amino group present on a side chain of an amino acid constituting the antibody or at the terminal amino group.
[4] The antibody-drug conjugate according to any one of [1] to [3], wherein the peptide residue of L^P is an amino acid residue comprising an amino acid selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid.
[5] The antibody-drug conjugate according to any one of [1] to [3], wherein L^P is a peptide residue consisting of 4 amino acids.
[6] The antibody-drug conjugate according to any one of [1] to [3], wherein L^P is -GGFG-.
[7] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 5]



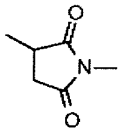
is conjugated to an antibody via a linker having a structure represented by the following formula:

$$-L^1-L^2-L^P-NH-(CH_2)\ n^1-L^a-L^b-L^c-.$$

Here, the antibody is connected to the terminal of L^1, the antitumor compound is connected to the terminal of L^c with the nitrogen atom of the amino group at position 1 as a connecting position,
wherein

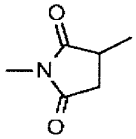
n^1 represents an integer of 0 to 6,
L^1 represents -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, -CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-, -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-, or -C(=O)-$(CH_2)n^4$-C(=O)-,
wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8,
L^2 represents -NH-$(CH_2$-CH_2-$O)n^5$-CH_2-CH_2-C(=O)-, -S-$(CH_2)n^6$-C(=O)-, or a single bond,
wherein n^5 represents an integer of 1 to 6, n^6 represents an integer of 1 to 6,
L^P represents a tetrapeptide residue of GGFG,
L^a represents -O- or a single bond,
L^b represents -CR^2(-R^3)- or a single bond,
wherein R^2 and R^3 each represents a hydrogen atom, L^c represents -C(=O)-,
-(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 6]



which is connected to the antibody at position 3 thereof and is connedted to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
-(N-ly-3-diminiccuS)- has a structure represented by the following formula:

[Formula 7]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and when L^2 is -S-$(CH_2)n^6$-C(=O)-, L^1 is -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-.

[8] The antibody-drug conjugate according to any one of [1] to [7], wherein L^1 is -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)- or -CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-.
[9] The antibody-drug conjugate according to any one of [1] to [7], wherein L^1 is -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-.
[10] The antibody-drug conjugate according to any one of [1] to [7], wherein L^1 is -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)- or -C(=O)-$(CH_2)n^4$-C(=O)-.
[11] The antibody-drug conjugate according to any one of [1] to [9], wherein n^2 is an integer of 2 to 5, and L^2 is a single bond.
[12] The antibody-drug conjugate according to any one of [1] to [9], wherein n^2 is an integer of 2 to 5, L^2 is -NH-$(CH_2CH_2O)n^5$-CH_2-CH_2-C(=O)-, and n^5 is 2 or 4.
[13] The antibody-drug conjugate according to any one of [1] to [12], wherein -NH-$(CH_2)n^1$-L^a-L^b-L^c- is a partial structure having a chain length of 4 to 7 atoms.

[14] The antibody-drug conjugate according to any one of [1] to [12], wherein -NH-$(CH_2)n^1$-L^a-L^b-L^c- is a partial structure having a chain length of 5 or 6 atoms.

[15] The antibody-drug conjugate according to any one of [1] to [14], wherein -NH-$(CH_2)n^1$-L^a-L^b-L^c- is

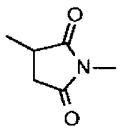
-NH-$(CH_2)_3$-C(=O)-,
-NH-CH_2-O-CH_2-C(=O)-, or
-NH-$(CH_2)_2$-O-CH_2-C(=O)-.

[16] The antibody-drug conjugate according to any one of [1] to [15], wherein the drug-linker structure moiety is one drug-linker structure selected from the group consisting of the following drug-linker structures:

-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

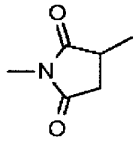
Wherein, -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 8]



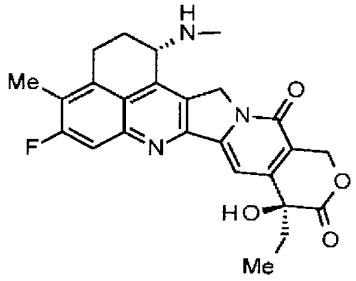
which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
-(N-Iy-3-diminiccuS)- has a structure represented by the following formula:

[Formula 9]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
cyc.Hex(1,4) represents a 1,4-cyclohexylene group,- (NH-DX) represents a group represented by the following formula:

[Formula 10]



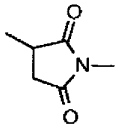
wherein the nitrogen atom of the amino group at position 1 is the connectig position, and
-GGFG- represents a peptide residue of -Gly-Gly-Phe-Gly-.

[17] The antibody-drug conjugate according to any one of [1] to [9] and [11] to [14], wherein the drug-linker structure moiety having a drug connected to $-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$ is one drug-linker structure selected from the following group:

- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX).

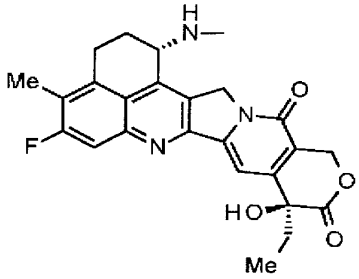
In the above, -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 11]



which is connected to the antibody at position 3 thereof and connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and
-(NH-DX) represents a group represented by the following formula:

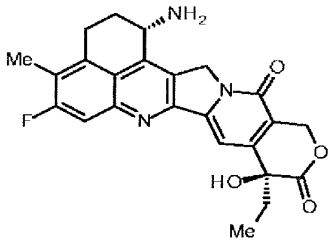
[Formula 12]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[18] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 13]



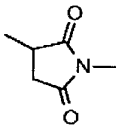
is conjugated to an antibody via a linker having a structure represented by the following formula:

$$-L^1-L^2-L^P-NH-(CH_2)\ n^1-L^a-L^b-L^c-.$$

Here, the antibody is connected to the terminal of L^1, the antitumor compound is connected to the terminal of L^c, wherein

n^1 represents an integer of 0 to 6,
L^1 represents -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)- and is connected to the antibody via a thioether bond which is formed at a disulfide bond site present in a hinge part of the antibody,
wherein n^2 represents an integer of 2 to 8,
L^2 represents -NH-$(CH_2$-CH_2-O$)n^5$-CH_2-CH_2-C(=O) -or a single bond,
wherein n^5 represents an integer of 1 to 6,
L^P represents a tetrapeptide residue of GGFG,
L^a represents -O- or a single bond,
L^b represents -CR^2(-R^3)- or a single bond,
wherein R^2 and R^3 each represents a hydrogen atom,
L^c represents -C(=O)-, and
-(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 14]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1.

[19] The antibody-drug conjugate according to [18], wherein

n^2 is 2, L^2 is -NH-$(CH_2$-CH_2-O$)n^5$-CH_2-CH_2-C(=O)-, n^5 is 2, n^1 is 3, and both of L^a and L^b are single bonds,
n^2 is 5, L^2 is a single bond, n^1 is 1, L^a is -O-, and L^b is -CR^2(-R^3)-, or
n^2 is 5, L^2 is a single bond, n^1 is 2, L^a is -O-, and L^b is -CR^2(-R^3)-.

[20] The antibody-drug conjugate according to [18] or [19], wherein n^2 is an integer of 2 to 5, and L^2 is a single bond.
[21] The antibody-drug conjugate according to [18] or [19], wherein n^2 is an integer of 2 to 5, L^2 is -NH-$(CH_2CH_2O)n^5$-CH_2-CH_2-C(=O)-, and n^5 is 2 or 4.
[22] The antibody-drug conjugate according to any one of [18] to [21], wherein -NH-$(CH_2)n^1$-L^a-L^b-L^c- is

-NH-$(CH_2)_3$-C(=O)-,
-NH-CH_2-O-CH_2-C(=O)-, or

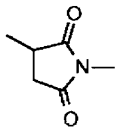
-NH-$(CH_2)_2$-O-CH_2-C(=O)-.

[23] The antibody-drug conjugate according to any one of [18] to [22], wherein the drug-linker structure moiety is one drug-linker structure selected from the group consisting of the following drug-linker structures:

-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)

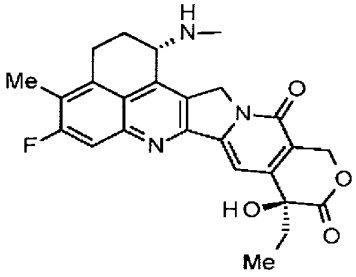
In the above, -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 15]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and -(NH-DX) represents a group represented by the following formula:

[Formula 16]



wherein the nitrogen atom of the amino group at position 1 is a connection position.

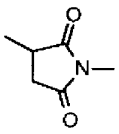
[24] The antibody-drug conjugate according to [23], wherein the drug-linker structure moiety having a drug connected to -L^1-L^2-L^P-NH-$(CH_2)n^1$-L^a-L^b-L^c- is one drug-linker structure selected from the following group:

-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)

- (Succinimid-3-yl-N) -CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX).

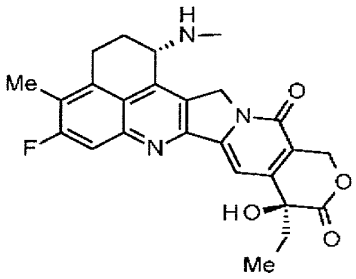
In the above, -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 17]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and -(NH-DX) represents a group represented by the following formula:

[Formula 18]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[25] The antibody-drug conjugate according to any one of [1] to [24], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 1 to 10.

[26] The antibody-drug conjugate according to any one of [1] to [24], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 2 to 8.

[27] The antibody-drug conjugate according to any one of [1] to [24], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 3 to 8.

[28] The antibody-drug conjugate according to any one of [1] to [27], wherein the antibody is an antibody having one or more of a property of recognizing a target cell, a property of binding to a target cell, a property of internalizing in a target cell, and a property of damaging a target cell.

[29] The antibody-drug conjugate according to any one of [1] to [27], wherein a cell which is targeted by the antibody-drug conjugate is a tumor cell.

[30] The antibody-drug conjugate according to any one of [1] to [27], wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.

[31] The antibody-drug conjugate according to any one of [1] to [27], wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

[32] The antibody-drug conjugate according to any one of [1] to [27], wherein the antibody is an anti-B7-H3 antibody.

[33] A drug containing the antibody-drug conjugate according to any one of [1] to [32], a salt thereof or a hydrate thereof.

[34] An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to any one of [1] to [32], a salt thereof or a hydrate thereof.

[35] The antitumor drug and/or anticancer drug according to [34], which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

[36] A pharmaceutical composition containing the antibody-drug conjugate according to any one of [1] to [32], a salt thereof or a hydrate thereof as an active component, and a pharmaceutically acceptable formulation component.

[37] The pharmaceutical composition according to [36], which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

[38] A method for treating tumor and/or cancer comprising administering the antibody-drug conjugate according to any one of [1] to [32], a salt thereof or a hydrate thereof.

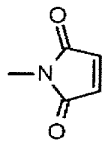
[39] A drug-linker intermediate compound represented by the following formula:

$$Q\text{-}(CH_2)_{n^Q}\text{-}C(=O)\text{-}L^{2a}\text{-}L^P\text{-}NH\text{-}(CH_2)_{n^1}\text{-}L^a\text{-}L^b\text{-}L^c\text{-}(NH\text{-}DX).$$

In the formula, Q represents (maleimid-N-yl)-, HS-, X-CH_2-C(=O)-NH-, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-,

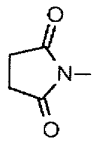
X represents a bromine atom or an iodine atom,
n^Q represents an integer of 2 to 8,
L^{2a} represents -NH-(CH_2-CH_2-O)n^5-CH_2-CH_2-C(=O)- or a single bond,
wherein n^5 represents an integer of 1 to 6,
L^P represents a peptide residue consisting of 2 to 7 amino acids selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid,
n^1 represents an integer of 0 to 6,
L^a represents -C(=O)-NH-, -NR^1-(CH_2)n^7-, -O-, or a single bond,
wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH_2)n^8-COOH, or -(CH_2)n^9-OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,
L^b represents -CR^2(-R^3)-, -O-, -NR^4-, or a single bond,
wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH_2)n^a-NH_2, -(CH_2)n^b-COOH, or -(CH_2)n^c-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other,
L^c represents -CH_2- or -C(=O)-,
(maleimid-N-yl)- is a group represented by the following formula:

[Formula 19]



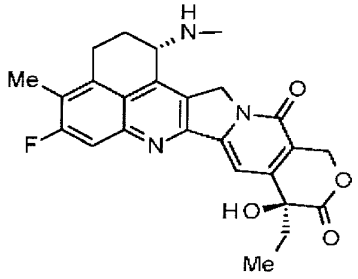
whherein the nitrogen atom is a connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

[Formula 20]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 21]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[40] The drug-linker intermediate compound according to [39], wherein L^c is -C(=O)-.
[41] The drug-linker intermediate compound according to [39] or [40], wherein L^P is a peptide residue consisting of 4 amino acids.
[42] The drug-linker intermediate compound according to any one of [39] to [41], wherein L^P is -GGFG-.
[43] The drug-linker intermediate compound according to any one of [39] to [42], wherein -NH-$(CH_2)n^1$-L^a-L^b- is

-NH-CH_2CH_2-,
-NH-$CH_2CH_2CH_2$-,
-NH-$CH_2CH_2CH_2CH_2$-,
-NH-$CH_2CH_2CH_2CH_2CH_2$-,
-NH-CH_2-O-CH_2-, or
-NH-CH_2CH_2-O-CH_2-.

[44] The drug-linker intermediate compound according to any one of [39] to [42], wherein -NH-$(CH_2)n^1$-L^a-L^b- is

-NH-$CH_2CH_2CH_2$-,
-NH-CH_2-O-CH_2-, or
-NH- $(CH_2)_2$-O-CH_2-.

[45] The drug-linker intermediate compound according to any one of [39] to [44], wherein n^Q is an integer of 2 to 6.
[46] The drug-linker intermediate compound according to [43], wherein

Q is (maleimid-N-yl)-,
n^Q is an integer of 2 to 5, and
L^{2a} is a single bond.

[47] The drug-linker intermediate compound according to [44], wherein

Q is (maleimid-N-yl)-,
n^Q is an integer of 2 to 5, and
L^{2a} is a single bond.

[48] The drug-linker intermediate compound according to any one of [39] to [42], wherein

Q is (maleimid-N-yl)-,
n^Q is an integer of 2 to 5,
L^{2a} is -NH-$(CH_2$-CH_2-O$)n^5$-CH_2-CH_2-C(=O)-,
n^5 is an integer of 2 to 4, and
-NH-$(CH_2)n^1$-L^a-L^b- is
-NH-CH_2CH_2-,
-NH-$CH_2CH_2CH_2$-,
-NH-$CH_2CH_2CH_2CH_2$-,

-NH-$CH_2CH_2CH_2CH_2CH_2$-,
-NH-CH_2-O-CH_2-, or
-NH-CH_2CH_2-O-CH_2-.

[49] The drug-linker intermediate compound according to [48], wherein

n^5 is an integer of 2 or 4, and
-NH- (CH_2) n^1-L^a-L^b- is
-NH-$CH_2CH_2CH_2$-,
-NH-CH_2-O-CH_2-, or
-NH-CH_2CH_2-O-CH_2-.

[50] A compound of the following:

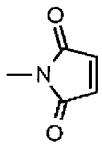
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl) -$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C (=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C (=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)

X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX), or
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX).

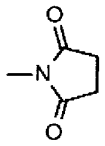
In the above, (maleimid-N-yl)- is a group represented by the following formula:

[Formula 22]



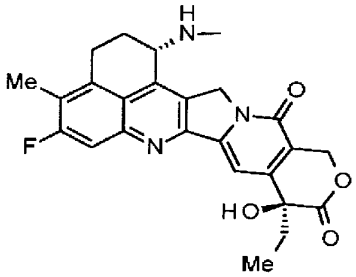
wherein the nitrogen atom is a connecting position, X represents a halogen atom, (Pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

[Formula 23]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 24]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

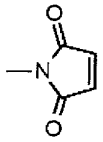
[51] A compound of the following:

(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-

$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX), or (maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX).

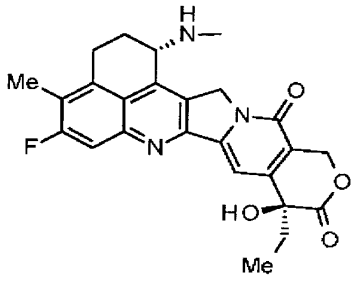
In the above, (maleimid-N-yl)- is a group represented by the following formula:

[Formula 25]



wherein the nitrogen atom is a connecting position, and- (NH-DX) is a group represented by the following formula:

[Formula 26]

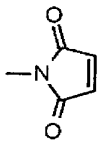


wherein the nitrogen atom of the amino group at position 1 is a connecting position.
[52] A compound of the following:

(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX),
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX) or
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX).

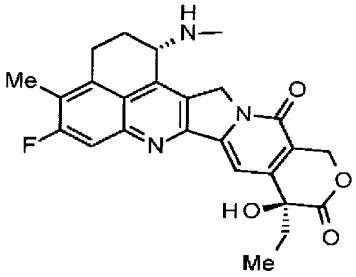
In the above, (maleimid-N-yl)- is a group represented by the following formula:

[Formula 27]



wherein the nitrogen atom is a connecting position, and- (NH-DX) is a group represented by the following formula:

[Formula 28]



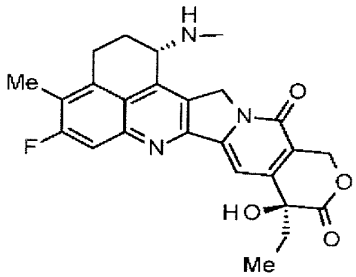
wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[53] A compound selected from the following group:

$NH2\text{-}CH_2CH_2\text{-}C(=O)\text{-}(NH\text{-}DX)$,
$NH2\text{-}CH_2CH_2CH_2\text{-}C(=O)(NH\text{-}DX)$
$NH_2\text{-}CH_2\text{-}O\text{-}CH_2\text{-}C(=O)\text{-}(NH\text{-}DX)$,
$NH_2\text{-}CHCH_2\text{-}O\text{-}CH_2\text{-}C(=O)\text{-}(NH\text{-}DX)$, and
$HO\text{-}CH_2\text{-}C(=O)\text{-}(NH\text{-}DX)$

wherein -(NH-DX) is a group represented by the following formula:

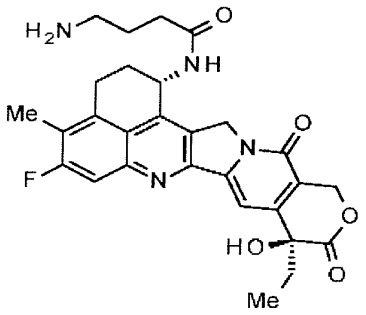
[Formula 29]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

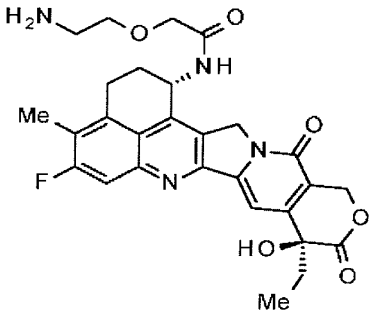
[54] A compound represented by the following formula:

[Formula 30]



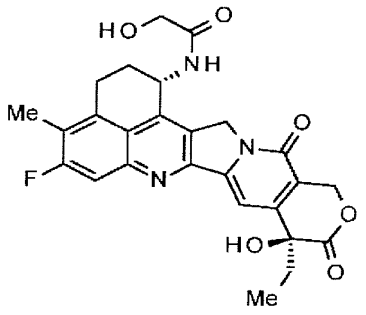
[55] A compound represented by the following formula:

[Formula 31]



[56] A compound represented by the following formula:

[Formula 32]



[57] A method for producing an antibody-drug conjugate comprising reacting a compound represented by the following formula:

$$Q\text{-}(CH_2)n^{Q}\text{-}C(=O)\text{-}L^{2a}\text{-}L^{P}\text{-}NH\text{-}(CH_2)n^{1}\text{-}L^{a}\text{-}L^{b}\text{-}L^{c}\text{-}(NH\text{-}DX)$$

with an antibody or a reactive derivative thereof and conjugating a drug-linker moiety to the antibody by a method

for forming a thioether bond at a disulfide bond site present in a hinge part of the antibody, or by a method for forming an amide bond at an amino group present on a side chain of an amino acid constituting the antibody or at the terminal amino group.

In the formula, Q represents (maleimid-N-yl)-, HS-, X-CH_2-C(=O)-NH-, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-,

X represents a bromine atom or an iodine atom,

n^Q represents an integer of 2 to 8,

L^{2a} represents -NH-(CH_2-CH_2-O)n^5-CH_2-CH_2-C(=O)- or a single bond,

wherein n^5 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 2 to 7 amino acids selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid,

n^1 represents an integer of 0 to 6,

L^a represents -C(=O)-NH-, -NR^1-(CH_2)n^7-, -O-, or a single bond,

wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH_2)n^8-COOH, or -(CH_2)n^9-OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,

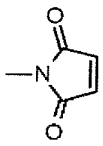
L^b represents -CR^2(-R^3)-, -O-, -NR^4-, or a single bond,

wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH_2)n^a-NH_2, -(CH_2)n^b-COOH, or -(CH_2)n^c-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other,

L^c represents -CH_2- or -C(=O)-,

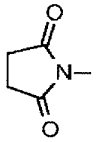
(maleimid-N-yl)- is a group represented by the following formula:

[Formula 33]



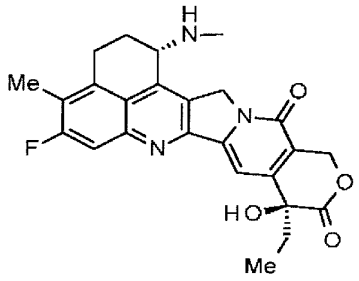
wherein the nitrogen atom is a connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

[Formula 34]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 35]

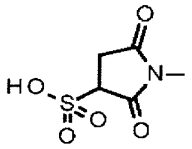


wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[58] The production method according to [57], wherein the method for conjugating a drug-linker moiety to an antibody is

a method of reducing the antibody and thereafter forming a thioether bond by the reaction with the compound in which Q is a maleimidyl group or X-CH_2-C(=O)-NH-,
a method of forming an amide bond by the reaction with the compound in which Q is (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, or
a method of reacting the antibody with a compound represented by the formula Q^1-L^{1a}-Q^2
[wherein Q^1 represents (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, R^Q-O-C(=N)-, or O=C=N-,
L^{1a}- represents -cyc.Hex(1,4)-CH_2-, an alkylene group having 1 to 10 carbon atoms, a phenylene group,-$(CH_2)n^4$-C(=O)-, - $(CH_2)n^{4a}$-NH-C(=O)-$(CH_2)n^{4b}$-, or - $(CH_2)n^{4a}$-NH-C(=O)-cyc.Hex(1,4)-CH_2-,
Q^2 represents (maleimid-N-yl), a halogen atom, or -S-S-(2-Pyridyl),
R^Q represents an alkyl group having 1 to 6 carbon atoms, n^4 represents an integer of 1 to 8,
n^{4a} represents an integer of 0 to 6, n^{4b} represents an integer of 1 to 6,
(3-Sulfo-pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

[Formula 36]



wherein the nitrogen atom is a connecting position, and this sulfonic acid is capable of forming a lithium salt, sodium salt, or potassium salt,
cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and (2-Pyridyl) represents a 2-pyridyl group]
and thereafter reacting with the compound in which Q is SH to form a drug-linker structure by an amide bond.

[59] The production method according to [57] or [58], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 1 to 10.
[60] The production method according to [57] or [58], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 2 to 8.
[61] The production method according to [57] or [58], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 3 to 8.
[62] The production method according to any one of [57] to [61], wherein a cell which is targeted by the antibody-drug conjugate is a tumor cell.
[63] The production method according to any one of [57] to [61], wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody,

an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.

[64] The production method according to any one of [57] to [61], wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

[65] The production method according to any one of [57] to [61], wherein the antibody is an anti-B7-H3 antibody.

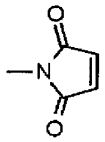
[66] An antibody-drug conjugate obtained by the production method according to any of [57] to [65].

[67] An antibody-drug conjugate obtained by forming a thioether bond at a sulfide bond site in a hinge part of an antibody, wherein the antibody is treated in a reducing condition and thereafter reacted with a compound selected from the compound group shown below:

(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX), or
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX).

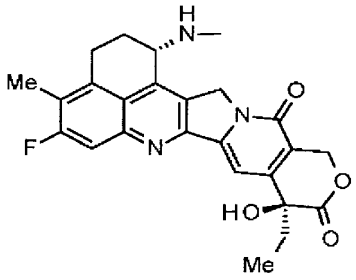
In the above, (maleimid-N-yl)- is a group represented by the following formula:

[Formula 37]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 38]



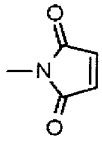
wherein the nitrogen atom of the amino group at position 1 is a connecting position.

[68] An antibody-drug conjugate obtained by forming a thioether bond at a sulfide bond site present in a hinge part of an antibody, wherein the antibody is treated in a reducing condition and thereafter reacted with a compound selected from the compound group shown below:

(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX),
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX), or
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX).

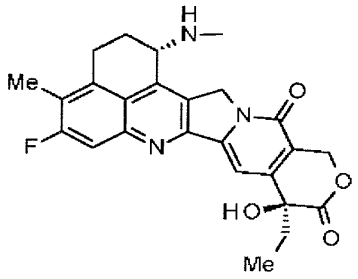
In the above, (maleimid-N-yl)- is a group represented by the following formula:

[Formula 39]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 40]



wherein the nitrogen atom of the amino group at position 1 is a connceting position.

[69] The antibody-drug conjugate according to [67] or [68], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 1 to 10.

[70] The antibody-drug conjugate according to [67] or [68], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 2 to 8.

[71] The antibody-drug conjugate according to [67] or [68], wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 3 to 8.

[72] The antibody-drug conjugate according to any one of [67] to [71], wherein a cell which is targeted by the antibody-drug conjugate is a tumor cell.

[73] The antibody-drug conjugate according to any one of [67] to [71], wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.

[74] The antibody-drug conjugate according to any one of [67] to [71], wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

[75] The antibody-drug conjugate according to any one of [67] to [71], wherein the antibody is an anti-B7-H3 antibody.

[76] A linker represented by the following formula:

$$-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$$

for obtaining an antibody-drug conjugate in which a drug is conjugated to an antibody via the linker.

In the above, L^1 is a connecting position for the antibody, L^c is a connectiong position for an antitumor compound, wherein

n^1 represents an integer of 0 to 6,

L^1 represents -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-, -CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-, -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-, or -C(=O)-$(CH_2)n^4$-C(=O)-,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8,

L^2 represents -NH-(CH_2-CH_2-O)n^5-CH_2-CH_2-C(=O) -, -S-$(CH_2)n^6$-C(=O)-, or a single bond,

wherein n^5 represents an integer of 1 to 6, n^6 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 2 to 7 amino acids,

L^a represents -C(=O)-NH-, -NR^1-$(CH_2)n^7$-, -O-, or a single bond,

wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, - $(CH_2)n^8$-COOH, or -$(CH_2)n^9$-OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,

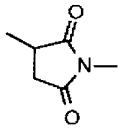
L^b represents -CR^2(-R^3)-, -O-, -NR^4-, or a single bond,

wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -$(CH_2)n^a$-NH_2, -$(CH_2)n^b$-COOH, or -$(CH_2)n^c$-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same each other,

L^c represents -CH_2- or -C(=O)-,

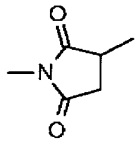
-(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 41]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
-(N-ly-3-diminiccuS)- has a structure represented by the following formula:

[Formula 42]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and when L^2 is -S-$(CH_2)n^6$-C(=O)-, L^1 is -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-.
[77] The linker according to [76], which is selected from the following group, provided that the left terminal is a connectiong position with the antibody and the right terminal is a connecting position with the antitumor compound:

-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)--(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-

$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)--CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-

-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH2-(N-Iy-3-diminiccuS)-S-CH2CH2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-C(=O)-cye.Hex(1,4)-CH2-(N-Iy-3-diminiccuS)-S-CH2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cye.Hex(1,4)-CH2-(N-Iy-3-diminiccuS)-S-CH2CH2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-eye.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-.

[78] The linker according to [76], which is selected from the following group, provided that the left terminal is a connectiong position with the antibody and the right terminal is a connecting position with the antitumor compound:

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2cH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH2CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O) -.

[79] The linker according to [76], which is selected from the following group, provided that the left terminal is a connecting position with the antibody and the right terminal is a connecting position with the antitumor compound:

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O) -
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O) -
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-.

[80] The linker according to [76], which is selected from the following group, provided that the left terminal is a connecting position with the antibody and the right terminal is a connecting position with the antitumor compound:

- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-.

Advantageous Effects of Invention

[0012] With an antibody-drug conjugate having an antitumor compound exatecan conjugated via a linker with a specific structure, an excellent antitumor effect and safety can be achieved.

Brief Description of Drawings

[0013]

[Figure 1] Figure 1 shows an amino acid sequence of B7-H3 variant 1 (SEQ ID NO: 1).
[Figure 2] Figure 2 shows an amino acid sequence of B7-H3 variant 2 (SEQ ID NO: 2).
[Figure 3] Figure 3 shows an amino acid sequence of an M30-H1-type heavy chain (SEQ ID NO: 9).
[Figure 4] Figure 4 shows an amino acid sequence of an M30-H2-type heavy chain (SEQ ID NO: 10).
[Figure 5] Figure 5 shows an amino acid sequence of an M30-H3-type heavy chain (SEQ ID NO: 11).
[Figure 6] Figure 6 shows an amino acid sequence of an M30-H4-type heavy chain (SEQ ID NO: 12).
[Figure 7] Figure 7 shows an amino acid sequence of an M30-L1-type light chain (SEQ ID NO: 13).
[Figure 8] Figure 8 shows an amino acid sequence of an M30-L2-type light chain (SEQ ID NO: 14).
[Figure 9] Figure 9 shows an amino acid sequence of an M30-L3-type light chain (SEQ ID NO: 15).
[Figure 10] Figure 10 shows an amino acid sequence of an M30-L4-type light chain (SEQ ID NO: 16).
[Figure 11] Figure 11 shows an amino acid sequence of an M30-L5-type light chain (SEQ ID NO: 17).
[Figure 12] Figure 12 shows an amino acid sequence of an M30-L6-type light chain (SEQ ID NO: 18).
[Figure 13] Figure 13 shows an amino acid sequence of an M30-L7-type light chain (SEQ ID NO: 19).
[Figure 14] Figure 14 shows an amino acid sequence of an M30 antibody heavy chain (SEQ ID NO: 20).
[Figure 15] Figure 15 shows an amino acid sequence of an M30 antibody light chain (SEQ ID NO: 21).

[Figure 16] Figure 16 shows a nucleotide sequence of B7-H3 variant 1 (SEQ ID NO: 26).

[Figure 17] Figure 17 shows the effect of an antibody-drug conjugate (2) on subcutaneously transplanted human melanoma line A375 cells. In the drawing, the line with open rhombuses depicts results about untreated tumor, the line with open triangles depicts the effect of an M30-H1-L4P antibody, and the line with open circles depicts the effect of the antibody-drug conjugate (2).

[Figure 18] Figure 18 shows the effect of the antibody-drug conjugate (2) on subcutaneously transplanted human melanoma line A375 cells. The line with open rhombuses depicts results about untreated tumor, the line with filled squares depicts the effect of the antibody-drug conjugate (2) administered at 0.1 mg/kg, the line with X marks depicts the effect of the antibody-drug conjugate (2) administered at 0.3 mg/kg, the line with filled triangles depicts the effect of the antibody-drug conjugate (2) administered at 1 mg/kg, and the line with open circles depicts the effect of the antibody-drug conjugate (2) administered at 3 mg/kg.

[Figure 19] Figure 19 shows the effect of the antibody-drug conjugate (2) on subcutaneously transplanted human non-small cell lung cancer line Calu-6 cells. The line with open rhombuses depicts results about untreated tumor, the line with open triangles depicts the effect of an M30-H1-L4P antibody, and the line with open circles depicts the effect of the antibody-drug conjugate (2).

[Figure 20] Figure 20 shows the effects of antibody-drug conjugates (1), (13), (41), and (55) on subcutaneously transplanted human melanoma line A375 cells. In the drawing, the line with open rhombuses depicts results about untreated tumor, the line with open circles depicts the effect of the antibody-drug conjugate (1), the line with open triangles depicts the effect of the antibody-drug conjugate (13), the line with X marks depicts the effect of the antibody-drug conjugate (41), and the line with open squares depicts the effect of the antibody-drug conjugate (55).

[Figure 21] Figure 21 shows the effects of antibody-drug conjugates (13), (41), and (55) on subcutaneously transplanted human non-small cell lung cancer line Calu-6 cells. The line with open rhombuses depicts results about untreated tumor, the line with open circles depicts the effect of DE-310, the line with open triangles depicts the effect of the antibody-drug conjugate (13), the line with X marks depicts the effect of the antibody-drug conjugate (41), and the line with open squares depicts the effect of the antibody-drug conjugate (55).

[Figure 22] Figure 22 shows the effects of antibody-drug conjugates (17), (18), (19), (59), (60), and (61) on subcutaneously transplanted human melanoma line A375 cells. In the drawing, the line with filled rhombuses depicts results about untreated tumor, the line with filled squares depicts the effect of the antibody-drug conjugate (17), the line with open squares depicts the effect of the antibody-drug conjugate (18), the line with open circles depicts the effect of the antibody-drug conjugate (19), the line with filled triangles depicts the effect of the antibody-drug conjugate (59), the line with open triangles depicts the effect of the antibody-drug conjugate (60), and the line with X marks depicts the effect of the antibody-drug conjugate (61).

Description of Embodiments

[0014] The antibody-drug conjugate of the present invention is an antitumor drug in which an antitumor antibody is conjugated to an antitumor compound via a linker structure moiety and explained in detail hereinbelow.

[Antibody]

[0015] The antibody used in the antibody-drug conjugate of the present invention means an immunoglobulin and is a molecule containing an antigen-binding site immunospecifically binding to an antigen. The class of the antibody of the present invention may be any of IgG, IgE, IgM, IgD, IgA, and IgY and is preferably IgG. The subclass of the antibody of the present invention may be any of IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2 and is preferably IgG1 or IgG2. The antibody may be derived from any species, and preferred examples of the species can include humans, rats, mice, and rabbits. In case when derived from other than human species, it is preferably chimerized or humanized using a well known technique. The antibody of the present invention may be a polyclonal antibody or a monoclonal antibody and is preferably a monoclonal antibody.

[0016] The antibody of the present invention may be those which is capable of targeting tumor cells. Since the antibody of the present invention is conjugated with a drug having antitumor activity via a linker, the antibody preferably possesses one or more of a property of recognizing a tumor cell, a property of binding to a tumor cell, a property of internalizing in a tumor cell, and a property of damaging a tumor cell.

[0017] The binding activity of the antibody against tumor cells can be confirmed using flow cytometry. The internalization of the antibody into tumor cells can be confirmed using (1) an assay of visualizing an antibody incorporated in cells under a fluorescence microscope using a secondary antibody (fluorescently labeled) binding to the therapeutic antibody (Cell Death and Differentiation (2008) 15, 751-761), (2) an assay of measuring the amount of fluorescence incorporated in cells using a secondary antibody (fluorescently labeled) binding to the therapeutic antibody (Molecular Biology of the Cell, Vol. 15, 5268-5282, December 2004), or (3) a Mab-ZAP assay using an immunotoxin binding to the therapeutic

antibody wherein the toxin is released upon incorporation into cells to inhibit cell growth (Bio Techniques 28: 162-165, January 2000).

[0018] The antitumor activity of the antibody refers to a cytotoxic activity or cytocidal effect against tumor cells and can be confirmed *in vitro* by determining inhibitory activity against cell growth. For example, a cancer cell line overexpressing a target protein for the antibody is cultured, and the antibody is added at varying concentrations into the culture system to determin an inhibitory activity against focus formation, colony formation, and spheroid growth. The antitumor activity can be confirmed *in vivo,* for example, by administering the antibody to a nude mouse with a transplanted tumor cell line highly expressing the target protein, and determining change in the cancer cell. Since the drug conjugated in the antibody-drug conjugate exerts an antitumor effect, it is more preferred but not essential that the antibody itself should have an antitumor effect. For exerting the antitumor effect and also for specifically and selectively damaging tumor cells by the drug, it is important and also preferred that the antibody should have the property of internalizing to migrate into tumor cells.

[0019] Examples of such an antibody can include, but not limited to, an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, and an anti-mesothelin antibody.

[0020] The antibody of the present invention is preferably an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD70 antibody, or an anti-B7-H3 antibody, and more preferably an anti-B7-H3 antibody.

[0021] The antibody of the present invention can be obtained using a method usually carried out in the art, which involves immunizing animals with an antigenic polypeptide and collecting and purifying antibodies produced *in vivo.* The origin of the antigen is not limited to humans, and the animals may be immunized with an antigen derived from a non-human animal such as a mouse, a rat and the like. In this case, the cross-reactivity of antibodies binding to the obtained heterologous antigen with human antigens can be tested to screen for an antibody applicable to a human disease.

[0022] Alternatively, antibody-producing cells which produce antibodies against the antigen are fused with myeloma cells according to a method known in the art (e.g., Kohler and Milstein, Nature (1975) 256, p. 495-497; and Kennet, R. ed., Monoclonal Antibodies, p. 365-367, Plenum Press, N.Y. (1980)) to establish hybridomas, from which monoclonal antibodies can in turn be obtained.

[0023] The antigen can be obtained by genetically engineering host cells to produce a gene encoding the antigenic protein. Specifically, vectors that permit expression of the antigen gene are prepared and transferred to host cells so that the gene is expressed. The antigen thus expressed can be purified.

[0024] The anti-CD30 antibody, the anti-CD33 antibody, and the anti-CD70 antibody can obtained by an approach known in the art with reference to WO2002/043661, U.S. Patent No. 5,773,001, and WO2006/113909, respectively.

[0025] The B7-H3 antibody used in the present invention is preferably those having properties as described below.

(1) An antibody having the following properties:

(a) specifically binding to B7-H3,
(b) having antibody-dependent cell-mediated phagocytosis (ADCP) activity, and
(c) having antitumor activity *in vivo.*

(2) The antibody according to (1), wherein B7-H3 is a molecule comprising the amino acid sequence represented by SEQ ID NO: 1 or 2.

(3) The antibody according to (1) or (2), wherein the antibody has CDRH1 comprising the amino acid sequence represented by SEQ ID NO: 3, CDRH2 comprising the amino acid sequence represented by SEQ ID NO: 4, and CDRH3 comprising the amino acid sequence represented by SEQ ID NO: 5 as heavy chain complementarity determining regions, and CDRL1 comprising the amino acid sequence represented by SEQ ID NO: 6, CDRL2 comprising the amino acid sequence represented by SEQ ID NO: 7, and CDRL3 comprising the amino acid sequence represented by SEQ ID NO: 8 as light chain complementarity determining regions.

(4) The antibody according to any of (1) to (3), wherein the constant region thereof is a human-derived constant region.

(5) The antibody according to any of (1) to (4), wherein the antibody is a humanized antibody.

(6) The antibody according to (5), wherein the antibody has a heavy chain variable region comprising an amino acid sequence selected from the group consisting of (a) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9, (b) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 10, (c) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 11, (d) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12, (e) an amino acid sequence having at least 95% or higher homology to any of the sequences (a) to (d), and (f) an amino acid sequence derived from any of the sequences (a) to (d) by the deletions, replacements, or additions of at least one amino acid, and a light chain

variable region comprising an amino acid sequence selected from the group consisting of (g) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 13, (h) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 14, (i) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 15, (j) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 16, (k) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 17, (l) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 18, (m) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 19, (n) an amino acid sequence having at least 95% or higher homology to any of the sequences (g) to (m), and (o) an amino acid sequence derived from any of the sequences (g) to (m) by the deletions, replacements, or additions of at least one amino acid.

(7) The antibody according to (6), wherein the antibody has a heavy chain variable region and a light chain variable region selected from the group consisting of a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 13, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 14, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 15, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 16, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 17, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 18, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 19, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 13, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 14, a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 15, and a heavy chain variable region comprising an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12 and a light chain variable region comprising an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 16.

(8) The antibody according to (6) or (7), wherein the antibody comprises a heavy chain and a light chain selected from the group consisting of a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 13, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 14, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 15, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 16, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 17, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 18, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 19, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 12 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 13, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 12 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 14, a heavy chain comprising an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 12 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 15, and a heavy chain comprising an amino acid sequence described in amino acid positions

20 to 471 in SEQ ID NO: 12 and a light chain comprising an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 16.

(9) The antibody according to any of (14) to (16), wherein the antibody comprises a heavy chain and a light chain selected from the group consisting of a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 13, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 14, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 15, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 16, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 17, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 18, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 19, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 13, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 14, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 15, and a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 16.

(10) The antibody according to (8) or (9), wherein the antibody lacks an amino acid at the carboxy terminus of the amino acid sequence represented by SEQ ID NO: 9 or 12 in the heavy chain.

(11) An antibody obtained by a method for producing the antibody according to any of (1) to (10), the method comprising the steps of: culturing a host cell transformed with an expression vector containing a polynucleotide encoding the antibody; and collecting the antibody of interest from the cultures obtained in the preceding step.

(12) The antibody according to any of (1) to (11), wherein the modification of a glycan is regulated in order to enhance antibody-dependent cytotoxic activity.

[0026] Hereinafter, the B7-H3 antibody used in the invention is described.

[0027] The terms "cancer" and "tumor" as used herein are used with the same meaning.

[0028] The term "gene" as used herein includes not only DNA, but also mRNA thereof, cDNA thereof and cRNA thereof.

[0029] The term "polynucleotide" as used herein is used with the same meaning as a nucleic acid and also includes DNA, RNA, probes, oligonucleotides, and primers.

[0030] The terms "polypeptide" and "protein" as used herein are used without distinction.

[0031] The term "cell" as used herein also includes cells in an animal individual and cultured cells.

[0032] The term "B7-H3" as used herein is used in the same meaning as B7-H3 protein, and also refers to B7-H3 variant 1 and/or B7-H3 variant 2.

[0033] The term "CDR" as used herein refers to a complementarity determining region (CDR), and it is known that each heavy and light chain of an antibody molecule has three complementarity determining regions (CDRs). The CDR is also called the hypervariable region, and is present in a variable region of each heavy and light chain of an antibody. It is a site which has unusually high variability in its primary structure, and there are three separate CDRs in the primary structure of each heavy and light polypeptide chain. In this specification, as for the CDRs of an antibody, the CDRs of the heavy chain are represented by CDRH1, CDRH2, and CDRH3 from the amino-terminal side of the amino acid sequence of the heavy chain, and the CDRs of the light chain are represented by CDRL1, CDRL2, and CDRL3 from the amino-terminal side of the amino acid sequence of the light chain. These sites are proximate to one another in the tertiary structure and determine the specificity for an antigen to which the antibody binds.

[0034] The phrase "hybridization is performed under stringent conditions" as used herein refers to a process in which hybridization is performed under conditions under which identification can be achieved by performing hybridization at 68°C in a commercially available hybridization solution ExpressHyb Hybridization Solution (manufactured by Clontech, Inc.) or by performing hybridization at 68°C in the presence of 0.7 to 1.0 M NaCl using a filter having DNA immobilized thereon, followed by performing washing at 68°C using 0.1 to 2 x SSC solution (1 x SSC solution is composed of 150 mM NaCl and 15 mM sodium citrate) or under conditions equivalent thereto.

1. B7-H3

[0035] B7-H3 is a member of the B7 family expressed on antigen-presenting cells as a co-stimulatory molecule, and is considered to act on a receptor on T cells to enhance or suppress immune activity.

[0036] B7-H3 is a protein having a single-pass transmembrane structure, and the N-terminal extracellular domain of

B7-H3 contains two variants. The B7-H3 variant 1 (4Ig-B7-H3) contains a V-like or C-like Ig domain at two sites, respectively, and the B7-H3 variant 2 (2Ig-B7-H3) contains a V-like or C-like Ig domain at one site, respectively.

[0037] As for B7-H3 to be used in the invention, B7-H3 can be directly purified from B7-H3-expressing cells of a human or a non-human mammal (such as a rat or a mouse) and used, or a cell membrane fraction of the above-described cells can be prepared and used. Further, B7-H3 can be obtained by in vitro synthesis thereof or production thereof in a host cell through genetic engineering. In the genetic engineering, specifically, after B7-H3 cDNA is integrated into a vector capable of expressing B7-H3 cDNA, B7-H3 can be obtained by synthesizing it in a solution containing an enzyme, a substrate and an energy substance required for transcription and translation, or by expressing B7-H3 in another prokaryotic or eucaryotic transformed host cell.

[0038] The amino acid sequence of an open reading frame (ORF) of a human B7-H3 variant 1 gene is represented by SEQ ID NO: 1 in the Sequence Listing. Further, the sequence of SEQ ID NO: 1 is shown in Fig. 1.

[0039] The amino acid sequence of an ORF of a human B7-H3 variant 2 gene is represented by SEQ ID NO: 2 in the Sequence Listing. Further, the sequence of SEQ ID NO: 2 is shown in Fig. 2.

[0040] Further, a protein which consists of an amino acid sequence wherein one or several amino acids are substituted, deleted and/or added in any of the above-described amino acid sequences of B7-H3 and also has a biological activity equivalent to that of the protein is also included in B7-H3.

[0041] Mature human B7-H3 variant 1 from which the signal sequence has been removed corresponds to an amino acid sequence consisting of amino acid residues 27 to 534 of the amino acid sequence represented by SEQ ID NO: 1. Further, mature human B7-H3 variant 2 from which the signal sequence has been removed corresponds to an amino acid sequence consisting of amino acid residues 27 to 316 of the amino acid sequence represented by SEQ ID NO: 2.

2. Production of anti-B7-H3 antibody

[0042] The antibody against B7-H3 of the invention can be obtained by immunizing an animal with B7-H3 or an arbitrary polypeptide selected from the amino acid sequence of B7-H3, and collecting and purifying the antibody produced in vivo according to a common procedure. The biological species of B7-H3 to be used as an antigen is not limited to being human, and an animal can be immunized with B7-H3 derived from an animal other than humans such as a mouse or a rat. In this case, by examining the cross-reactivity between an antibody binding to the obtained heterologous B7-H3 and human B7-H3, an antibody applicable to a human disease can be selected.

[0043] Further, a monoclonal antibody can be obtained from a hybridoma established by fusing antibody-producing cells which produce an antibody against B7-H3 with myeloma cells according to a known method (for example, Kohler and Milstein, Nature, (1975) 256, pp. 495-497; Kennet, R. ed., Monoclonal Antibodies, pp. 365-367, Plenum Press, N.Y. (1980)).

[0044] B7-H3 to be used as an antigen can be obtained by expressing B7-H3 gene in a host cell using genetic enginering.

[0045] Specifically, a vector capable of expressing B7-H3 gene is produced, and the resulting vector is transfected into a host cell to express the gene, and then, the expressed B7-H3 is purified. Hereinafter, a method of obtaining an antibody against B7-H3 is specifically described.

(1) Preparation of antigen

[0046] Examples of the antigen to be used for producing the anti-B7-H3 antibody include B7-H3, a polypeptide consisting of a partial amino acid sequence comprising at least 6 consecutive amino acids of B7-H3, and a derivative obtained by adding a given amino acid sequence or carrier thereto.

[0047] B7-H3 can be purified directly from human tumor tissues or tumor cells and used. Further, B7-H3 can be obtained by synthesizing it in vitro or by producing it in a host cell by genetic engineering.

[0048] With respect to the genetic engineering, specifically, after B7-H3 cDNA is integrated into a vector capable of expressing B7-H3 cDNA, B7-H3 can be obtained by synthesizing it in a solution containing an enzyme, a substrate and an energy substance required for transcription and translation, or by expressing B7-H3 in another prokaryotic or eucaryotic transformed host cell.

[0049] Further, the antigen can also be obtained as a secretory protein by expressing a fusion protein obtained by ligating the extracellular domain of B7-H3, which is a membrane protein, to the constant region of an antibody in an appropriate host-vector system.

[0050] B7-H3 cDNA can be obtained by, for example, a so-called PCR method in which a polymerase chain reaction (hereinafter referred to as "PCR") is performed using a cDNA library expressing B7-H3 cDNA as a template and primers which specifically amplify B7-H3 cDNA (see Saiki, R. K., et al., Science, (1988) 239, pp. 487-489).

[0051] As the in vitro synthesis of the polypeptide, for example, Rapid Translation System (RTS) manufactured by Roche Diagnostics, Inc. can be exemplified, but it is not limited thereto.

[0052] Examples of the prokaryotic host cells include Escherichia coli and Bacillus subtilis. In order to transform the

host cells with a target gene, the host cells are transformed by a plasmid vector comprising a replicon, i.e., a replication origin derived from a species compatible with the host, and a regulatory sequence. Further, the vector preferably has a sequence capable of imposing phenotypic selectivity on the transformed cell.

[0053] Examples of the eucaryotic host cells include vertebrate cells, insect cells, and yeast cells. As the vertebrate cells, for example, simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61); and the like are often used, however, the cells are not limited thereto.

[0054] The thus obtained transformant can be cultured according to a common procedure, and by the culturing of the transformant, a target polypeptide is produced intracellularly or extracellularly.

[0055] A suitable medium to be used for the culturing can be selected from various commonly used culture media depending on the employed host cells. If Escherichia coli is employed, for example, an LB medium supplemented with an antibiotic such as ampicillin or IPMG as needed can be used.

[0056] A recombinant protein produced intracellularly or extracellularly by the transformant through such culturing can be separated and purified by any of various known separation methods utilizing the physical or chemical property of the protein.

[0057] Specific examples of the methods include treatment with a common protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, and affinity chromatography, dialysis, and a combination thereof.

[0058] Further, by attaching a tag of six histidine residues to a recombinant protein to be expressed, the protein can be efficiently purified with a nickel affinity column. Alternatively, by attaching the IgG Fc region to a recombinant protein to be expressed, the protein can be efficiently purified with a protein A column.

[0059] By combining the above-described methods, a large amount of a target polypeptide can be easily produced in high yield and high purity.

(2) Production of anti-B7-H3 monoclonal antibody

[0060] Examples of the antibody specific binding to B7-H3 include a monoclonal antibody specific binding to B7-H3, and a method of obtaining the antibody is as described below.

[0061] The production of a monoclonal antibody generally requires the following operational steps of:

(a) purifying a biopolymer to be used as an antigen;
(b) preparing antibody-producing cells by immunizing an animal by injection of the antigen, collecting the blood, assaying its antibody titer to determine when the spleen is excised;
(c) preparing myeloma cells (hereinafter referred to as "myeloma");
(d) fusing the antibody-producing cells with the myeloma;
(e) screening a group of hybridomas producing a desired antibody;
(f) dividing the hybridomas into single cell clones (cloning);
(g) optionally, culturing the hybridoma or rearing an animal implanted with the hybridoma for producing a large amount of a monoclonal antibody;
(h) examining the thus produced monoclonal antibody for biological activity and binding specificity, or assaying the same for properties as a labeled reagent; and the like.

[0062] Hereinafter, the method of producing a monoclonal antibody will be described in detail following the above steps, however, the method is not limited thereto, and, for example, antibody-producing cells other than spleen cells and myeloma can be used.

(a) Purification of antigen

[0063] As the antigen, B7-H3 prepared by the method as described above or a partial peptide thereof can be used.

[0064] Further, a membrane fraction prepared from recombinant cells expressing B7-H3 or the recombinant cells expressing B7-H3 themselves, and also a partial peptide of the protein of the invention chemically synthesized by a method known to those skilled in the art can also be used as the antigen.

(b) Preparation of antibody-producing cells

[0065] The antigen obtained in the step (a) is mixed with an adjuvant such as Freund's complete or incomplete adjuvant or aluminum potassium sulfate and the resulting mixture is used as an immunogen to immunize an experimental animal.

As the experimental animal, any animal used in a known hybridoma production method can be used without any trouble. Specifically, for example, a mouse, a rat, a goat, sheep, cattle, a horse, or the like can be used. However, from the viewpoint of ease of availability of myeloma cells to be fused with the extracted antibody-producing cells, a mouse or a rat is preferably used as the animal to be immunized.

[0066] Further, the strain of a mouse or a rat to be used is not particularly limited, and in the case of a mouse, for example, various strains such as A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BL, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, R III, SJL, SWR, WB, and 129 and the like can be used, and in the case of a rat, for example, Wistar, Low, Lewis, Sprague, Dawley, ACI, BN, Fischer and the like can be used.

[0067] These mice and rats are commercially available from breeders/distributors of experimental animals, for example, CLEA Japan, Inc. and Charles River Laboratories Japan, Inc.

[0068] Among these, in consideration of compatibility of fusing with myeloma cells described below, in the case of a mouse, BALB/c strain, and in the case of a rat, Wistar and Low strains are particularly preferred as the animal to be immunized.

[0069] Further, in consideration of antigenic homology between humans and mice, it is also preferred to use a mouse having decreased biological function to remove auto-antibodies, that is, a mouse with an autoimmune disease.

[0070] The age of such mouse or rat at the time of immunization is preferably 5 to 12 weeks of age, more preferably 6 to 8 weeks of age.

[0071] In order to immunize an animal with B7-H3 or a recombinant thereof, for example, a known method described in detail in, for example, Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher Springfield, Illinois (1964) or the like can be used.

[0072] Among these immunization methods, a preferred specific method in the invention is, for example, as follows.

[0073] That is, first, a membrane protein fraction serving as the antigen or cells caused to express the antigen is/are intradermally or intraperitoneally administrated to an animal.

[0074] However, the combination of both routes of administration is preferred for increasing the immunization efficiency, and when intradermal administration is performed in the first half and intraperitoneal administration is performed in the latter half or only at the last dosing, the immunization efficiency can be particularly increased.

[0075] The administration schedule of the antigen varies depending on the type of animal to be immunized, individual difference or the like. However, in general, an administration schedule in which the frequency of administration of the antigen is 3 to 6 times and the dosing interval is 2 to 6 weeks is preferred, and an administration schedule in which the frequency of administration of the antigen is 3 to 4 times and the dosing interval is 2 to 4 weeks is more preferred.

[0076] Further, the dose of the antigen varies depending on the type of animal, individual differences or the like, however, the dose is generally set to 0.05 to 5 mg, preferably about 0.1 to 0.5 mg.

[0077] A booster immunization is performed 1 to 6 weeks, preferably 2 to 4 weeks, more preferably 2 to 3 weeks after the administration of the antigen as described above.

[0078] The dose of the antigen at the time of performing the booster immunization varies depending on the type or size of animal or the like, however, in the case of, for example, a mouse, the dose is generally set to 0.05 to 5 mg, preferably 0.1 to 0.5 mg, more preferably about 0.1 to 0.2 mg.

[0079] Spleen cells or lymphocytes including antibody-producing cells are aseptically removed from the immunized animal 1 to 10 days, preferably 2 to 5 days, more preferably 2 to 3 days after the booster immunization. At this time, the antibody titer is measured, and if an animal having a sufficiently increased antibody titer is used as a supply source of the antibody-producing cells, the subsequent procedure can be carried out more efficiently.

[0080] Examples of the method of measuring the antibody titer to be used here include an RIA method and an ELISA method, but the method is not limited thereto.

[0081] For example, if an ELISA method is employed, the measurement of the antibody titer in the invention can be carried out according to the procedures as described below.

[0082] First, a purified or partially purified antigen is adsorbed to the surface of a solid phase such as a 96-well plate for ELISA, and the surface of the solid phase having no antigen adsorbed thereto is covered with a protein unrelated to the antigen such as bovine serum albumin (hereinafter referred to as "BSA"). After washing the surface, the surface is brought into contact with a serially-diluted sample (for example, mouse serum) as a primary antibody to allow the antibody in the sample to bind to the antigen.

[0083] Further, as a secondary antibody, an antibody labeled with an enzyme against a mouse antibody is added and is allowed to bind to the mouse antibody. After washing, a substrate for the enzyme is added and a change in absorbance which occurs due to color development induced by degradation of the substrate or the like is measured and the antibody titer is calculated based on the measurement.

[0084] The separation of the antibody-producing cells from the spleen cells or lymphocytes of the immunized animal can be carried out according to a known method (for example, Kohler et al., Nature (1975), 256, p. 495; Kohler et al., Eur. J. Immunol. (1977), 6, p. 511; Milstein et al., Nature (1977), 266, p. 550; Walsh, Nature (1977), 266, p. 495). For

example, in the case of spleen cells, a general method in which the antibody-producing cells are separated by homogenizing the spleen to obtain the cells through filtration with a stainless steel mesh and suspending the cells in Eagle's Minimum Essential Medium (MEM) can be employed.

(c) Preparation of myeloma cells (hereinafter referred to as "myeloma")

[0085] The myeloma cells to be used for cell fusion are not particularly limited and suitable cells can be selected from known cell lines. However, in consideration of convenience when a hybridoma is selected from fused cells, it is preferred to use an HGPRT (hypoxanthine-guanine phosphoribosyl transferase) deficient strain whose selection procedure has been established.

[0086] More specifically, examples of the HGPRT-deficient strain include X63-Ag8 (X63), NS1-ANS/1(NS1), P3X63-Ag8.U1(P3U1), X63-Ag8.653(X63.653), SP2/0-Agl4(SP2/0), MPC11-45.6TG1.7(45.6TG), FO, S149/5XXO, and BU.1 derived from mice; 210.RSY3.Ag.1.2.3(Y3) derived from rats; and U266AR(SKO-007), GM1500·GTG-A12(GM1500) , UC729-6, LICR-LOW-HMy2(HMy2) and 8226AR/NIP4-1(NP41) derived from humans. These HGPRT-deficient strains are available from, for example, the American Type Culture Collection (ATCC) or the like.

[0087] These cell strains are subcultured in an appropriate medium such as an 8-azaguanine medium [a medium obtained by adding 8-azaguanine to an RPMI 1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, and fetal calf serum (hereinafter referred to as "FCS")], Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM"), or Dulbecco's Modified Eagle Medium (hereinafter referred to as "DMEM"). In this case, 3 to 4 days before performing cell fusion, the cells are subcultured in a normal medium [for example, an ASF104 medium (manufactured by Ajinomoto Co., Ltd.) containing 10% FCS] to ensure not less than 2×10^7 cells on the day of cell fusion.

(d) Cell fusion

[0088] Fusion between the antibody-producing cells and the myeloma cells can be appropriately performed according to a known method (Weir, D. M. Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher, Springfield, Illinois (1964), etc.), under conditions such that the survival rate of cells is not excessively reduced.

[0089] As such a method, for example, a chemical method in which the antibody-producing cells and the myeloma cells are mixed in a solution containing a polymer such as polyethylene glycol at a high concentration, a physical method using electric stimulation, or the like can be used. Among these methods, a specific example of the chemical method is as described below.

[0090] That is, in the case where polyethylene glycol is used in the solution containing a polymer at a high concentration, the antibody-producing cells and the myeloma cells are mixed in a solution of polyethylene glycol having a molecular weight of 1500 to 6000, more preferably 2000 to 4000 at a temperature of from 30 to 40°C, preferably from 35 to 38°C for 1 to 10 minutes, preferably 5 to 8 minutes.

(e) Selection of a group of hybridomas

[0091] The method of selecting hybridomas obtained by the above-described cell fusion is not particularly limited. Usually, an HAT (hypoxanthine, aminopterin, thymidine) selection method (Kohler et al., Nature (1975), 256, p. 495; Milstein et al., Nature (1977), 266, p. 550) is used.

[0092] This method is effective when hybridomas are obtained using the myeloma cells of an HGPRT-deficient strain which cannot survive in the presence of aminopterin.

[0093] That is, by culturing unfused cells and hybridomas in an HAT medium, only hybridomas resistant to aminopterin are selectively allowed to survive and proliferate.

(f) Division into single cell clone (cloning)

[0094] As a cloning method for hybridomas, a known method such as a methylcellulose method, a soft agarose method, or a limiting dilution method can be used (see, for example, Barbara, B. M. and Stanley, M. S.: Selected Methods in Cellular Immunology, W. H. Freeman and Company, San Francisco (1980)). Among these methods, particularly, a three-dimensional culture method such as a methylcellulose method is preferred. For example, the group of hybridomas produced by cell fusion are suspended in a methylcellulose medium such as ClonaCell-HY Selection Medium D (manufactured by StemCell Technologies, inc., #03804) and cultured. Then, the formed hybridoma colonies are collected, whereby monoclonal hybridomas can be obtained. The collected respective hybridoma colonies are cultured, and a hybridoma which has been confirmed to have a stable antibody titer in an obtained hybridoma culture supernatant is selected as a B7-H3 monoclonal antibody-producing hybridoma strain.

[0095] Examples of the thus established hybridoma strain include B7-H3 hybridoma M30. In this specification, an antibody produced by the B7-H3 hybridoma M30 is referred to as "M30 antibody" or simply "M30".

[0096] The heavy chain of the M30 antibody has an amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing. Further, the light chain of the M30 antibody has an amino acid sequence represented by SEQ ID NO: 21 in the Sequence Listing. In the heavy chain amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 19 is a signal sequence, an amino acid sequence consisting of amino acid residues 20 to 141 is a variable region, and an amino acid sequence consisting of amino acid residues 142 to 471 is a constant region. Further, in the light chain amino acid sequence represented by SEQ ID NO: 21 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 22 is a signal sequence, an amino acid sequence consisting of amino acid residues 23 to 130 is a variable region, and an amino acid sequence consisting of amino acid residues 131 to 235 is a constant region.

(g) Preparation of monoclonal antibody by culturing hybridoma

[0097] By culturing the thus selected hybridoma, a monoclonal antibody can be efficiently obtained. However, prior to culturing, it is preferred to perform screening of a hybridoma which produces a target monoclonal antibody.

[0098] In such screening, a known method can be employed.

[0099] The measurement of the antibody titer in the invention can be carried out by, for example, an ELISA method explained in item (b) described above.

[0100] The hybridoma obtained by the method described above can be stored in a frozen state in liquid nitrogen or in a freezer at -80°C or below.

[0101] After completion of cloning, the medium is changed from an HT medium to a normal medium, and the hybridoma is cultured.

[0102] Large-scale culture is performed by rotation culture using a large culture bottle or by spinner culture. From the supernatant obtained by the large-scale culture, a monoclonal antibody which specifically binds to the protein of the invention can be obtained by purification using a method known to those skilled in the art such as gel filtration.

[0103] Further, the hybridoma is injected into the abdominal cavity of a mouse of the same strain as the hybridoma (for example, the above-described BALB/c) or a Nu/Nu mouse to proliferate the hybridoma, whereby the ascites containing a large amount of the monoclonal antibody of the invention can be obtained.

[0104] In the case where the hybridoma is administrated in the abdominal cavity, if a mineral oil such as 2,6,10,14-tetramethyl pentadecane (pristane) is administrated 3 to 7 days prior thereto, a larger amount of the ascites can be obtained.

[0105] For example, an immunosuppressant is previously injected into the abdominal cavity of a mouse of the same strain as the hybridoma to inactivate T cells. 20 days thereafter, 10^6 to 10^7 hybridoma clone cells are suspended in a serum-free medium (0.5 ml), and the suspension is administrated in the abdominal cavity of the mouse. In general, when the abdomen is expanded and filled with the ascites, the ascites is collected from the mouse. By this method, the monoclonal antibody can be obtained at a concentration which is about 100 times or much higher than that in the culture solution.

[0106] The monoclonal antibody obtained by the above-described method can be purified by a method described in, for example, Weir, D. M.: Handbook of Experimental Immunology Vol. I, II, III, Blackwell Scientific Publications, Oxford (1978).

[0107] The thus obtained monoclonal antibody has high antigen specificity for B7-H3.

(h) Assay of monoclonal antibody

[0108] The isotype and subclass of the thus obtained monoclonal antibody can be determined as follows.

[0109] First, examples of the identification method include an Ouchterlony method, an ELISA method, and an RIA method.

[0110] An Ouchterlony method is simple, but when the concentration of the monoclonal antibody is low, a condensation operation is required.

[0111] On the other hand, when an ELISA method or an RIA method is used, by directly reacting the culture supernatant with an antigen-adsorbed solid phase and using antibodies corresponding to various types of immunoglobulin isotypes and subclasses as secondary antibodies, the isotype and subclass of the monoclonal antibody can be identified.

[0112] In addition, as a simpler method, a commercially available identification kit (for example, Mouse Typer Kit manufactured by Bio-Rad Laboratories, Inc.) or the like can also be used.

[0113] Further, the quantitative determination of a protein can be performed by the Folin Lowry method and a method of calculation based on the absorbance at 280 nm [1.4 (OD 280) = Immunoglobulin 1 mg/ml].

[0114] Further, even when the monoclonal antibody is separately and independently obtained by performing again

the steps of (a) to (h) in (2), it is possible to obtain an antibody having a cytotoxic activity equivalent to that of the M30 antibody. As one example of such an antibody, an antibody which binds to the same epitope as the M30 antibody can be exemplified. The M30 recognizes an epitope in the IgC1 or IgC2 domain, which is a domain in the B7-H3 extracellular domain, and binds to the IgC1 domain or the IgC2 domain or both. Therefore, as the epitope for the antibody of the invention, particularly, an epitope present in the IgC1 or IgC2 domain of B7-H3 can be exemplified. If a newly produced monoclonal antibody binds to a partial peptide or a partial tertiary structure to which the M30 antibody binds, it can be determined that the monoclonal antibody binds to the same epitope as the M30 antibody. Further, by confirming that the monoclonal antibody competes with the M30 antibody for the binding to B7-H3 (that is, the monoclonal antibody inhibits the binding between the M30 antibody and B7-H3), it can be determined that the monoclonal antibody binds to the same epitope as the M30 antibody even if the specific epitope sequence or structure has not been determined. When it is confirmed that the monoclonal antibody binds to the same epitope as the M30 antibody, the monoclonal antibody is strongly expected to have a cytotoxic activity equivalent to that of the M30 antibody.

(3) Other antibodies

[0115] The antibody of the invention includes not only the above-described monoclonal antibody against B7-H3 but also a recombinant antibody obtained by artificial modification for the purpose of decreasing heterologous antigenicity to humans such as a chimeric antibody, a humanized antibody and a human antibody. These antibodies can be produced using a known method.

[0116] As the chimeric antibody, an antibody in which antibody variable and constant regions are derived from different species, for example, a chimeric antibody in which a mouse- or rat-derived antibody variable region is connected to a human-derived antibody constant region can be exemplified (see Proc. Natl. Acad. Sci. USA, 81, 6851-6855, (1984)).

[0117] As the humanized antibody, an antibody obtained by integrating only a complementarity determining region (CDR) into a human-derived antibody (see Nature (1986) 321, pp. 522-525), and an antibody obtained by grafting a part of the amino acid residues of the framework as well as the CDR sequence to a human antibody by a CDR-grafting method (WO 90/07861) can be exemplified.

[0118] However, the humanized antibody derived from the M30 antibody is not limited to a specific humanized antibody as long as the humanized antibody has all 6 types of CDR sequences of the M30 antibody and has an antitumor activity. The heavy chain variable region of the M30 antibody has CDRH1 (NYVMH) consisting of an amino acid sequence represented by SEQ ID NO: 3 in the Sequence Listing, CDRH2 (YINPYNDDVKYNEKFKG) consisting of an amino acid sequence represented by SEQ ID NO: 4 in the Sequence Listing, and CDRH3 (WGYYGSPLYYFDY) consisting of an amino acid sequence represented by SEQ ID NO: 5 in the Sequence Listing. Further, the light chain variable region of the M30 antibody has CDRL1 (RASSRLIYMH) consisting of an amino acid sequence represented by SEQ ID NO: 6 in the Sequence Listing, CDRL2 (ATSNLAS) consisting of an amino acid sequence represented by SEQ ID NO: 7 in the Sequence Listing, and CDRL3 (QQWNSNPPT) consisting of an amino acid sequence represented by SEQ ID NO: 8 in the Sequence Listing.

[0119] As an example of the humanized antibody of a mouse antibody M30, an arbitrary combination of a heavy chain comprising a heavy chain variable region consisting of any one of (1) an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9, 10, 11, or 12 in the Sequence Listing, (2) an amino acid sequence having a homology of at least 95% or more with the amino acid sequence (1) described above, and (3) an amino acid sequence wherein one or several amino acids in the amino acid sequence (1) described above are deleted, substituted or added and a light chain comprising a light chain variable region consisting of any one of (4) an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 13, 14, 15, 16, 17, 18, or 19 in the Sequence Listing, (5) an amino acid sequence having a homology of at least 95% or more with the amino acid sequence (4) described above, and (6) an amino acid sequence wherein one or several amino acids in the amino acid sequence (4) described above are deleted, substituted or added can be exemplified.

[0120] The term "several" as used herein refers to 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 or 2.

[0121] As the amino acid substitution in this specification, a conservative amino acid substitution is preferred. The conservative amino acid substitution refers to a substitution occurring within a group of amino acids related to amino acid side chains. Preferred amino acid groups are as follows: an acidic group (aspartic acid and glutamic acid); a basic group (lysine, arginine, and histidine); a non-polar group (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan); and an uncharged polar family (glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine). More preferred amino acid groups are as follows: an aliphatic hydroxy group (serine and threonine); an amide-containing group (asparagine and glutamine); an aliphatic group (alanine, valine, leucine, and isoleucine); and an aromatic group (phenylalanine, tryptophan, and tyrosine). Such an amino acid substitution is preferably performed within a range which does not impair the properties of a substance having the original amino acid sequence.

[0122] As an antibody which has a preferred combination of a heavy chain and a light chain described above, an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence

consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 13; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 14; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 15; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 16; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 17; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 18; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 19; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 12 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 13; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 12 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 14; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 12 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 15; and an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 12 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 16 can be exemplified.

[0123] Further, as an antibody which has a more preferred combination of a heavy chain and a light chain described above, an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 13; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 14; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 15; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 16; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 17; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 18; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 19; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 13; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 14; an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 15; and an antibody consisting of a heavy chain consisting of an amino acid sequence consisting of amino acid residues 20 to 471 of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence consisting of amino acid residues 21 to 233 of SEQ ID NO: 16 can be exemplified.

[0124] Furthermore, as an antibody which has another more preferred combination of a heavy chain and a light chain

described above, an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 13; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 14; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 15; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 16; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 17; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 18; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 9 and a light chain consisting of an amino acid sequence of SEQ ID NO: 19; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 13; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 14; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 15; and an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 16 can be exemplified.

[0125] By combining a sequence having a high homology with the above-described heavy chain amino acid sequence with a sequence having a high homology with the above-described light chain amino acid sequence, it is possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies. Such a homology is generally a homology of 80% or more, preferably a homology of 90% or more, more preferably a homology of 95% or more, most preferably a homology of 99% or more. Further, by combining an amino acid sequence wherein one to several amino acid residues are substituted, deleted or added in the heavy chain or light chain amino acid sequence, it is also possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies.

[0126] The homology between two amino acid sequences can be determined using default parameters of Blast algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25: 3389-3402). The Blast algorithm can be used also through the Internet by accessing the site www.ncbi.nlm.nih.gov/blast.

[0127] In the heavy chain amino acid sequence represented by SEQ ID NO: 9, 10, 11 or 12 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 19 is a signal sequence, an amino acid sequence consisting of amino acid residues 20 to 141 is a variable region, and an amino acid sequence consisting of amino acid residues 142 to 471 is a constant region. The sequence of SEQ ID NO: 9, 10, 11 and 12 are shown in Fig. 3, 4, 5 and 6 respectively.

[0128] Further, in the light chain amino acid sequence represented by SEQ ID NO: 13, 14, 15, 16, 17, 18 or 19 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 20 is a signal sequence, an amino acid sequence consisting of amino acid residues 21 to 128 is a variable region, and an amino acid sequence consisting of amino acid residues 129 to 233 is a constant region. The sequence of SEQ ID NO: 13, 14, 15, 16, 17, 18 and 19 are shown in Fig. 7, 8, 9, 10, 11, 12 and 13 respectively.

[0129] Further, the antibody of the invention includes a human antibody which binds to the same epitope as the M30 antibody. An anti-B7-H3 human antibody refers to a human antibody having only a sequence of an antibody derived from a human chromosome. The anti-B7-H3 human antibody can be obtained by a method using a human antibody-producing mouse having a human chromosome fragment comprising heavy and light chain genes of a human antibody (see Tomizuka, K. et al., Nature Genetics (1997) 16, pp. 133-143; Kuroiwa, Y. et al., Nucl. Acids Res. (1998) 26, pp. 3447-3448; Yoshida, H. et al., Animal Cell Technology: Basic and Applied Aspects vol. 10, pp. 69-73 (Kitagawa, Y., Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999; Tomizuka, K. et al., Proc. Natl. Acad. Sci. USA (2000) 97, pp. 722-727, etc.).

[0130] Such a human antibody-producing mouse can be created specifically as follows. A genetically modified animal in which endogenous immunoglobulin heavy and light chain gene loci have been disrupted, and instead, human immunoglobulin heavy and light chain gene loci have been introduced via a yeast artificial chromosome (YAC) vector or the like is created by producing a knockout animal and a transgenic animal and mating these animals.

[0131] Further, according to a recombinant DNA technique, by using cDNAs encoding each of such a heavy chain and a light chain of a human antibody, and preferably a vector comprising such cDNAs, eukaryotic cells are transformed, and a transformant cell which produces a recombinant human monoclonal antibody is cultured, whereby the antibody can also be obtained from the culture supernatant.

[0132] Here, as the host, for example, eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes, or myeloma cells can be used.

[0133] Further, a method of obtaining a phage display-derived human antibody selected from a human antibody library (see Wormstone, I. M. et al., Investigative Ophthalmology & Visual Science. (2002) 43 (7), pp. 2301-2308; Carmen, S. et al., Briefings in Functional Genomics and Proteomics (2002), 1 (2), pp. 189-203; Siriwardena, D. et al., Ophthalmology (2002) 109 (3), pp. 427-431, etc.) is also known.

[0134] For example, a phage display method in which a variable region of a human antibody is expressed on the surface of a phage as a single-chain antibody (scFv), and a phage which binds to an antigen is selected (Nature Biotechnology (2005), 23, (9), pp. 1105-1116) can be used.

[0135] By analyzing the gene of the phage selected based on the binding to an antigen, a DNA sequence encoding the variable region of a human antibody which binds to an antigen can be determined.

[0136] If the DNA sequence of scFv which binds to an antigen is determined, a human antibody can be obtained by preparing an expression vector comprising the sequence and introducing the vector into an appropriate host to express it (WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, WO 95/15388, Annu. Rev. Immunol. (1994) 12, pp. 433-455, Nature Biotechnology (2005) 23 (9), pp. 1105-1116).

[0137] If a newly produced human antibody binds to a partial peptide or a partial tertiary structure to which the M30 antibody binds, it can be determined that the human antibody binds to the same epitope as the M30 antibody. Further, by confirming that the human antibody competes with the M30 antibody for the binding to B7-H3 (that is, the human antibody inhibits the binding between the M30 antibody and B7-H3), it can be determined that the human antibody binds to the same epitope as the M30 antibody even if the specific epitope sequence or structure has not been determined. When it is confirmed that the human antibody binds to the same epitope as the M30 antibody, the human antibody is strongly expected to have a cytotoxic activity equivalent to that of the M30 antibody.

[0138] The chimeric antibodies, humanized antibodies, or human antibodies obtained by the above-described method are evaluated for the binding property to an antigen by a known method or the like, and a preferred antibody can be selected.

[0139] As one example of another index for use in the comparison of the properties of antibodies, the stability of antibodies can be exemplified. The differential scanning calorimetry (DSC) is a device capable of quickly and accurately measuring a thermal denaturation midpoint temperature (Tm) to be used as a favorable index of the relative conformational stability of proteins. By measuring the Tm values using DSC and comparing the values, a difference in thermal stability can be compared. It is known that the storage stability of antibodies shows some correlation with the thermal stability of antibodies (Lori Burton, et. al., Pharmaceutical Development and Technology (2007) 12, pp. 265-273), and a preferred antibody can be selected by using thermal stability as an index. Examples of other indices for selecting antibodies include the following features: the yield in an appropriate host cell is high; and the aggregability in an aqueous solution is low. For example, an antibody which shows the highest yield does not always show the highest thermal stability, and therefore, it is necessary to select an antibody most suitable for the administration to humans by making comprehensive evaluation based on the above-described indices.

[0140] In the invention, a modified variant of the antibody is also included. The modified variant refers to a variant obtained by subjecting the antibody of the invention to chemical or biological modification. Examples of the chemically modified variant include variants chemically modified by linking a chemical moiety to an amino acid skeleton, variants chemically modified with an N-linked or O-linked carbohydrate chain, etc. Examples of the biologically modified variant include variants obtained by post-translational modification (such as N-linked or O-linked glycosylation, N- or C-terminal processing, deamidation, isomerization of aspartic acid, or oxidation of methionine), and variants in which a methionine residue has been added to the N terminus by being expressed in a prokaryotic host cell.

[0141] Further, an antibody labeled so as to enable the detection or isolation of the antibody or an antigen of the invention, for example, an enzyme-labeled antibody, a fluorescence-labeled antibody, and an affinity-labeled antibody are also included in the meaning of the modified variant. Such a modified variant of the antibody of the invention is useful for improving the stability and blood retention of the original antibody of the invention, reducing the antigenicity thereof, detecting or isolating such an antibody or an antigen, and so on.

[0142] Further, by regulating the modification of a glycan which is linked to the antibody of the invention (glycosylation, defucosylation, etc.), it is possible to enhance an antibody-dependent cellular cytotoxic activity. As the technique for regulating the modification of a glycan of antibodies, WO 99/54342, WO 00/61739, WO 02/31140, etc. are known. However, the technique is not limited thereto. In the antibody of the invention, an antibody in which the modification of a glycan is regulated is also included.

[0143] In the case where an antibody is produced by first isolating an antibody gene and then introducing the gene into an appropriate host, a combination of an appropriate host and an appropriate expression vector can be used. Specific examples of the antibody gene include a combination of a gene encoding a heavy chain sequence of an antibody described in this specification and a gene encoding a light chain sequence thereof. When a host cell is transformed, it is possible to insert the heavy chain sequence gene and the light chain sequence gene into the same expression vector, and also into different expression vectors separately.

[0144] In the case where eukaryotic cells are used as the host, animal cells, plant cells, and eukaryotic microorganisms

can be used. As the animal cells, mammalian cells, for example, simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61) can be exemplified.

[0145] In the case where prokaryotic cells are used, for example, Escherichia coli and Bacillus subtilis can be exemplified.

[0146] By introducing a desired antibody gene into these cells through transformation, and culturing the thus transformed cells in vitro, the antibody can be obtained. In the above-described culture method, the yield may sometimes vary depending on the sequence of the antibody, and therefore, it is possible to select an antibody which is easily produced as a pharmaceutical by using the yield as an index among the antibodies having an equivalent binding activity. Therefore, in the antibody of the invention, an antibody obtained by a method of producing an antibody, characterized by including a step of culturing the transformed host cell and a step of collecting a desired antibody from a cultured product obtained in the culturing step is also included.

[0147] It is known that a lysine residue at the carboxyl terminus of the heavy chain of an antibody produced in a cultured mammalian cell is deleted (Journal of Chromatography A, 705: 129-134 (1995)), and it is also known that two amino acid residues (glycine and lysine) at the carboxyl terminus of the heavy chain of an antibody produced in a cultured mammalian cell are deleted and a proline residue newly located at the carboxyl terminus is amidated (Analytical Biochemistry, 360: 75-83 (2007)). However, such deletion and modification of the heavy chain sequence do not affect the antigen-binding affinity and the effector function (the activation of a complement, the antibody-dependent cellular cytotoxicity, etc.) of the antibody. Therefore, in the invention, an antibody subjected to such modification is also included, and a deletion variant in which one or two amino acids have been deleted at the carboxyl terminus of the heavy chain, a variant obtained by amidation of the deletion variant (for example, a heavy chain in which the carboxyl terminal proline residue has been amidated), and the like can be exemplified. The type of deletion variant having a deletion at the carboxyl terminus of the heavy chain of the antibody according to the invention is not limited to the above variants as long as the antigen-binding affinity and the effector function are conserved. The two heavy chains constituting the antibody according to the invention may be of one type selected from the group consisting of a full-length heavy chain and the above-described deletion variant, or may be of two types in combination selected therefrom. The ratio of the amount of each deletion variant can be affected by the type of cultured mammalian cells which produce the antibody according to the invention and the culture conditions, however, a case where one amino acid residue at the carboxyl terminus has been deleted in both of the two heavy chains contained as main components in the antibody according to the invention can be exemplified.

[0148] As isotype of the antibody of the invention, for example, IgG (IgG1, IgG2, IgG3, IgG4) can be exemplified, and IgG1 or IgG2 can be exemplified preferably.

[0149] As the function of the antibody, generally an antigen-binding activity, an activity of neutralizing the activity of an antigen, an activity of enhancing the activity of an antigen, an antibody-dependent cellular cytotoxicity (ADCC) activity and a complement-dependent cytotoxicity (CDC) activity can be exemplified. The function of the antibody of the invention is a binding activity to B7-H3, preferably an antibody-dependent cell-mediated phagocytosis (ADCP) activity, more preferably a cytotoxicity activity (antitumor activity) to tumor cell mediated by an ADCP activity. Further, the antibody of the invention may have an ADCC activity and/or a CDC activity in addition to an ADCP activity.

[0150] The obtained antibody can be purified to homogeneity. The separation and purification of the antibody may be performed employing a conventional protein separation and purification method. For example, the antibody can be separated and purified by appropriately selecting and combining column chromatography, filter filtration, ultrafiltration, salt precipitation, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing electrophoresis, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the method is not limited thereto.

[0151] Examples of such chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and adsorption chromatography.

[0152] Such chromatography can be performed employing liquid chromatography such as HPLC or FPLC.

[0153] As a column to be used in affinity chromatography, a Protein A column and a Protein G column can be exemplified. For example, as a column using a Protein A column, Hyper D, POROS, Sepharose FF (Pharmacia) and the like can be exemplified.

[0154] Further, by using a carrier having an antigen immobilized thereon, the antibody can also be purified utilizing the binding property of the antibody to the antigen.

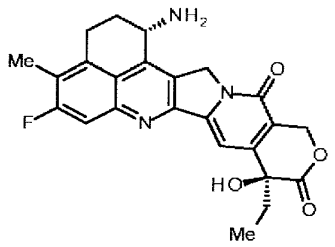
[Antitumor compound]

[0155] The antitumor compound to be conjugated to the antibody-drug conjugate of the present invention is explained.

The antitumor compound is not particularly limited if it is a compound having an antitumor effect and a substituent group or a partial structure allowing connecting to a linker structure. When a part or whole linker is cleaved in tumor cells, the antitumor compound moiety is released to exhibit the antitumor effect of the antitumor compound. As the linker is cleaved at a connecting position to drug, the antitumor compound is released in its intrinsic structure to exhibit its intrinsic antitumor effect.

[0156] Examples of the antitumor compound can include doxorubicin, daunorubicin, mitomycin C, bleomycin, cyclocytidine, vincristine, vinblastine, methotrexate, platinum-based antitumor agent (cisplatin or derivatives thereof), taxol or derivatives thereof, and camptothecin or derivatives thereof (antitumor agent described in Japanese Patent Laid-Open No. 6-87746). In the antibody-drug conjugate of the present invention, exatecan as a camptothecin derivative (((1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione; shown in the following formula) can be preferably used.

[Formula 43]



[0157] Although having an excellent antitumor effect, exatecan has not been commercialized as an antitumor drug. The compound can be easily obtained by a known method and the amino group at position 1 can be preferably used as a connecting position to the linker structure. Further, although exatecan can be also released in tumor cells while part of the linker is still attached thereto, it is an excellent compound exhibiting an excellent antitumor effect even in such case.

[0158] With regard to the antibody-drug conjugate, the number of conjugated drug molecules per antibody molecule is a key factor having an influence on the efficacy and safety. Production of the antibody-drug conjugate is performed by defining the reaction condition including the amounts of use of raw materials and reagents for reaction so as to have a constant number of conjugated drug molecules, a mixture containing different numbers of conjugated drug molecules is generally obtained unlike the chemical reaction of a low-molecular-weight compound. The number of drugs conjugated in an antibody molecule is expressed or specified by the average value, that is, the average number of conjugated drug molecules. Unless specifically described otherwise as a principle, the number of conjugated drug molecules means an average value except in a case in which it represents an antibody-drug conjugate having a specific number of conjugated drug molecules that is included in an antibody-drug conjugate mixture having different number of conjugated drug molecules. The number of exatecan molecules conjugated to an antibody molecule is controllable, and as an average number of conjugated drug molecules per antibody, about 1 to 10 exatecans can be bound. Preferably, it is 2 to 8, and more preferably 3 to 8. Meanwhile, a person skilled in the art can design a reaction for conjugating a required number of drug molecules to an antibody molecule based on the description of the Examples of the present application and can obtain an antibody conjugated with a controlled number of exatecan molecules.

[0159] Because exatecan has a camptothecin structure, it is known that the equilibrium shifts to a structure with a closed lactone ring (closed ring) in an aqueous acidic medium (for example, pH 3 or so) but it shifts to a structure with an open lactone ring (open ring) in an aqueous basic medium (for example, pH 10 or so). A drug conjugate being introduced with an exatecan residue corresponding to the closed ring structure and the open ring structure is also expected to have the same antitumor effect and it is needless to say that any of them is within the scope of the present invention.

[Linker structure]

[0160] With regard to the antibody-drug conjugate of the present invention, the linker structure for conjugating an antitumor drug to the antibody is explained. The linker has a structure of the following structure:

$$-L^1-L^2-L^P-NH-(CH2)n^1-L^a-L^b-L^c-.$$

The antibody is connected to the terminal of L^1 (terminal opposite to the connection to L^2), and the antitumor drug is connected to the terminal of L^c (terminal opposite to the connection to L^b).

[0161] n^1 represents an integer of 0 to 6 and is preferably an integer of 1 to 5, and more preferably 1 to 3.

1. L^1

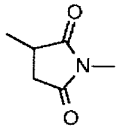
[0162] L^1 is a moiety in the linker represented by the following structure:

-(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-,
-CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-,
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-, or
-C(=O)-$(CH_2)n^4$-C(=O)-

In the above, n^2 is an integer of 2 to 8, n^3 is an integer of 1 to 8, and n^4 is an integer of 1 to 8.

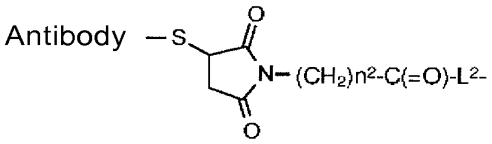
[0163] In the linker having a structure represented by - (Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)-of L^1, "- (Succinimid-3-yl-N)-" has a structure represented by the following formula:

[Formula 44]



[0164] Position 3 of the above partial structure is a connecting position to the antibody. The bond to the antibody at position 3 is characterized by bonding with thioether formation. On the other hand, the nitrogen atom at position 1 of the structure moiety is connected to the carbon atom of methylene which is present within the linker including the structure. Specifically,-(Succinimid-3-yl-N)-$(CH_2)n^2$-C (=O)-L^2- is a structure represented by the following formula (herein, "antibody-S-" originates from an antibody).

[Formula 45]



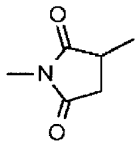
[0165] In the formula, n^2 is an integer of 2 to 8, and preferably 2 to 5.

[0166] In the linker having a structure represented by-CH_2-C (=O) -NH- (CH_2) n^3-C (=O) - of L^1, n^3 is an integer of 1 to 8, preferably 2 to 6. This linker is connected to the antibody at its carbon atom of terminal methylene and has the following structure for connecting by thioether formation, as with the preceding linker (herein, "antibody-S-" originates from an antibody).

Antibody-S-CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-L^2-.

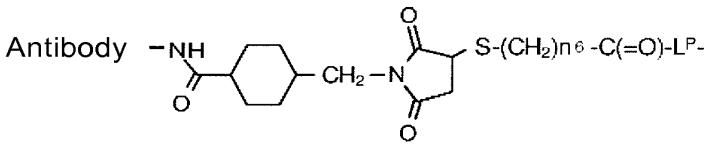
[0167] In the linker having a structure represented by - C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)- of L^1, "-(N-ly-3-diminiccuS)-" has a structure represented by the following formula:

[Formula 46]



[0168] In this structure moiety, the nitrogen atom at position 1 is connected to the carbon atom of methylene present in the linker structure containing this structure. The carbon atom at position 3 is connected to the terminal sulfur atom of -S-$(CH_2)_n$6-C(=O)- of L^2 in the linker. This moiety -S-$(CH_2)n^6$-C(=O)- of L^2 in the linker forms a combined linker structure only with -C(=O)-cyc.Hex(1, 4)-CH_2-(N-ly-3-diminiccuS)- of L^1 in the linker. In the above, "-Cyc.Hex(1,4)-" contained in the linker represents a 1,4-cyclohexylene group. In the linker, -C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)- is connected to the antibody with amide bond formation at its terminal carbonyl carbon (herein, "antibody-NH-" originates from an antibody).

[Formula 47]



[0169] The amino group of the antibody for this amide bond formation is the terminal amino group of a side chain of a lysine residue in the antibody or an amino group at the N terminal of the antibody. Said linker of a structure can connect by forming ester bond with the hydroxy group of an amino acid in the antibody other than such amide bond.

[0170] The structure moiety "-cyc.Hex(1,4)-" contained in said linker may be a divalent saturated cyclic alkylene group other than the 1,4-cyclohexylene group, i.e., a divalent cyclic saturated hydrocarbon group such as a cyclobutylene group, a cyclopentylene group, a cycloheptalene group, or a cyclooctalene group, a divalent aromatic hydrocarbon group such as a phenylene group or a naphthylene group, or a 5- or 6-membered saturated, partially saturated, or aromatic divalent heterocyclic group containing 1 or 2 heteroatoms. Alternatively, this moiety may be a divalent alkylene group having 1 to 4 carbon atoms. The connection to the divalent group may occur at adjacent positions or at distant positions.

[0171] In the linker having a structure represented by - C (=O)- (CH_2) n^4-C (=O)- as L^1, n^4 is an integer of 1 to 8, and preferably 2 to 6. This linker is also connected by amide bond formation at its terminal carbonyl group with an amino group of the antibody, as with the linkers mentioned above (see the following formula; in the structure thereof, "antibody-NH-" originates from an antibody).

Antibody-NH-C(=O)-$(CH_2)n^4$-C(=O)-L^2-.

[0172] Specific examples of L^1 in the linker can include

-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-CH_2C(=O)NH-CH_2-C(=O)-,
-CH_2C(=O)NH-CH_2CH_2-C(=O)-
-CH_2C(=O)NH-$CH_2CH_2CH_2$-C(=O)-
-CH_2C(=O)NH-$CH_2CH_2CH_2CH_2$-C(=O)-
-CH_2C(=O)NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-
-C(=O)-Aryl-CH_2-(N-ly-3-diminiccuS)-
-C(=O)-cyc.Het-CH_2-(N-ly-3-diminiccuS)-
-C(=O)-CH_2CH_2-C(=O)-
-C(=O)-$CH_2CH_2CH_2$-C(=O)-

-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-.
(Aryl represents a divalent aromatic hydrocarbon group, and cyc.Het represents a divalent cyclic heterocyclic group).

2. L^2

[0173] L^2 is a linker represented by the following structure:

- NH-$(CH_2CH_2O)n^5$-CH_2-CH_2-C(=O)-,or
-S-$(CH_2)n^6$-C(=O)-,
L^2 may not be present, and in such a case, L^2 is a single bond. In the above, n^5 is an integer of 1 to 6, and n^6 is an integer of 1 to 6.

[0174] In the linker having a structure of -NH-$(CH_2CH_2O)n^5$-CH_2-CH2-C(=O)- as L^2, n^5 is an integer of 1 to 6, and preferably 2 to 4. This moiety in the linker is connected to L^1 at its terminal amino group and is connected to L^P at its carbonyl group at the other terminal.
[0175] In the linker having a structure of -S-$(CH_2)n^6$-C(=O)- as L^2, n^6 is an integer of 1 to 6, and preferably 2 to 4.
[0176] Specific examples of L^2 can include

-NH-CH_2CH_2O-CH_2CH_2-C (=O) -,
-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O) -,
-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C (=O) -,
-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C (=O) -,
-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O) -,
-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-.

[0177] When L^2 is -S-$(CH_2)n^6$-C(=O)-, L^1 to be combined therewith is -C(=O)-cyc.Hex(1, 4)-CH_2-(N-ly-3-diminiccuS)-. Specific examples of -L^1-L^2- can include

-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-CH_2CH_2-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-.

3. L^P

[0178] The linker L^p is a peptide residue consisting of 2 to 7 amino acids. Specifically, it consists of an oligopeptide residue in which 2 to 6 amino acids are linked by a peptide bonding. The linker L^p is connected to L^2 at its N terminal and is connected to the amino group of -NH-$(CH_2)n^1$-L^a-L^b-L^c-moiety of the linker at its C terminal. The amino acid constituting L^p in the linker is not particularly limited, however, examples thereof include an L- or a D-amino acid, preferably an L-amino acid. And, it can be an amino acid having a structure such as β-alanine, ε-aminocaproic acid, or γ-aminobutyric acid in addition to an α-amino acid, further, it can be a non-natural type amino acid such as N-methylated amino acid.
[0179] The amino acid sequence of L^p is not particularly limited, but examples of the constituting amino acid include phenylalanine (Phe; F), tyrosine (Tyr; Y), leucine (Leu; L), glycine (Gly; G), alanine (Ala; A), valine (Val; V), lysine (Lys; K), citrulline (Cit), serine (Ser; S), glutamic acid (Glu; E), and aspartic acid (Asp; D). Among them, preferred examples include phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Depending on the type of the amino acid, drug release pattern can be controlled. The number of the amino acid can be between 2 to 7.
[0180] Specific examples of L^P can include

-GGF-
-DGGF-
-(D-)D-GGF-
-EGGF-
-GGFG-
-SGGF-

-KGGF-
-DGGFG-
-GGFGG-
-DDGGFG-
-KDGGFG-
-GGFGGGF-

[in the above, "(D-)D" represents a D-aspartic acid]. Particularly preferred examples of L^P for the antibody-drug conjugate of the present invention can include - GGFG-.

[0181] In the structure represented by -NH-$(CH_2)n^1$- within the linker, n^1 is an integer of 0 to 6 and is preferably an integer of 1 to 5, and more preferably 1 to 3. The amino group of this moiety is connected to the C terminal of L^P in the linker.

4. L^a

[0182] The linker L^a is represented by any of structures -C(=O)-NH-, -NR^1- (CH_2) n^7-, and -O- or is a single bond. In the above, n^7 is an integer of 1 to 6, R^1 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -$(CH_2)n^8$-COOH, or -$(CH_2)n^9$-OH, n^8 is an integer of 1 to 4, and n^9 is an integer of 1 to 6.

[0183] The amide structure -C(=O)-NH- within likner L^a is connected to L^b at its nitrogen atom side. In the structure moiety of -NR^1-$(CH_2)n^7$- witthin L^a, n^7 is an integer of 1 to 6, and preferably 1 to 3. This moiety is connected to L^b at its methylene side. R^1 is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms. The alkyl group having 1 to 6 carbon atoms may be linear or branched. Examples thereof can include a methyl group, an ethyl group, a propyl group, an isopropyl group, a butyl group, an isobutyl group, a sec-butyl group, a tert-butyl group, a pentyl group, an isopentyl group, a 2-methylbutyl group, a neopentyl group, a 1-ethylpropyl group, a hexyl group, an isohexyl group, a 4-methylpentyl group, a 3-methylpentyl group, a 2-methylpentyl group, a 1-methylpentyl group, a 3,3-dimethylbutyl group, a 2,2-dimethylbutyl group, a 1,1-dimethylbutyl group, a 1,2-dimethylbutyl group, a 1,3-dimethylbutyl group, a 2,3-dimethylbutyl group, and a 2-ethylbutyl group. Of them, a methyl group or an ethyl group is preferred. When R^1 has a structure represented by -$(CH_2)n^8$-COOH, n^8 is an integer of 1 to 4, and preferably 1 or 2. When R^1 has a structure represented by -$(CH_2)n^9$-OH, n^9 is an integer of 1 to 6, and preferably 1 or 2. R^1 is preferably a hydrogen atom, a methyl group, an ethyl group, -CH_2COOH, -CH_2CH_2-COOH, or -CH_2CH_2-OH, and more preferably a hydrogen atom, a methyl group, or -CH_2COOH. It is further preferably a hydrogen atom. The L^a moiety of the linker may be -O- or a single bond.

5. L^b

[0184] The linker L^b is any of structures -$CR^2(-R^3)$-, -O-, and -NR^4- or is a single bond. In the above, R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -$(CH_2)n^a$-NH_2,-$(CH_2)n^b$-COOH, or -$(CH_2)n^c$-OH, R^4 is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a is an integer of 0 to 6, n^b is an integer of 1 to 4, and n^c is an integer of 0 to 4. When n^a or n^c is 0, R^2 and R^3 are not the same each other.

[0185] When each of R^2 and R^3 is an alkyl group, this alkyl group is interpreted as defined in the alkyl group of R^1. When R^2 and R^3 has a structure of -$(CH_2)n^a$-NH_2, n^a is an integer of 0 to 6, and preferably 0, or is 3 to 5. When n^a is 0, R^2 and R^3 are not the same as each other. When R^2 and R^3 has a structure of -$(CH_2)n^b$-COOH, n^b is an integer of 1 to 4, and preferably 1 or 2. When R^2 and R^3 has a structure of -$(CH_2)n^c$-OH, n^c is an integer of 0 to 4, and preferably 1 or 2.

[0186] Each of R^2 and R^3 is preferably a hydrogen atom, a methyl group, an ethyl group, -NH_2, -$CH_2CH_2CH_2NH_2$,-$CH_2CH_2CH_2CH_2NH_2$, -$CH_2CH_2CH_2CH_2CH_2CH_2NH_2$, -$CH_2$COOH, -$CH_2CH_2$-COOH, -$CH_2$OH, or -$CH_2CH_2$-OH, and more preferably a hydrogen atom, a methyl group, -NH_2, -$CH_2CH_2CH_2CH_2NH_2$,-CH_2COOH, -CH_2CH_2-COOH, -CH_2OH, or -CH_2CH_2-OH. They are further preferably hydrogen atoms.

[0187] When R^4 is an alkyl group having 1 to 6 carbon atoms, this alkyl group is interpreted as defined in the alkyl group of R^1. R^4 is preferably a hydrogen atom or a methyl group, and more preferably a hydrogen atom.

[0188] Specific examples of the structure represented by

-NH-$(CH_2)n^1$-L^a-L^b- as the linker can include
-NH-CH_2-
-NH-CH(-Me)-
-NH-C(-Me)$_2$-
-NH-CH_2-CHMe-
-NH-CH(-CH_2OH)-
-NH-CH (-CH_2COOH)-
-NH-CH(-CH_2CH_2COOH)-
-NH-CH(-$CH_2CH_2CH_2CH_2NH_2$)-

-NH-CH_2CH_2-
-NH-CH_2-O-CH_2-
-NH-CH_2CH_2-O-
-NH-CH_2CH_2-O-CH_2-
-NH-$CH_2CH_2C(-Me)_2$-
-NH-CH_2CH_2NH-
-NH-CH_2CH_2NH-CH_2-
-NH-CH_2CH_2NMe-CH_2-
-NH-CH_2CH_2NH-CH_2CH_2-
-NH-CH_2CH_2NMe-CH_2CH_2-
-NH-$CH_2CH_2N(-CH_2COOH)$-CH_2-
-NH-$CH_2CH_2N(-CH_2CH_2OH)$-CH_2-
-NH-$CH_2CH_2N(-CH_2CH_2OH)$-CH_2CH_2-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-CH_2OH)-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-CH_2COOH)-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-$CH_2CH_2CH_2CH_2NH_2$)-
-NH-$CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2CH(NH_2)$-.

[0189] Of them, preferred examples thereof can include

-NH-CH_2-
-NH-CH_2-CH(Me)-
-NH-CH(-CH_2OH)-
-NH-CH(-CH_2CH_2COOH)-
-NH-CH_2CH_2-
-NH-CH_2-O-CH_2-
-NH-CH_2CH_2-O-
-NH-CH_2CH_2-O-CH_2-
-NH-$CH_2CH_2C(-Me)_2$-
-NH-CH_2CH_2NH-
-NH-CH_2CH_2NH-CH_2-
-NH-CH_2CH_2NMe-CH_2-
-NH-CH_2CH_2NMe-CH_2CH_2-
-NH-$CH_2CH_2N(-CH_2COOH)$-CH_2-
-NH-$CH_2CH_2N(-CH_2CH_2OH)$-CH_2-
-NH-$CH_2CH_2N(-CH_2CH_2OH)$-CH_2CH_2-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-CH_2OH)-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-CH_2COOH)-
-NH-$CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2CH_2$-.

[0190] More preferred examples thereof can include

-NH-CH_2-
-NH-CH_2CH_2-
-NH-CH_2-O-CH_2-
-NH-CH2CH2-O-
-NH-CH_2CH_2-O-CH_2-
-NH-CH_2CH_2NH-
-NH-CH_2CH_2NH-CH_2-
-NH-$CH_2CH_2N(-CH_2COOH)$-CH_2-
-NH-$CH_2CH_2N(-CH_2CH_2OH)$-CH_2CH_2-
-NH-$CH_2CH_2CH_2C(=O)$-NHCH(-CH_2COOH)-
-NH-$CH_2CH_2CH_2$-

-NH-$CH_2CH_2CH_2CH_2$-
-NH-$CH_2CH_2CH_2CH_2CH_2$-.

[0191] Further preferred examples thereof can include

-NH-$(CH_2)_3$-,
-NH-CH_2-O-CH_2-, and
-NH-$(CH_2)_2$-O-CH_2-.

6. L^c

[0192] The linker L^c is -CH_2- or -C(=O)-. Said linker is connected to the antitumor compound. L^c of the linker is more preferably -C(=O)-.

[0193] In the linker, the chain length of -NH-$(CH_2)n^1$-L^a-L^b-L^c is preferably a chain length of 4 to 7 atoms, and more preferably a chain length of 5 or 6 atoms.

[0194] With regard to the antibody-drug conjugate of the present invention, when it is transferred to the inside of tumor cells, the linker moiety is cleaved and the drug derivative having a structure represented by NH_2-$(CH_2)n^1$-L^a-L^b-L^c- (NH-DX) is released to express an antitumor action. Examples of the antitumor derivative exhibiting an antitumor effect by releasing from the antibody-drug conjugate of the present invention include an antitumor derivative having a structure moiety in which the structure represented by -NH-$(CH_2)n^1$-L^a-L^b- of the linker is bound with L^c and has a terminal amino group, and the particularly preferred include the followings.

NH_2-CH_2CH_2-C(=O)-(NH-DX)
NH_2-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
NH_2-CH_2-O-CH_2-C(=O)-(NH-DX)
NH_2-$CHCH_2$-O-CH_2-C(=O)-(NH-DX)

[0195] Meanwhile, in case of NH_2-CH_2-O-CH_2-C (=O) - (NH-DX), it was confirmed that, as the aminal structure in the molecule is unstable, it again undergoes a self-degradation to release the following HO-CH_2-C(=O)-(NH-DX). Those compounds can be also preferably used as a production intermediate of the antibody-drug conjugate of the present invention.

[0196] For the antibody-drug conjugate of the present invention in which exatecan is used as a drug, it is preferable that the drug-linker structure moiety having the following structure [-L^1-L^2-L^P-NH-(CH_2) n^1-L^a-L^b-L^c-(NH-DX)] is connected to an antibody. The average conjugated number of the drug-linker structure moiety per antibody can be 1 to 10. Preferably, it is 2 to 8, and more preferably 3 to 8.

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N) -CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
- CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

[0197] Among them, the more preferred are the followings.

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX).

[0198] The particularly preferred are the followings.

- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX).

[0199] With regard to the linker structure for conjugating the antibody and a drug in the antibody-drug conjugate of the present application, the preferred linker can be constructed by connecting preferred structures shown for each part of the linker explained above. As for the linker structure, those with the following structure can be preferably used. Meanwhile, the left terminal of the structure is a connectiong position with the antibody and the right terminal is a connecting position with the drug.

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
-(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-(Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-
-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
-C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-

[0200] Among them, the more preferred are the followings.

- (Succinimid-3-yl-N)-CH_2CH_2-C (=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2CH2-C(=O)-
- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-
- C(=O)-cyc.Hex(1,4)-CH_2-(N-Iy-3-diminiccuS)-S-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-.

[0201] The particularly preferred include the followings.

- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-
- (Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-

- (Succinimid-3-yl-N)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-.

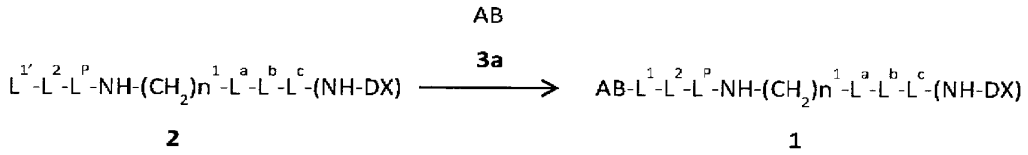
[Production method]

[0202] Next, explanations are given for the representative method for producing the antibody-drug conjugate of the present invention or a production intermediate thereof. Meanwhile, the compounds are hereinbelow described with the compound number shown in each reaction formula. Specifically, they are referred to as a "compound of the formula (1)", a "compound (1)", or the like. The compounds with numbers other than those are also described similarly.

1. Production method 1

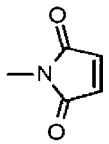
[0203] The antibody-drug conjugate represented by the formula (1) in which the antibody is bound to the linker structure via thioether can be produced by the following method, for example.

[Formula 48]



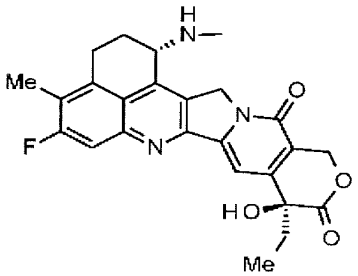
[In the formula, AB represents an antibody with a sulfhydryl group, and $L^{1'}$ represents L^1 linker structure in which the linker terminal is a maleimidyl group (formula shown below)

[Formula 49]



(in the formula, the nitrogen atom is the connecting position)
or the terminal is halogen, and represents a group in which the -(Succinimid-3-yl-N)- moiety in -(Succinimid-3-yl-N)-$(CH_2)n^2$-C(=O)- of L^1 is a maleimidyl group or a halogen-$CH_2C(=O)NH$-$(CH_2)n^3$-C(=O)- group in which terminal methylene in -$CH_2C(=O)NH$-$(CH_2)n^3$-C(=O)- of L^1 is halogenated to form haloacetamide. Further, the - (NH-DX) represents a structure represented by the following formula:

[Formula 50]



and it represents a group that is derived by removing one hydrogen atom of the amino group at position 1 of exatecan. Further, the compound of the formula (1) in the above reaction formula is described as a structure in which one structure moiety from drug to the linker terminal connects to one antibody. However, it is only the description given for the sake

of convenience, and there are actually many cases in which a plurality of the structure moieties are connected to one antibody molecule. The same applies to the explanation of the production method described below.]

[0204] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2), which is obtainable by the method described below, with the antibody (3a) having a sulfhydryl group.

[0205] The antibody (3a) having a sulfhydryl group can be obtained by a method well known in the art (Hermanson, G.T, Bioconjugate Techniques, pp. 56-136, pp. 456-493, Academic Press (1996)). Examples include: Traut's reagent is reacted with the amino group of the antibody; N-succinimidyl S-acetylthioalkanoates are reacted with the amino group of the antibody followed by reaction with hydroxylamine; after reacting with N-succinimidyl 3-(pyridyldithio)propionate, the antibody is reacted with a reducing agent; the antibody is reacted with a reducing agent such as dithiothreitol, 2-mercaptoethanol, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce the disulfide bond in a hinge part in the antibody to form a sulfhydryl group, but it is not limited thereto.

[0206] Specifically, using 0.3 to 3 molar equivalents of TCEP as a reducing agent per disulfide in hinge part in the antibody and reacting with the antibody in a buffer solution containing a chelating agent, the antibody with partially or completely reduced disulfide in hinge part in the antibody can be obtained. Examples of the chelating agent include ethylenediamine tetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA). It can be used at concentration of 1 mM to 20 mM. Examples of the buffer solution which may be used include a solution of sodium phosphate, sodium borate, or sodium acetate. As a specific example, by reacting the antibody with TCEP for 1 to 4 hours at 4°C to 37°C, the antibody (3a) having partially or completely reduced sulfhydryl group can be obtained.

[0207] Meanwhile, by performing the reaction for adding a sulfhydryl group to a drug-linker moiety, the drug-linker moiety can be conjugated by a thioether bond.

[0208] Next, using 2 to 20 molar equivalents of the compound (2) per the antibody (3a) having a sulfhydryl group, the antibody-drug conjugate (1) in which 2 to 8 drug molecules are conjugated per antibody can be produced. Specifically, it is sufficient that the solution containing the compound (2) dissolved therein is added to a buffer solution containing the antibody (3a) having a sulfhydryl group for the reaction. Herein, examples of the buffer solution which may be used include sodium acetate solution, sodium phosphate, and sodium borate. pH for the reaction is 5 to 9, and more preferably the reaction is performed near pH 7. Examples of the solvent for dissolving the compound (2) include an organic solvent such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), dimethyl acetamide (DMA), and N-methyl-2-pyridone (NMP). It is sufficient that the organic solvent solution containing the compound (2) dissolved therein is added at 1 to 20% v/v to a buffer solution containing the antibody (3a) having a sulfhydryl group for the reaction. The reaction temperature is 0 to 37°C, more preferably 10 to 25°C, and the reaction time is 0.5 to 2 hours. The reaction can be terminated by deactivating the reactivity of unreacted compound (2) with a thiol-containing reagent. Examples of the thiol-containing reagent include cysteine and N-acetyl-L-cysteine (NAC). More specifically, 1 to 2 molar equivalents of NAC are added to the compound (2) used and, by incubating at room temperature for 10 to 30 minutes, the reaction can be terminated.

[0209] The produced antibody-drug conjugate (1) can be subjected to, after concentration, buffer exchange, purification, and measurement of antibody concentration and average number of conjugated drug molecules per antibody molecule according to common procedures described below, identification of the antibody-drug conjugate (1).

Common procedure A: Concentration of aqueous solution of antibody or antibody-drug conjugate

[0210] To a Amicon Ultra (50,000 MWCO, Millipore Corporation) container, a solution of antibody or antibody-drug conjugate was added and the solution of the antibody or antibody-drug conjugate was concentrated by centrifugation (centrifuge for 5 to 20 minutes at 2000 G to 3800 G) using a centrifuge (Allegra X-15R, Beckman Coulter, Inc.).

Common procedure B: Measurement of antibody concentration

[0211] Using a UV detector (Nanodrop 1000, Thermo Fisher Scientific Inc.), measurement of the antibody concentration was performed according to the method defined by the manufacturer. At that time, 280 nm absorption coefficient different for each antibody was used (1.3 $mLmg^{-1}cm^{-1}$ to 1.8 $mLmg^{-1}cm^{-1}$).

Common procedure C-1: Buffer Exchange for antibody

[0212] NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation) using Sephadex G-25 carrier was equilibrated with phosphate buffer (10 mM, pH 6.0) (it is referred to as PBS6.0/EDTA in the specification) containing sodium chloride (137 mM) and ethylene diamine tetraacetic acid (EDTA, 5 mM) according to the method defined by the manufacturer's instruction manual. Aqueous solution of the antibody was applied in an amount of 2.5 mL to single NAP-25 column, and then the fraction (3.5 mL) eluted with 3.5 mL of PBS6.0/EDTA was collected. The resulting fraction was concentrated by the Common procedure A. After measuring the concentration of the antibody using the Common procedure B, the antibody concentration was adjusted to 10 mg/mL using PBS6.0/EDTA.

Common procedure C-2: Buffer Exchange for antibody

[0213] NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation) using Sephadex G-25 carrier was equilibrated with phosphate buffer (50 mM, pH 6.5) (it is referred to as PBS6.5/EDTA in the specification) containing sodium chloride (50 mM) and EDTA (2 mM) according to the method defined by the manufacturer. Aqueous solution of the antibody was applied in an amount of 2.5 mL to single NAP-25 column, and then the fraction (3.5 mL) eluted with 3.5 mL of PBS6.5/EDTA was collected. The resulting fraction was concentrated by the Common procedure A. After measuring the concentration of the antibody using the Common procedure B, the antibody concentration was adjusted to 20 mg/mL using PBS6.5/EDTA.

Common procedure D-1: Purification of antibody-drug conjugate

[0214] NAP-25 column was equilibrated with any buffer selected from commercially available phosphate buffer (PBS7.4, Cat. No. 10010-023, Invitrogen), sodium phosphate buffer (10 mM, pH 6.0; it is referred to as PBS6.0) containing sodium chloride (137 mM), and acetate buffer containing sorbitol (5%) (10 mM, pH 5.5; it is referred to as ABS in the specification). Aqueous solution of the antibody-drug conjugate reaction was applied in an amount of about 1.5 mL to the NAP-25 column, and then eluted with the buffer in an amount defined by the manufacturer to collect the antibody fraction. The collected fraction was again applied to the NAP-25 column and, by repeating 2 to 3 times in total the gel filtration purification process for eluting with buffer, the antibody-drug conjugate excluding non-conjugated drug linker and a low-molecular-weight compound (tris(2-carboxyethyl)phosphine hydrochloride (TCEP), N-acetyl-L-cysteine (NAC), and dimethyl sulfoxide) was obtained. Common procedure E: Measurement of antibody concentration in antibody-drug conjugate and average number of conjugated drug molecules per antibody molecule.

[0215] The conjugated drug concentration in the antibody-drug conjugate can be calculated by measuring UV absorbance of an aqueous solution of the antibody-drug conjugate at two wavelengths of 280 nm and 370 nm, followed by performing the calculation shown below.

[0216] Because the total absorbance at any wavelength is equal to the sum of the absorbance of every light-absorbing chemical species that are present in a system [additivity of absorbance], when the molar absorption coefficients of the antibody and the drug remain the same before and after conjugation between the antibody and the drug, the antibody concentration and the drug concentration in the antibody-drug conjugate are expressed with the following equations.

$$A_{280} = A_{D,280} + A_{A,280} = \varepsilon_{D,280}C_D + \varepsilon_{A,280}C_A \qquad \text{Equation (1)}$$

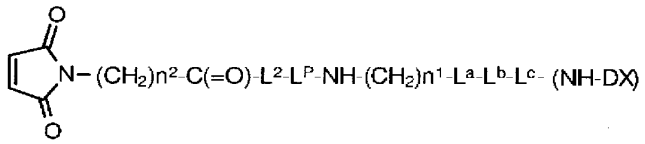
$$A_{370} = A_{D,370} + A_{A,370} = \varepsilon_{D,370}C_D + \varepsilon_{A,370}C_A \qquad \text{Equation (2)}$$

[0217] In the above, A_{280} represents the absorbance of an aqueous solution of the antibody-drug conjugate at 280 nm, A_{370} represents the absorbance of an aqueous solution of the antibody-drug conjugate at 370 nm, $A_{A,280}$ represents the absorbance of an antibody at 280 nm, $A_{A,370}$ represents the absorbance of an antibody at 370 nm, $A_{D,280}$ represents the absorbance of a conjugate precursor at 280 nm, $A_{D,370}$ represents the absorbance of a conjugate precursor at 370 nm, $\varepsilon_{A,280}$ represents the molar absorption coefficient of an antibody at 280 nm, $\varepsilon_{A,370}$ represents the molar absorption coefficient of an antibody at 370 nm, $\varepsilon_{D,280}$ represents the molar absorption coefficient of a conjugate precursor at 280 nm, $E_{D,370}$ represents the molar absorption coefficient of a conjugate precursor at 370 nm, C_A represents the antibody concentration in an antibody-drug conjugate, and C_D represent the drug concentration in an antibody-drug conjugate.

[0218] As for $\varepsilon_{A,280}$, $\varepsilon_{A,370}$ $\varepsilon_{D,280}$, and $\varepsilon_{D,370}$ in the above, previously prepared values (estimated value based on calculation or measurement value obtained by UV measurement of the compound) are used. For example, $\varepsilon_{A,280}$ can be estimated from the amino acid sequence of an antibody using a known calculation method (Protein Science, 1995, vol. 4, 2411-2423). $\varepsilon_{A,370}$ is generally zero. $\varepsilon_{D,280}$ and $\varepsilon_{D,370}$ can be obtained based on Lambert-Beer's law (Absorbance = molar concentration x molar absorption coefficient x cell path length) by measuring the absorbance of a solution in which the conjugate precursor to be used is dissolved at a certain molar concentration. By measuring A_{280} and A_{370} of an aqueous solution of the antibody-drug conjugate and solving the simultaneous equations (1) and (2) using the values, C_A and C_D can be obtained. Further, by diving C_D by C_A, the average number of conjugated drug per antibody can be obtained.

[0219] The compound represented by the formula (2) in Production method 1 is any compound represented by the following formula:

[Formula 51]



Halogen-$CH_2C(=O)NH$-$(CH_2)n^3$-$C(=O)$-L^2-L^P-NH-$(CH_2)n^1$-L^a-L^b-L^c- (NH-DX)

[0220] In the formula, n^1, n^2, n^3, L^2, L^P, L^a, L^b, and L^c are as already defined, and L^c is a conneting position for the drug.

[0221] In an intermediate useful in producing such a compound of the present invention, preferably, n^2 is an integer of 2 to 5, L^2 is -NH-$(CH_2CH_2O)n^5$-CH_2CH_2-C(=O) - or a single bond, n^5 is an integer of 2 to 4, L^P is GGFG, and -NH-$(CH_2)n^1$-L^a-L^b-L^c- is a partial structure of -NH-CH_2CH_2-C(=O)-, NH-$CH_2CH_2CH_2$-C(=O)-, -NH-CH_2-O-CH_2-C(=O)-, or -NH-CH_2CH_2-O-CH_2-C(=O)-. Halogen is preferably bromine or iodine. Specific examples of these compounds can include the followings [herein, (maleimid-N-yl) represents a maleimidyl group (2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl group)].

(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)- (NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)

X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
X-CH_2-C(=O)-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH2-C(=O)-(NH-DX)

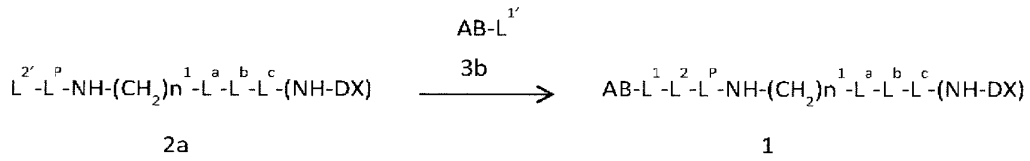
[0222] In the formula, X represents a bromine atom or an iodine atom. All of these bromine and iodine compounds can be preferably used as production intermediates.

[0223] In order to secure the amount of the conjugate, a plurality of conjugates obtained under similar production conditions to have an equivalent number of drugs (e.g., about ± 1) can be mixed to prepare new lots. In this case, the average number of drugs falls between the average numbers of drugs in the conjugates before the mixing.

2. Production method 2

[0224] The antibody-drug conjugate represented by the formula (1) in which the antibody is connected via an amide group to a linker and having a thioether bond within the linker, specifically, a structure in which-L^1-L^2- is -C(=0)-eye . Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-S-$(CH_2)n^6$-C(=O)-, can be also produced by the following method.

[Formula 52]



2a 1

[0225] In the formula, AB-$L^{1'}$ represents a group which the antibody and linker L^1 are connected and, further, the terminal of L^1 is converted to a N-maleimidyl group. This group specifically has a structure in which -(N-ly-3-diminiccuS)- in AB-C(=0)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)- is converted to a maleimidyl group. $L^{2'}$ represents a HS-$(CH_2)n^6$-C(=O)- group in which the terminal is a mercapto group, and AB represents the antibody.

[0226] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2a), which is obtainable by the method described below, with the antibody (3b) which is connected to the linker having a maleimidyl group.

[0227] The antibody (3b) having a maleimidyl group can be also obtained by a method well known in the art (Hermanson, G.T, Bioconjugate Techniques, pp. 56-136, pp. 456-493, Academic Press (1996)). Examples include: a bifunctional linker, such as succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), which is capable of bonding to an amino group or a hydroxyl group and has a maleimidyl group is allowed to react on the amino group of the ligand to introduce a maleimidyl group, but it is not limited thereto.

[0228] For example, a compound having an amino group-reactive moiety and a thiol group-reactive moiety bound via a linker can be preferably used. Here, the amino group-reactive moiety can be active ester, imide ester, or the like, and the thiol-reactive moiety can be maleimidyl, acetyl halide, alkyl halide, dithiopyridyl, or the like.

[0229] As a method for constructing the linker with amino goroup or hydroxy group of an amino acid constituting the antibody, particularly via an amide bond with the amino group, the compound to be first reacted with the antibody can be a compound represented by the following formula:

$$Q_1\text{-}L^{1a}\text{-}Q^2.$$

[In the formula, Q^1 represents (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, R^Q-O-C(=N)-, or O=C=N-,
L^{1a}- represents -cyc.Hex(1,4)-CH_2-, an alkylene group having 1 to 10 carbon atoms, a phenylene group,-$(CH_2)n^4$-C(=O)-, -$(CH_2)n^{4a}$-NH-C(=O)-$(CH_2)n^{4b}$-,or - $(CH_2)n^{4a}$-NH-C(=O)-cyc.Hex(1,4)-CH_2-,
Q^2 represents (maleimid-N-yl), a halogen atom, or -S-S-(2-Pyridyl),
R^Q represents an alkyl group having 1 to 6 carbon atoms, n^4 represents an integer of 1 to 8, n^{4a} represents an integer of 0 to 6, and n^{4b} represents an integer of 1 to 6.]

[0230] In the above, R^Q is an alkyl group having 1 to 6 carbon atoms, and more preferably a methyl group or an ethyl group.

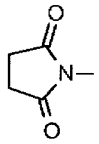
[0231] The alkylene group of L^{1a} may be those having 1 to 10 carbon atoms. The phenylene group may be any of ortho, meta, and para configurations and is more preferably a para- or meta-phenylene group.

[0232] Preferred examples of L^{1a} can include - cyc.Hex(1,4)-CH_2-, -$(CH_2)_5$-NH-C(=O)-cyc.Hex(1,4)-CH_2-,-(CH_2)2_NH-C(=0)-CH2-, -(CH2)5-$_N$H-$_C$(=0)-(CH2)2-, -CH_2-,-$(CH_2)_2$-, -$(CH_2)_3$-, -$(CH_2)_5$-, -(-CH2)$_{10}$-, -(para-Ph)-, - (meta-Ph)-, -(para-Ph)-CH(-CH_3)-, -$(CH_2)_3$-(meta-Ph)-, and -(meta-Ph)-NH-C(=O)-CH_2-.

[0233] Q^1 is preferably (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, Q^2 is preferably (maleimid-N-yl), or -S-S-(2-Pyridyl) can be used when a disulfide bond is to be formed.

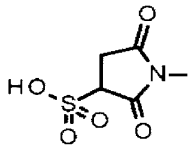
[0234] In the above, (Pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

[Formula 53]



wherein the nitrogen atom as a connecting position, and (3-Sulfo-pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

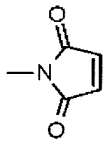
[Formula 54]



wherein the nitrogen atom is a connectiong position, and this sulfonic acid is capable of forming a lithium salt, sodium salt, or potassium salt, and preferably sodium salt,

cyc.Hex(1,4) represents a 1,4-cyclohexylene group, (maleimid-N-yl) is a group represented by the following formula:

[Formula 55]



wherein the nitrogen atom is a connecting position, (2-Pyridyl) represents a 2-pyridyl group, (para-Ph) represents a para-phenylene group, and (meta-Ph) represents a meta-phenylene group.

[0235] Examples of such a compound include sulfosuccinimidyl-4-(N-maleimidylmethyl)cyclohexane-1-carboxylate (sulfo-SMCC), N-succinimidyl-4-(N-maleimidylmethyl)-cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC), κ-maleimidyl undecanoic acid N-succinimidyl ester (KMUA), γ-maleimidyl butyric acid N-succinimidyl ester (GMBS), ε-maleimidyl caproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidylbenzoyl-N-hydroxysuccinimide ester (MBS), N-(α-maleimidylacetoxy)-succinimide ester (AMAS), succinimidyl-6-(β-maleimidylpropionamide)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidylphenyl)-butyrate (SMPB), N-(p-maleimidylphenyl)isocyanate (PMPI), N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), N-succinimidyl 3-(bromoacetamide)propionate (SBAP), N-succinimidyl-3-(2-pyridodithio)propionate (SPDP), and succinimidyloxycarbonyl-a-methyl-a-(2-pyridyldithio)toluene (SMPT).

[0236] Specifically, for example, by reacting 2 to 6 equivalents of SMCC with the antibody (3) in a phosphate buffer of pH 6 to 7 for 1 to 6 hours at room temperature, the active ester of SMCC can react with the antibody to yield the

antibody (3b) having a maleimidyl group. The obtained antibody (3b) can be purified by Common procedure D-2 described below, and used for the next reaction with the compound (2a). Common procedure D-2: Purification of succinimidyl 4-(N-maleimidylmethyl)-cyclohexane-1-carboxylate (SMCC)-derivatized antibody

[0237] NAP-25 column was equilibrated with PBS6.5/EDTA. Reaction solution containing the succinimidyl 4-(N-maleimidylmethyl)-cyclohexane-1-carboxylate (herein, referred to as SMCC)-derivatized antibody was applied in an amount of about 0.5 mL to the NAP-25 column, and then eluted with the buffer in an amount defined by the manufacturer to collect the antibody fraction for purification.

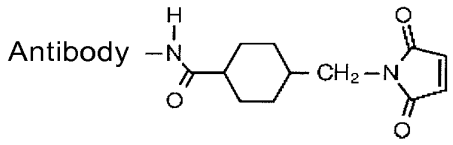
[0238] The amino group of the antibody for connectiong to the linker can be a N-terminal amino group and/or an amino group carried by a lysine residue, but it is not limited thereto. Alternatively, the antibody may be connected to the linker with ester bond formation by use of a hydroxy group carried by a serine residue.

[0239] The reaction of the compound (2a) with the antibody (3b) connected to the linker having a maleimidyl group can be performed in the same manner as the method for reacting the compound (2) with the antibody (3a) having a sulfhydryl group as mentioned in Production method 1.

[0240] For the antibody-drug conjugate (1) prepared, concentration, buffer exchange, purification, and identification of the antibody-drug conjugate (1) by the measurement of antibody concentration and an average number of conjugated drug molecules per antibody molecule can be performed in the same manner as Production method 1.

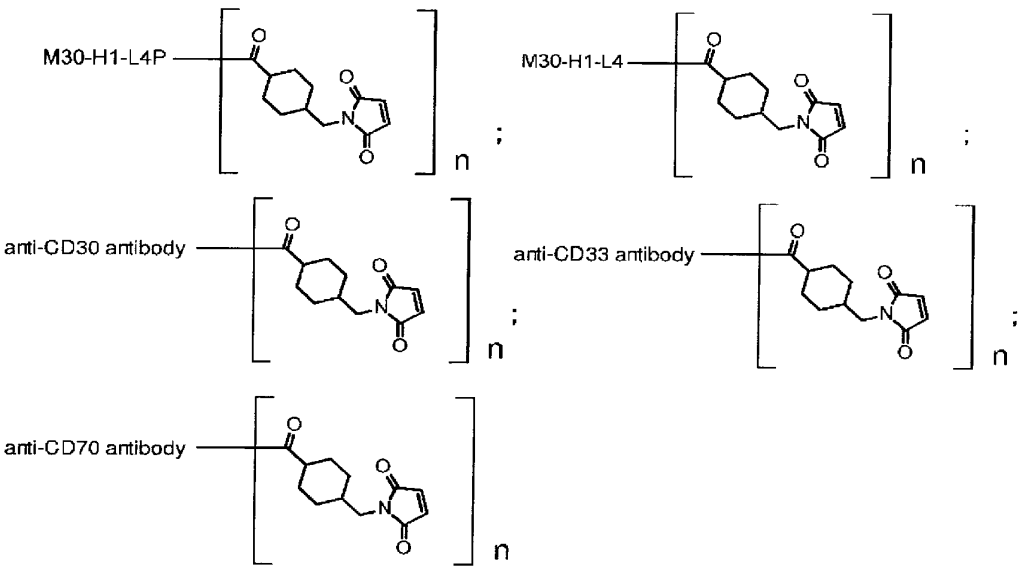
[0241] The compound represented by the formula (3b) in Production method 2 has the following structure (see the following formula; in the structure thereof, "antibody -NH-" originates from an antibody).

[Formula 56]



[0242] A compound which is an intermediate for producing the antibody-drug conjugate of the present invention and has the above structure is as described below (in the formula, n is an integer of 1 to 10, preferably 2 to 8, and more preferably 3 to 8).

[Formula 57]



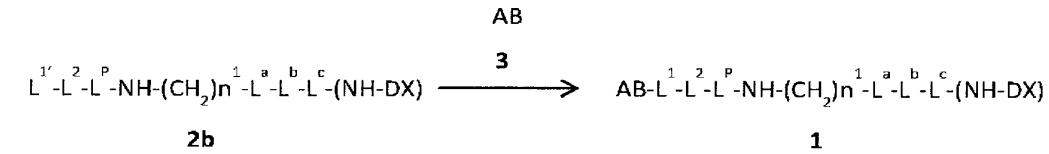
[0243] Further, examples of the compound of the present invention in which the terminal is a mercapto group can include the followings.

HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

3. Production method 3

[0244] The antibody-drug conjugate represented by the formula (1) in which the antibody is conjugated to the drug linker moiety via an amide bond can be produced by a method described below. For example, as for -C(=O)-$(CH_2)n^4$-C(=O)- of L^1, its active ester $L^{1'}$, for example, (Pyrrolidine-2,5-dione-N-yl) -O-C(=O)-$(CH_2)n^4$-C(=O)-, can be preferably used. When L^2 is a single bond, the antibody-drug conjugate (1) can be produced by the following method, for example.

[Formula 58]



[0245] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2b), which is obtainable by the method described below, with the antibody (3).

[0246] The compound (2b) is capable of connecting to the amino group or hydroxyl group of the antibody. The amino group and hydroxyl group of the antibody refer to, as described in Production method 2, for example, a N-terminal amino group carried by the antibody and/or an amino group carried by a lysine residue and a hydroxy group carried by a serine residue, respectively, but they are not limited thereto.

[0247] The compound (2b) is activie ester composed of a N-hydroxysuccinimidyl ester group. Alternatively, other active esters, for example, a sulfosuccinimidyl ester group, N-hydroxyphthalimidyl ester, N-hydroxysulfophthalimidyl ester, ortho-nitrophenyl ester, para-nitrophenyl ester, 2,4-dinitrophenyl ester, 3-sulfonyl-4-nitrophenyl ester, 3-carboxy-4-nitrophenyl ester, and pentafluorophenyl ester, may be used.

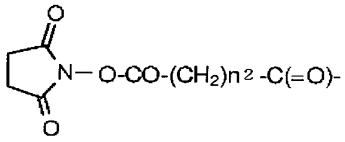
[0248] By using 2 to 20 molar equivalents of the compound (2b) per the antibody (3) in the reaction of the compound (2b) with the antibody(3), the antibody-drug conjugate (1) in which 1 to 10 drug molecules are conjugated per antibody can be produced. Specifically, the solution containing the compound (2b) dissolved therein can be added to a buffer solution containing the antibody (3) for the reaction to yield the antibody-drug conjugate (1). Herein, examples of the buffer solution which may be used include sodium acetate solution, sodium phosphate, and sodium borate. pH for the reaction can be 5 to 9, and more preferably the reaction is performed near pH 7. Examples of the solvent for dissolving the compound (2b) include an organic solvent such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), dimethyl acetamide (DMA), and N-methyl-2-pyridone (NMP). It is sufficient that the organic solvent solution containing the compound (2b) dissolved therein is added at 1 to 20% v/v to a buffer solution containing the antibody (3) for the reaction.

The reaction temperature is 0 to 37°C, more preferably 10 to 25°C, and the reaction time is 0.5 to 20 hours.

[0249] For the produced antibody-drug conjugate (1), concentration, buffer exchange, purification, and identification of the antibody-drug conjugate (1) by the measurement of antibody concentration and an average number of conjugated drug molecules per antibody molecule can be performed in the same manner as Production method 1.

[0250] The moiety (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$(CH_2)n^4$-C(=O)- in Production method 3 has the following structure.

[Formula 59]



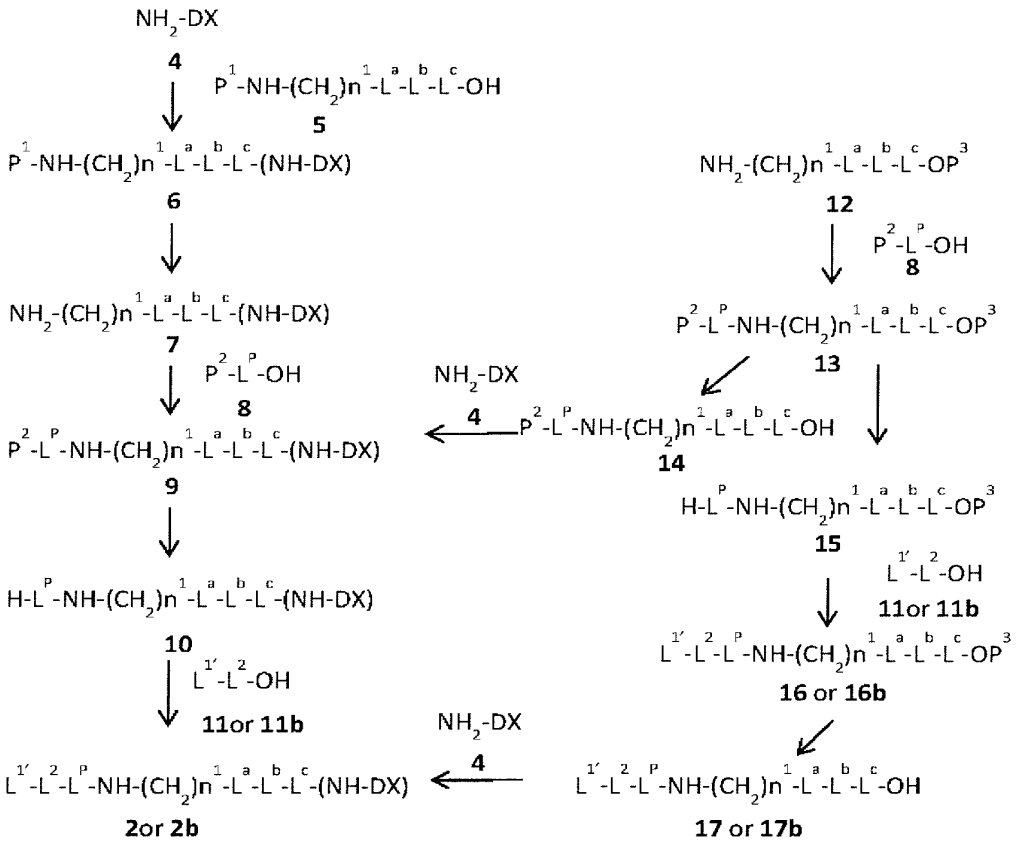
[0251] Examples of the compound of the present invention having the above partial structure can include the followings.

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-0-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

4. Production method 4

[0252] The compound represented by the formula (2) or (2b) as an intermediate used in the previous production method and a pharmacologically acceptable salt thereof can be produced by the following method, for example.

[Formula 60]



[0253] In the formula, L^C is -C(=O)- and is connected to -(NH-DX) with formation of amide bond, $L^{1'}$ represents L^1 structure in which the terminal is converted to a maleimidyl group or a haloacetyl group, or to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$(CH_2)n^4$-C(=O)-, and P^1, P^2, and P^3 each represents a protecting group.

[0254] The compound (6) can be produced by derivatizing the carboxylic acid (5) into an active ester, mixed acid anhydride, acid halide, or the like and reacting it with NH_2-DX [indicating exatecan; chemical name: (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-10,13(9H,15H)-dione] (4) or a pharmacologically acceptable salt thereof.

[0255] Reaction reagents and conditions that are commonly used for peptide synthesis can be employed for the reaction. There are various kinds of active ester. For example, it can be produced by reacting phenols such as p-nitrophenol, N-hydroxy benzotriazole, N-hydroxy succinimide, or the like, with the carboxylic acid (5) using a condensing agent such as N,N'-dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. Further, the active ester can be also produced by a reaction of the carboxylic acid (5) with pentafluorophenyl trifluoroacetate or the like; a reaction of the carboxylic acid (5) with 1-benzotriazolyl oxytripyrrolidinophosphonium hexafluorophosphite; a reaction of the carboxylic acid (5) with diethyl cyanophosphonate (salting-in method); a reaction of the carboxylic acid (5) with triphenylphosphine and 2,2'-dipyridyl disulfide (Mukaiyama's method); a reaction of the carboxylic acid (5) with a triazine derivative such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM); or the like. Further, the reaction can be also performed by, e.g., an acid halide method by which the carboxylic acid (5) is treated with acid halide such as thionyl chloride and oxalyl chloride in the presence of a base. By reacting the active ester, mixed acid anhydride, or acid halide of the carboxylic acid (5) obtained accordingly with the compound (4) in the presence of a suitable base in an inert solvent at -78°C to 150°C, the compound (6) can be produced. (Meanwhile, "inert solvent" indicates a solvent which does not inhibit a reaction for which the solvent is used.)

[0256] Specific examples of the base used for each step described above include carbonate of an alkali metal or an alkali earth metal, an alkali metal alkoxide, hydroxide or hydride of an alkali metal including sodium carbonate, potassium

carbonate, sodium ethoxide, potassium butoxide, sodium hydroxide, potassium hydroxide, sodium hydride, and potassium hydride, organometallic base represented by an alkyl lithium including n-butyl lithium, dialkylamino lithium including lithium diisopropylamide; organometallic base of bissilylamine including lithium bis(trimethylsilyl)amide; and organic base including pyridine, 2,6-lutidine, collidine, 4-dimethylaminopyridine, triethylamine, N-methyl morpholine, diisopropylethylamine, and diazabicyclo[5.4.0]undec-7-ene (DBU).

[0257] Examples of the inert solvent which is used for the reaction of the present invention include a halogenated hydrocarbon solvent such as dichloromethane, chloroform, and carbon tetrachloride; an ether solvent such as tetrahydrofuran, 1,2-dimethoxyethane, and dioxane; an aromatic hydrocarbon solvent such as benzene and toluene; and an amide solvent such as N,N-dimethyl formamide, N,N-dimethyl acetamide, and N-methylpyrrolidin-2-one. In addition to them, a sulfoxide solvent such as dimethyl sulfoxide and sulfolane; and a ketone solvent such as acetone and methyl ethyl ketone may be used depending on a case.

[0258] The hydroxy group, carboxy group, amino group, or the like of L^a and L^b in the compound (6) may be protected with a protecting group which is commonly used in organic compound synthesis, as mentioned later. Specifically, examples of the protecting group for a hydroxyl group include an alkoxymethyl group such as methoxymethyl group; an arylmethyl group such as benzyl group, 4-methoxybenzyl group, and triphenylmethyl group; an alkanoyl group such as acetyl group; an aroyl group such as benzoyl group; and a silyl group such as tert-butyl diphenylsilyl group. Carboxy group can be protected, e.g., as an ester with an alkyl group such as methyl group, ethyl group, and tert-butyl group, an allyl group, or an arylmethyl group such as benzyl group. Amino group can be protected with a protecting group for an amino group which is generally used for peptide synthesis, for example, an alkyloxy carbonyl group such as tert-butyloxy carbonyl group, methoxycarbonyl group, and ethoxycarbonyl group; an arylmethyl group such as allyloxycarbonyl, 9-fluorenylmethyloxy carbonyl group, benzyloxy carbonyl group, paramethoxybenzyloxy carbonyl group, and para (or ortho)nitroybenzyloxy carbonyl group; an alkanoyl group such as acetyl group; an arylmethyl group such as benzyl group and triphenyl methyl group; an aroyl group such as benzoyl group; and an aryl sulfonyl group such as 2,4-dinitrobenzene sulfonyl group or orthonitrobenzene sulfonyl group. Protection with and deprotection of the protecting group can be performed according to a method commonly carried out.

[0259] As for the protecting group P^1 for the terminal amino group of the compound (6), a protecting group for an amino group which is generally used for peptide synthesis, for example, tert-butyloxy carbonyl group, 9-fluorenylmethyloxy carbonyl group, and benzyloxy carbonyl group, can be used. Examples of the other protecting group for an amino group include an alkanoyl group such as acetyl group; an alkoxycarbonyl group such as methoxycarbonyl group and ethoxycarbonyl group; an arylmethoxy carbonyl group such as paramethoxybenzyloxy carbonyl group, and para (or ortho)nitroybenzyloxy carbonyl group; an arylmethyl group such as benzyl group and triphenyl methyl group; an aroyl group such as benzoyl group; and an aryl sulfonyl group such as 2,4-dinitrobenzene sulfonyl group and orthonitrobenzene sulfonyl group. The protecting group P^1 can be selected depending on, e.g., properties of a compound having an amino group to be protected.

[0260] By deprotecting the protecting group P^1 for the terminal amino group of the compound (6) obtained, the compound (7) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0261] The compound (9) can be produced by derivatizing the peptide carboxylic acid (8) having the N terminal protected with P^2 into an active ester, mixed acid anhydride, or the like and reacting it with the compound (7) obtained. The reaction conditions, reagents, base, and inert solvent used for forming a peptide bond between the peptide carboxylic acid (8) and the compound (7) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^2 can be suitably selected from those described for the protecting group of the compound (6), and the selection can be made based on, e.g., the properties of the compound having an amino group to be protected. As it is generally used for peptide synthesis, by repeating sequentially the reaction and deprotection of the amino acid or peptide constituting the peptide carboxylic acid (8) for elongation, the compound (9) can be also produced.

[0262] By deprotecting P^2 as the protecting group for the amino group of the compound (9) obtained, the compound (10) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0263] It is possible to produce the compound (2) or (2b) by derivatizing the carboxylic acid (11) or (11b) into an active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (10) obtained. The reaction conditions, reagents, base, and inert solvent used for forming a peptide bond between the carboxylic acid (11) or (11b) and the compound (10) can be suitably selected from those described for the synthesis of the compound (6).

[0264] The compound (9) can be also produced by the following method, for example.

[0265] The compound (13) can be produced by derivatizing the peptide carboxylic acid (8) having the N terminal protected with P^2 into active ester, mixed acid anhydride, or the like and reacting it with the amine compound (12) having the carboxy group protected with P^3 in the presence of a base. The reaction conditions, reagents, base, and inert solvent used for forming a peptide bond between the peptide carboxylic acid (8) and the compound (12) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^2 for the amino group of the compound (13) can be suitably selected from those described for the protecting group of the compound (6). As for the protecting group P^3 for a carboxy group, a protecting group commonly used as a protecting group for a carboxy group in organic

synthetic chemistry, in particular, peptide synthesis can be used. Specifically, it can be suitably selected from those described for the protecting group of the compound (6), for example, esters with an alkyl group such as a methyl group, an ethyl group, or a tert-butyl, allyl esters, and benzyl esters. In such case, it is necessary that the protecting group for an amino group and the protecting group for a carboxy group can be removed by a different method or different conditions. For example, a representative example includes a combination in which P^2 is a tert-butyloxy carbonyl group and P^3 is a benzyl group. The protecting groups can be selected from the aforementioned ones depending on, e.g., the properties of a compound having an amino group and a carboxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group.

[0266] By deprotecting the protecting group P^3 for the carboxy group of the compound (13) obtained, the compound (14) can be produced. Reagents and conditions are selected depending on the protecting group.

[0267] The compound (9) can be produced by derivatizing the compound (14) obtained into active ester, mixed acid anhydride, acid halide, or the like and reacting with the compound (4) in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and inert solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6) .

[0268] The compound (2) or (2b) can be also produced by the following method, for example.

[0269] By deprotecting the protecting group P^2 for the amino group of the compound (13), the compound (15) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0270] The compound (16) or (16b) can be produced by derivatizing the carboxylic acid derivative (11) or (11b) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (15) obtained in the presence of a base. The reaction conditions, reagents, base, and inert solvent used for forming an amide bond between the peptide carboxylic acid (11) or (11b) and the compound (15) can be suitably selected from those described for the synthesis of the compound (6).

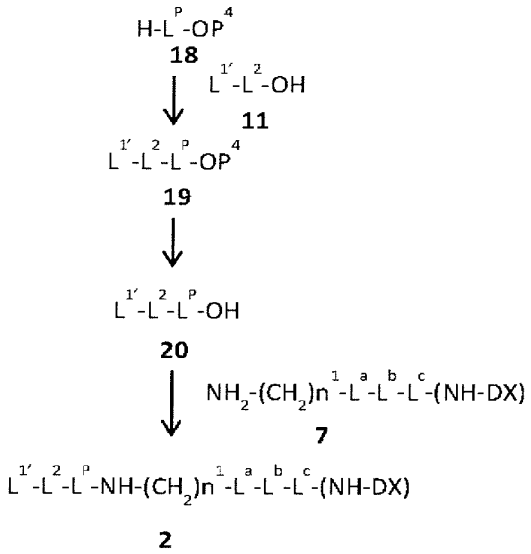
[0271] By deprotecting the protecting group for the carboxy group of the compound (16) or (16b) obtained, the compound (17) or (17b) can be produced. It can be carried out similar to deprotecting carboxy group for producing the compound (14).

[0272] The compound (2) or (2b) can be produced by derivatizing the compound (17) or (17b) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and inert solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

5. Production method 5

[0273] The compound represented by the formula (2) of an intermediate can be also produced by the following method.

[Formula 61]



[0274] In the formula, $L^{1'}$ corresponds to L^1 having a structure in which the terminal is converted to a maleimidyl group or a haloacetyl group, and P^4 represents a protecting group.

[0275] The compound (19) can be produced by derivatizing the compound (11) into active ester, mixed acid anhydride, or the like and reacting it with the peptide carboxylic acid (18) having the C terminal protected with P^4 in the presence of a base. The reaction conditions, reagents, base, and inert solvent used for forming a peptide bond between the peptide carboxylic acid (18) and the compound (11) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^4 for the carboxy group of the compound (18) can be suitably selected from those described for the protecting group of the compound (6) .

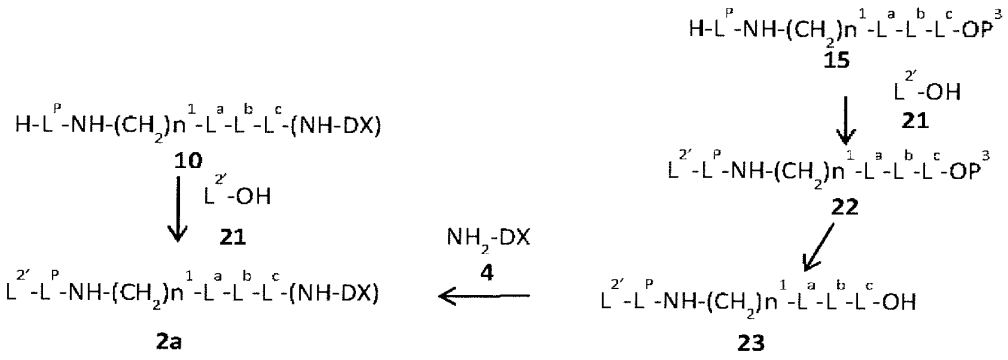
[0276] By deprotecting the protecting group for the carboxy group of the compound (19) obtained, the compound (20) can be produced. It can be performed similar to the deprotection of the carboxy group for producing the compound (14).

[0277] The compound (2) can be produced by derivatizing the compound (20) obtained into active ester, mixed acid anhydride, or the like and reacting it with the compound (7). For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and inert solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

6. Production method 6

[0278] The production intermediate (2a) described in Production method 2 in which $L^{2'}$ corresponds to L^2 having a structure in which the terminal is converted to a mercaptoalkanoyl group can be produced by the following method.

[Formula 62]



[0279] The compound (2a) can be produced by derivatizing the carboxylic acid (21) having a terminal mercapto group into active ester, mixed acid anhydride, or the like and reacting it with the compound (10). For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and inert solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (4) .

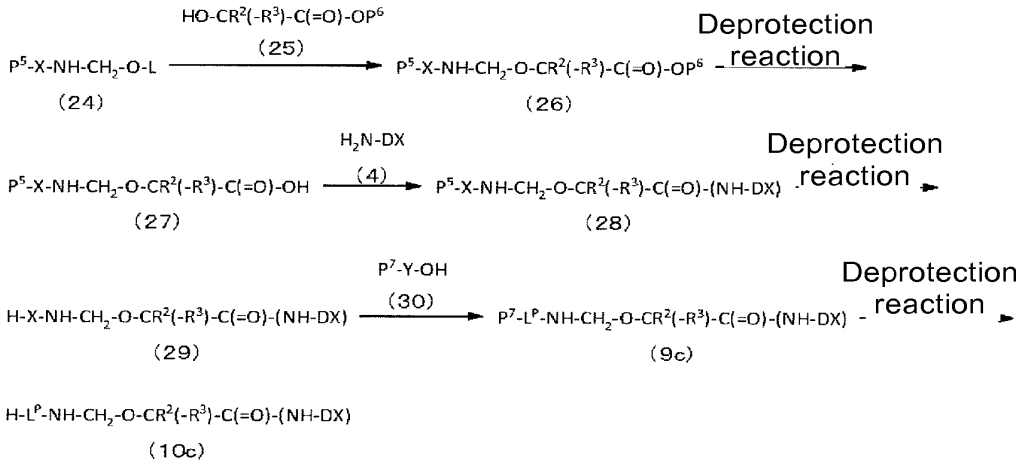
[0280] Further, the compound (23) can be produced by derivatizing the compound (21) into active ester, mixed acid anhydride, acid halide, or the like, reacting it with the compound (15), and deprotecting the protecting group for the carboxy group of the compound (22) obtained.

[0281] The compound (2a) can be produced by derivatizing the compound (23) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and inert solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6) .

7. Production method 7

[0282] Hereinbelow, the method for producing the compound (10c) having $n^1 = 1$, $L^a = O$, and $L^b = CR^2(-R^3)$ in the production intermediate (10) described in Production method 4 is described in detail. The compound represented by the formula (10c), a salt or a solvate thereof can be produced according to the following method, for example.

[Formula 63]



[0283] In the formula, L^P, R^2, and R^3 are as defined above, L represents an acetyl group, a hydrogen atom, or the like, X and Y each represent an oligopeptide consisting of 1 to 3 amino acids, P^5 and P^7 each represent a protecting group for an amino group, and P^6 represents a protecting group for a carboxy group.

[0284] A compound represented by the formula (24) can be produced by using or applying the method described in Japanese Patent Laid-Open No. 2002-60351 or the literature (J. Org. Chem., Vol. 51, page 3196, 1986), and if necessary, by removing the protecting groups or modifying the functional groups. Alternatively, it can be also obtained by treating an amino acid with a protected terminal amino group or acid amide of oligopeptide with protected amino group with aldehyde or ketone.

[0285] By reacting the compound (24) with the compound (25) having a hydroxyl group at a temperature ranging from under cooling to room temperature in an inert solvent in the presence of an acid or a base, the compound (26) can be produced. Examples of the acid which may be used include inorganic acid such as hydrofluoric acid, hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid, and boric acid; an organic acid such as acetic acid, citric acid, paratoluene sulfonic acid, and methane sulfonic acid; and a Lewis acid such as tetrafluoroborate, zinc chloride, tin chloride, aluminum chloride, and iron chloride. Paratoluene sulfonic acid is particularly preferable. As for the base to be used, any one of the aforementioned base can be suitably selected and used. Preferred examples thereof include an alkali metal alkoxide such as potassium tert-butoxide, an alkali metal hydroxide such as sodium hydroxide and potassium hydroxide; alkali metal hydride such as sodium hydride and potassium hydride; organometallic base represented by dialkylamino lithium such as lithium diisopropylamide; and organometallic base of bissilylamine such as lithium bis(trimethylsilyl)amide. Examples of the solvent to be used for the reaction include an ether solvent such as tetrahydrofuran and 1,4-dioxane; and an aromatic hydrocarbon solvent such as benzene and toluene. Those solvents can be prepared as a mixture with water. Further, the protecting group for an amino group as exemplified by P^5 is not particularly limited if it is a group commonly used for protection of an amino group. Representative examples include the protecting groups for an amino group that are described in Production method 4. However, in the present reaction, the protecting group for an amino group as exemplified by P^5 may be cleaved off. In such case, it is necessary to perform a reaction with a suitable reagent for protecting an amino group as it may be required.

[0286] The compound (27) can be produced by removing the protecting group P^6 of the compound (26). Herein, although the representative examples of the protecting group for a carboxy group as exemplified by P^6 are described in Production method 4, it is desirable in this case that the protecting group P^5 for an amino group and the protecting group P^6 for a carboxy group are the protecting groups that can be removed by a different method or different conditions. For example, a representative example includes a combination in which P^5 is a 9-fluorenylmethyloxy carbonyl group and P^6 is a benzyl group. The protecting groups can be selected depending on, e.g., the properties of a compound having an amino group and a carboxy group to be protected. For removal of the protecting groups, reagents and conditions are selected depending on the protecting group.

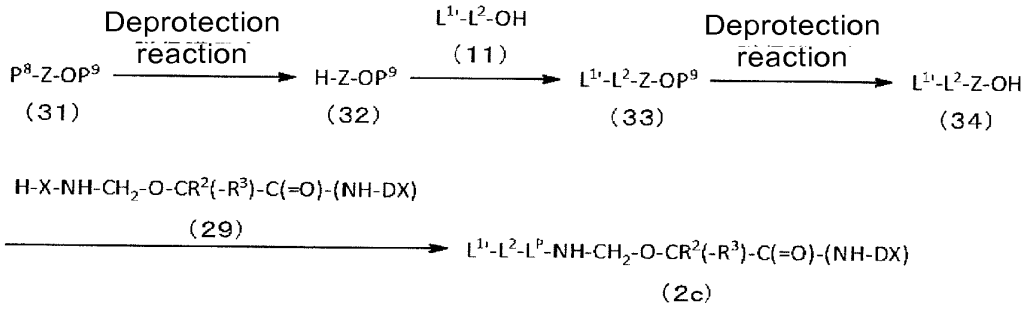
[0287] The compound (29) can be produced by derivatizing the carboxylic acid (27) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) and a pharmacologically acceptable salt thereof to produce the compound (28) followed by removing the protecting group P^5 of the compound (28) obtained. For the reaction between the compound (4) and the carboxylic acid (27) and the reaction for removing the protecting group P^6, the same reagents and reaction conditions as those described for Production method 4 can be used.

[0288] The compound (10c) can be produced by reacting the compound (29) with an amino acid with protected terminal amino group or the oligopeptide (30) with protected amino group to produce the compound (9c) and removing the protecting group P^7 of the compound (9c) obtained. The protecting group for an amino group as exemplified by P^7 is not particularly limited if it is generally used for protection of an amino group. Representative examples thereof include the protecting groups for an amino group that are described in Production method 4. For removing the protecting group, reagents and conditions are selected depending on the protecting group. For the reaction between the compound (29) and the compound (30), reaction reagents and conditions that are commonly used for peptide synthesis can be employed. The compound (10c) produced by the aforementioned method can be derivatized into the compound (1) of the present invention according to the method described above.

8. Production method 8

[0289] Hereinbelow, the method for producing the compound (2c) having $n^1 = 1$, $L^a = O$, and $L^b = CR^2(-R^3)$ in the production intermediate (2) described in Production method 4 is described in detail. The compound represented by the formula (2c), a salt or a solvate thereof can be produced according to the following method, for example.

[Formula 64]



[0290] In the formula, L^1, L^2, L^P, R^2, and R^3 are as defined above, Z represents an oligopeptide consisting of 1 to 3 amino acids, P^8 represents a protecting group for an amino group, and P^9 represents a protecting group for a carboxy group.

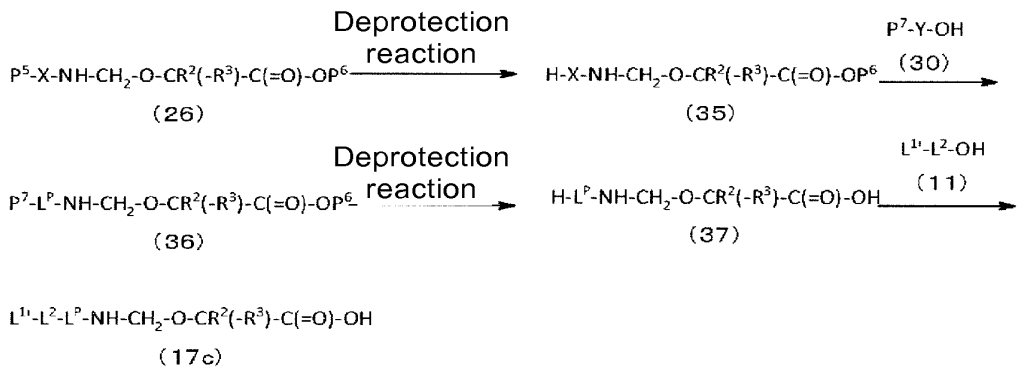
[0291] The compound (33) can be produced by removing the protecting group P^8 of the amino acid or oligopeptide (31) with protected terminal amino group and carboxy group to produce the compound (32) and reacting the obtained amine form (32) with the compound (11). The protecting group for an amino group as exemplified by P^8 is not particularly limited if it is a group commonly used for protection of an amino group. Representative examples include the protecting groups for an amino group that are described in Production method 4. Further, for removing the protecting group P^8, reagents and conditions can be selected depending on the protecting group. For the reaction between the compound (32) and the carboxylic acid (11), the same reagents and reaction conditions as those described for Production method 4 can be used.

[0292] The production intermediate (2c) can be produced by removing the protecting group P^9 of the compound (33) to produce the compound (34) and reacting the obtained carboxylic acid (34) with the compound (29). The representative examples of the protecting group for a carboxy group as exemplified by P^8 are described in Production method 4. For the deprotection reaction thereof, the same reagents and reaction conditions as those described for Production method 4 can be used. For the reaction between the compound (29) and the carboxylic acid (34), reaction reagents and conditions that are generally used for peptide synthesis can be also used. The compound (2c) produced by the aforementioned method can be derivatized into the compound (1) of the present invention according to the method described above.

9. Production method 9

[0293] Hereinbelow, the method for producing the compound (17c) having $n^1 = 1$, $L^a = O$, and $L^b = CR^2(-R^3)$ in the production intermediate (17) described in Production method 4 is described in detail. The compound represented by the formula (17c), a salt or a solvate thereof can be also produced according to the following method, for example.

[Formula 65]



[0294] In the formula, L^1, L^2, L^P, R^2, R^3, X, Y, P^5, P^6, and P^7 are as defined above.

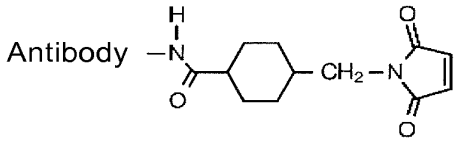
[0295] The compound (36) can be produced by deprotecting the protecting group P^5 for the amino group of the compound (26) with protected terminal amino group and carboxy group to produce the compound (35) and reacting the obtained amine form (35) with the oligopeptide (30) with protected terminal amino group or protected amino group. The protecting group for an amino group as exemplified by P^5 is not particularly limited if it is a group commonly used for protection of an amino group. Representative examples include the protecting groups for an amino group that are described in Production method 4. Further, for removing the protecting group P^5, reagents and conditions can be selected depending on the protecting group. Herein, although representative examples of the protecting group for a carboxy group as exemplified by P^6 and the protecting group for an amino group as exemplified by P^7 include the protecting groups for a carboxy group and an amino group that are described in Production method 4, it is desirable that the protecting group P^6 for a carboxy group and the protecting group P^7 for an amino group are the protecting groups that can be removed by the same method or the same conditions. For example, a representative example includes a combination in which P^6 is a benzyl ester group and P^7 is a benzyloxy carbonyl group.

[0296] The compound (37) can be produced by removing the protecting group P^6 for the carboxy group of the compound (36) and the protecting group P^7 for the amino group of the compound (36). The compound (37) can be also produced by sequentially removing the protecting group P^6 for the carboxy group and the protecting group P^7 for the amino group, or the compound (37) can be produced by removing at once both of the protecting groups P^6 and P^7 that can be removed by the same method or the same conditions.

[0297] The compound (17c) can be produced by reacting the obtained compound (37) with the compound (11). For the reaction between the compound (37) and the compound (11), the same reagents and reaction conditions as those described for Production method 4 can be used.

[0298] In the foregoing, the compound represented by the following formula:

[Formula 66]



is described as a production intermediate useful for producing the antibody-drug conjugate of the present invention. In addition, a group of compounds represented by the following formula:

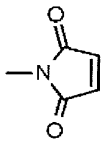
$$Q\text{-}(CH_2)n^Q\text{-}C(=O)\text{-}L^{2a}\text{-}L^P\text{-}NH\text{-}(CH_2)n^1\text{-}L^a\text{-}L^b\text{-}L^c\text{-}(NH\text{-}DX)$$

are also compounds that serve as production intermediates useful for producing the antibody-drug conjugate of the present invention.

[0299] Specifically, in the above formula, Q is (maleimid-N-yl)-, HS-, X-CH_2-C(=O)-NH-, or (pyrrolidine-2,5-dione-N-yl)-O-C(=O)-,

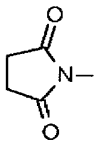
X is a bromine atom or an iodine atom,
n^Q is an integer of 2 to 8,
L^{2a} represents -NH-(CH_2-CH_2-O)n^5-CH_2-CH_2-C(=O)- or a single bond,
wherein n^5 represents an integer of 1 to 6,
L^P represents a peptide residue consisting of 2 to 7 amino acids,
n^1 represents an integer of 0 to 6,
L^a represents -C(=O)-NH-, -NR^1-(CH_2)n^7-, -O-, or a single bond,
wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, - (CH_2) n^8-COOH, or -(CH_2)n^9-OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,
L^b represents -CR^2(-R^3-, -O-, -NR^4-, or a single bond,
wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH_2)n^a-NH_2, - (CH_2)n^b-COOH, or -(CH_2)n^c-OH, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other,
L^C represents -CH_2- or -C(=O)-,
(maleimid-N-yl)- is a group having a structure represented by the following formula:

[Formula 67]



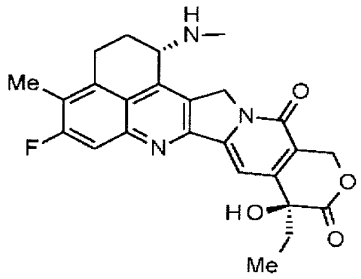
(in the formula, the nitrogen atom is the connecting position), (Pyrrolidine-2,5-dione-N-yl)- is a group having a structure represented by the following formula:

[Formula 68]



(in the formula, the nitrogen atom is the connecting position), -(NH-DX) is a group having a structure represented by the following formula:

[Formula 69]



(in the formula, the nitrogen atom of the amino group at position 1 is the connceting position).

[0300] A compound in which L^C is -C(=O)- is preferred as a production intermediate.

[0301] As for the peptide residue of L^P, a compound of an amino acid residue consisting of an amino acid selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid is preferred as a production intermediate. Among those peptide residues, a compound in which L^P is a peptide residue consisting of 4 amino acids is preferred as a production intermediate. More specifically, a compound in which L^P is -GGFG- is preferred as a production intermediate.

[0302] Further, as for the -NH-$(CH_2)n^1$-L^a-L^b-, a compound of -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is preferred as a production intermediate. A compound of NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-$(CH_2)_2$-O-CH_2-C(=O)-is more preferred.

[0303] As for n^Q, a compound in which it is an integer of 2 to 6 is preferred as a production intermediate.

[0304] A compound in which L^{2a} is a single bond or n^5 is an integer of 2 to 4 is preferred as a production intermediate.

[0305] When Q is (maleimid-N-yl) -, a compound in which n^Q is an integer of 2 to 5, L^{2a} is a single bond, and -NH-$(CH_2)n^1$-L^a-L^b- is -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or - NH-CH_2CH_2-O-CH_2- is preferred as a production intermediate. A compound in which -NH-$(CH_2)n^1$-L^a-L^b- is -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is more preferred. A compound in which n^Q is an integer of 2 or 5 is further preferred.

[0306] Also, when Q is (maleimid-N-yl)-, a compound in which n^Q is an integer of 2 to 5, L^{2a} is -NH-$(CH_2$-CH_2-O$)n^5$-CH_2-CH_2-C(=O)-, n^5 is an integer of 2 to 4, and - NH-$(CH_2)n^1$-L^a-L^b- is -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or - NH-CH_2CH_2-O-CH_2- is pre-

ferred as a production intermediate. A compound in which n^5 is an integer of 2 or 4 is more preferred. A compound in which -NH-$(CH_2)n^1$-L^a-L^b- is -NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is further preferred.

[0307] When Q is HS-, a compound in which n^Q is an integer of 2 to 5, L^{2a} is a single bond, and -NH-(CH_2) n^1-L^a-L^b- is -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or - NH-CH_2CH_2-O-CH_2- is preferred as a production intermediate. A compound in which -NH-$(CH_2)n^1$-L^a-L^b- is -NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is more preferred.

[0308] When Q is X-CH_2-C(=O)-NH-, a compound in which X is a bromine atom is preferred as a production intermediate. A compound in which n^Q is an integer of 2 to 8 is preferred, also a compound in which L^{2a} is a single bond is preferred, and a compound in which -NH-$(CH_2)n^1$-L^a-L^b- is -NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is preferred as a production intermediate.

[0309] When Q is (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, a compound in which n^Q is an integer of 2 to 5, L^{2a} is a single bond, and -NH-$(CH_2)n^1$-L^a-L^b- is -NH-CH_2CH_2-, -NH-$CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2$-, -NH-$CH_2CH_2CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is preferred as a production intermediate. A compound in which -NH-$(CH_2)n^1$-L^a-L^b- is -NH-$CH_2CH_2CH_2$-, -NH-CH_2-O-CH_2-, or -NH-CH_2CH_2-O-CH_2- is more preferred.

[0310] More specifically, the followings are compounds preferred as production intermediates.

(maleimid-N-yl)-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl) -CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl) -$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl) -$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)
(maleimid-N-yl)-CH_2CH_2-C(=O)-NH-CH_2CH_2O-CH_2CH_2O-CH_2CH_2O-
CH_2CH_2O-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
HS-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
Br-CH_2-C(=O)-NH-CH_2CH_2-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-$CH_2CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-$CH_2CH_2CH_2$-C(=O)-(NH-DX)

[0311] Meanwhile, the antibody-drug conjugate of the present invention, when it is left in air or recrystallized, may absorb moisture to have adsorption water or turn into a hydrate, and such a compound and a salt containing water are also included in the present invention.

[0312] A compound labeled with various radioactive or non-radioactive isotopes is also included in the present invention. One or more atoms constituting the antibody-drug conjugate of the present invention may contain an atomic isotope at non-natural ratio. Examples of the atomic isotope include deuterium (^2H), tritium (^3H), iodine-125 (^{12}I), and carbon-14 (^{14}C). Further, the compound of the present invention may be radioactive-labeled with a radioactive isotope such as tritium (^3H), iodine-125 (^{125}I), carbon-14 (^{14}C), copper-64 (^{64}Cu), zirconium-89 (^{89}Zr), iodine-124 (^{124}I), fluorine-18 (^{18}F), indium-111 (^{111}I), carbon-11 (^{11}C) and iodine-131 (^{131}I) . The compound labeled with a radioactive isotope is useful as a therapeutic or prophylactic agent, a reagent for research such as an assay reagent and an agent for diagnosis such as an in vivo diagnostic imaging agent. Without being related to radioactivity, any isotope variant type of the antibody-drug conjugate of the present invention is within the scope of the present invention.

[Drugs]

[0313] The antibody-drug conjugate of the present invention exhibits a cytotoxic activity against cancer cells, and thus, it can be used as a drug, particularly as a therapeutic agent and/or prophylactic agent for cancer.

[0314] Examples of the cancer type to which the antibody-drug conjugate of the present invention is applied include lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer, however, it is not limited to them as long as it is a cancer cell expressing, in a cancer cell as a treatment subject, a protein which the antibody within the antibody-drug conjugate can recognize.

[0315] The antibody-drug conjugate of the present invention can be preferably administered to a mammal, but it is more preferably administered to a human.

[0316] Substances used in a pharmaceutical composition containing antibody-drug conjugate of the present invention can be suitably selected and applied from formulation additives or the like that are generally used in the art, in view of

the dosage or administration concentration.

[0317] The antibody-drug conjugate of the present invention can be administered as a pharmaceutical composition containing at least one pharmaceutically suitable ingredient.

For example, the pharmaceutical composition above typically contains at least one pharmaceutical carrier (for example, sterilized liquid). for example, water and oil (petroleum oil and oil of animal origin, plant origin, or synthetic origin(the oil may be, for example, peanut oil, soybean oil, mineral oil, sesame oil or the like)). Water is a more typical carrier when the pharmaceutical composition above is intravenously administered. Saline solution, an aqueous dextrose solution, and an aqueous glycerol solution can be also used as a liquid carrier, in particular, for an injection solution. A suitable pharmaceutical vehicle is known in the art. If desired, the composition above may also contain a trace amount of a moisturizing agent, an emulsifying agent, or a pH buffering agent. Examples of suitable pharmaceutical carrier are disclosed in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulations correspond to an administration mode.

[0318] Various delivery systems are known and they can be used for administering the antibody-drug conjugate of the present invention. Examples of the administration route include intradermal, intramuscular, intraperitoneal, intravenous, and subcutaneous routes, but not limited thereto. The administration can be made by injection or bolus injection, for example. According to a specific preferred embodiment, the administration of the antibody- drug conjugate is performed by injection. Parenteral administration is a preferred administration route.

[0319] According to a representative embodiment, the pharmaceutical composition is prescribed, as a pharmaceutical composition suitable for intravenous administration to human, according to the conventional procedures. The composition for intravenous administration is typically a solution in a sterile and isotonic aqueous buffer solution. If necessary, the drug may contain a solubilizing agent and local anesthetics to alleviate pain at injection site (for example, lignocaine). Generally, the ingredient above is provided individually as any one of lyophilized powder or an anhydrous concentrate contained in a container which is obtained by sealing in an ampoule or a sachet having an amount of the active agent or as a mixture in a unit dosage form. When the drug is to be administered by injection, it may be administered from an injection bottle containing water or saline of sterile pharmaceutical grade. When the drug is administered by injection, an ampoule of sterile water or saline for injection may be provided such that the aforementioned ingredients are admixed with each other before administration.

[0320] The pharmaceutical composition of the present invention may be a pharmaceutical composition containing only the antibody-drug conjugate of the present invention or a pharmaceutical composition containing the antibody-drug conjugate and at least one cancer treating agent other than the conjugate. The antibody-drug conjugate of the present invention can be administered with other cancer treating agent. The anti-cancer effect may be enhanced accordingly. Another anti-cancer agent used for such purpose may be administered to an individual simultaneously with, separately from, or subsequently to the antibody-drug conjugate, and it may be administered while varying the administration interval for each. Examples of the cancer treating agent include abraxane, carboplatin, cisplatin, gemcitabine, irinotecan (CPT-11), paclitaxel, pemetrexed, sorafenib, vinorelbine, drugs described in International Publication No. WO 2003/038043, LH-RH analogues (leuprorelin, goserelin, or the like), estramustine phosphate, estrogen antagonist (tamoxifen, raloxifene, or the like), and an aromatase inhibitor (anastrozole, letrozole, exemestane, or the like), but it is not limited as long as it is a drug having an antitumor activity.

[0321] The pharmaceutical composition can be formulated into a lyophilization formulation or a liquid formulation as a formulation having desired composition and required purity. When formulated as a lyophilization formulation, it may be a formulation containing suitable formulation additives that are used in the art. Also for a liquid formulation, it can be formulated as a liquid formulation containing various formulation additives that are used in the art.

[0322] Composition and concentration of the pharmaceutical composition may vary depending on administration method. However, the antibody-drug conjugate contained in the pharmaceutical composition of the present invention can exhibit the pharmaceutical effect even at a small dosage when the antibody-drug conjugate has higher affinity for an antigen, that is, higher affinity (= lower Kd value) in terms of the dissociation constant (that is, Kd value) for the antigen. Thus, for determining dosage of the antibody-drug conjugate, the dosage can be determined in view of a situation relating to the affinity between the antibody-drug conjugate and antigen. When the antibody-drug conjugate of the present invention is administered to a human, for example, about 0.001 to 100 mg/kg can be administered once or administered several times with an interval of one time for 1 to 180 days.

[Examples]

[0323] The present invention is specifically described in view of the examples shown below. However, the present invention is not limited to them. Further, it is by no means interpreted in a limited sense. Further, unless specifically described otherwise, the reagent, solvent, and starting material described in the specification can be easily obtained from a commercial supplier.

Reference Example 1 M30-H1-L4 antibody

[0324] Of humanized antibodies of an anti-B7-H3 antibody, an antibody composed of a heavy chain consisting of an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain consisting of an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 16 was produced in accordance with a method known in the art to yield humanized anti-B7-H3 antibody designated as an M30-H1-L4 antibody (or simply referred to as "M30-H1-L4").

Reference Example 2 M30-H1-L4P antibody

[0325] The modification of a glycan bonded to the M30-H1-L4 antibody obtained above was regulated by defucosylation in accordance with a method known in the art to yield antibody with the regulated modification of a glycan designated as an M30-H1-L4P antibody (or simply referred to as "M30-H1-L4P").

Reference Example 3 Anti-CD30 antibody

[0326] An anti-CD30 antibody was produced with reference to National Publication of International Patent Application No. 2005-506035. Its sequence is shown in SEQ ID NOs: 27 and 28.

Reference Example 4 Anti-CD33 antibody

[0327] An anti-CD33 antibody was produced with reference to Japanese Patent Laid-Open No. 8-48637. Its sequence is shown in SEQ ID NOs: 29 and 30.

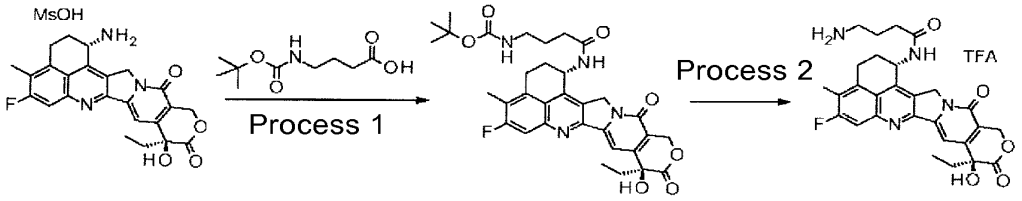
Reference Example 5 Anti-CD70 antibody

[0328] An anti-CD70 antibody was produced with reference to National Publication of International Patent Application No. 2008-538292. Its sequence is shown in SEQ ID NOs: 31 and 32.

Example 1 4-Amino-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]butanamide

[0329]

[Formula 70]



Process 1: Tert-butyl (4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-l-yl]amino}-4-oxobutyl)carbamate

[0330] 4-(Tert-butoxycarbonylamino)butanoic acid (0.237 g, 1.13 mmol) was dissolved in dichloromethane (10 mL), N-hydroxysuccinimide (0.130 g, 1.13 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.216 g, 1.13 mmol) were added, and stirred for 1 hour. The reaction solution was added dropwise to an N,N-dimethyl formamide solution (10 mL) charged with mesylate of the compound (4) (0.500 g, 0.94 mmol) and triethylamine (0.157 mL, 1.13 mmol), and stirred for 1 day at room temperature. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform-chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound (0.595 g, quantitative).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.2 Hz), 1.31 (9H, s), 1.58 (1H, t, J=7.2 Hz), 1.66 (2H, t, J=7.2 Hz), 1.82-1.89 (2H, m), 2.12-2.21 (3H, m), 2.39 (3H, s), 2.92 (2H, t, J=6. 5 Hz), 3.17 (2H, s), 5.16 (1H, d, J=18.8 Hz), 5.24 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.59-5.55 (1H, m), 6.53 (1H, s), 6.78 (1H, t, J=6.3 Hz), 7.30 (1H, s), 7.79 (1H, d, J=11.0

Hz), 8.40 (1H, d, J=8.6 Hz).
MS (APCI) m/z: 621 $(M+H)^+$

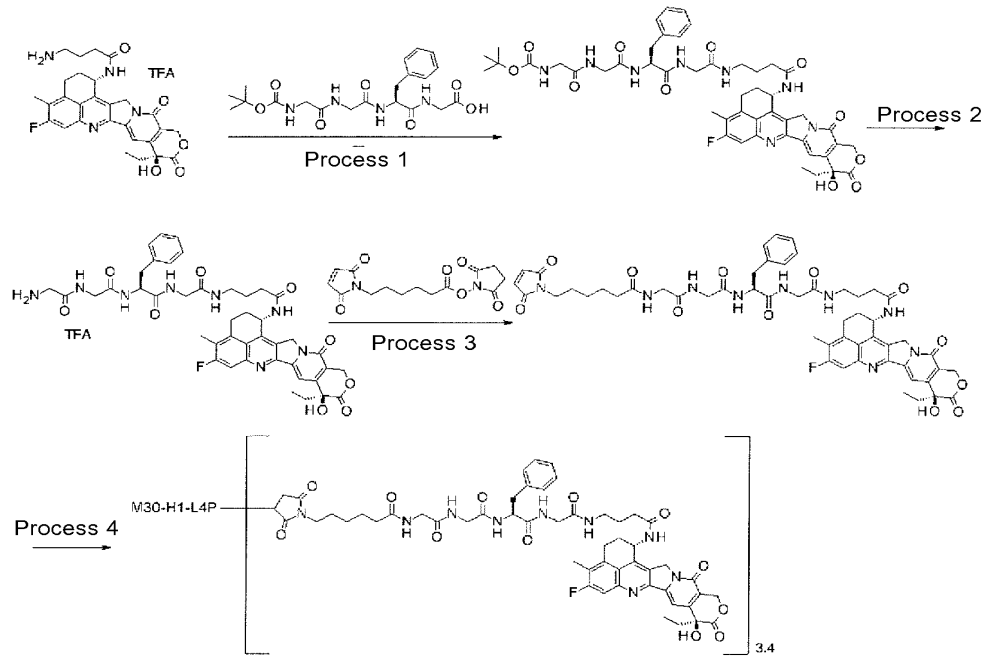
Process 2: 4-Amino-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]butanamide

[0331] The compound (0.388 g, 0.61 mmol) obtained in Process 1 was dissolved in dichloromethane (9 mL). Trifluoroacetic acid (9 mL) was added and it was stirred for 4 hours. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform-partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield trifluoroacetate of the titled compound (0.343 g, quantitative). This compound was confirmed in the tumor of a cancer-bearing mouse that received the antibody-drug conjugate (13) or (14).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.2 Hz), 1.79-1.92 (4H, m), 2.10-2.17 (2H, m), 2.27 (2H, t, J=7.0 Hz), 2.40 (3H, s), 2.80-2.86 (2H, m), 3.15-3.20 (2H, m), 5.15 (1H, d, J=18.8 Hz), 5.26 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.54-5.61 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.72 (3H, brs), 7.82 (1H, d, J=11.0 Hz), 8.54 (1H, d, J=8.6 Hz).
MS (APCI) m/z: 521 $(M+H)^+$

Example 2 Antibody-drug conjugate (1)

[0332]

[Formula 71]



Process 1: N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanyl-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0333] N-(Tert-butoxycarbonyl)glycylglycyl-L-phenylalanylglycine (0.081 g, 0.19 mmol) was dissolved in dichloromethane (3 mL), N-hydroxysuccinimide (0.021 g, 0.19 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.036 g, 0.19 mmol) were added and then stirred for 3.5 hours. The reaction solution was added dropwise to an N,N-dimethyl formamide solution (1.5 mL) charged with the compound (0.080 g, 0.15 mmol) of Example 1, and stirred for 4 hours at room temperature. The solvent was removed under reduced pressure and the residue obtained were

purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound (0.106 g, 73%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.36 (9H, s), 1.71 (2H, m), 1.86 (2H, t, J=7.8 Hz), 2.15-2.19 (4H, m), 2.40 (3H, s), 2.77 (1H, dd, J=12.7, 8.8 Hz), 3.02 (1H, dd, J=14.1, 4.7 Hz), 3.08-3.11 (2H, m), 3.16-3.19 (2H, m), 3.54 (2H, d, J=5.9 Hz), 3.57-3.77 (4H, m), 4.46-4.48 (1H, m), 5.16 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 6.53 (1H, s), 7.00 (1H, t, J=6.3 Hz), 7.17-7.26 (5H, m), 7.31 (1H, s), 7.71 (1H, t, J=5.7 Hz), 7.80 (1H, d, J=11.0 Hz), 7.92 (1H, t, J=5.7 Hz), 8.15 (1H, d, J=8.2 Hz), 8.27 (1H, t, J=5.5 Hz), 8.46 (1H, d, J=8.2 Hz).
MS (APCI) m/z: 939 $(M+H)^+$

Process 2: Glycylglycyl-L-phenylalanyl-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide trifluoroacetate

[0334] The compound (1.97 g, 2.10 mmol) obtained in Process 1 was dissolved in dichloromethane (7 mL). After adding trifluoroacetic acid (7 mL), it was stirred for 1 hour. The solvent was removed under reduced pressure, and it was charged with toluene for azeotropic distillation. The residue obtained were purified by silica gel column chromatography [chloroform - partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield the titled compound (1.97 g, 99%).
^{1}H-NMR (400 MHz, DMSO-d6) δ: 0.87 (3H, t, J=7.4 Hz), 1.71-1.73 (2H, m), 1.82-1.90 (2H, m), 2.12-2.20 (4H, m), 2.40 (3H, s), 2.75 (1H, dd, J=13.7, 9.4 Hz), 3.03-3.09 (3H, m), 3.18-3.19 (2H, m), 3.58-3.60 (2H, m), 3.64 (1H, d, J=5.9 Hz), 3.69 (1H, d, J=5.9 Hz), 3.72 (1H, d, J=5.5 Hz), 3.87 (1H, dd, J=16.8, 5.9 Hz), 4.50-4.56 (1H, m), 5.16 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 7.17-7.27 (5H, m), 7.32 (1H, s), 7.78-7.81 (2H, m), 7.95-7.97 (3H, m), 8.33-8.35 (2H, m), 8.48-8.51 (2H, m).
MS (APCI) m/z: 839 $(M+H)^+$

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0335] To an N,N-dimethyl formamide (1.2 mL) solution of the compound (337 mg, 0.353 mmol) obtained in Process 2, triethylamine (44.3 mL, 0.318 mmol) and N-succinimidyl 6-maleimide hexanoic acid (119.7 mg, 0.388 mmol) were added and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform : methanol = 5 : 1 (v/v)] to yield the titled compound as a pale yellow solid (278.0 mg, 76%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.3 Hz), 1.12-1.22 (2H, m), 1.40-1.51 (4H, m), 1.66-1.76 (2H, m), 1.80-1.91 (2H, m), 2.05-2.21 (6H, m), 2.39 (3H, s), 2.79 (1H, dd, J=14.0, 9.8 Hz), 2.98-3.21 (5H, m), 3.55-3.77 (8H, m), 4.41-4.48 (1H, m), 5.15 (1H, d, J=18.9 Hz), 5.24 (1H, d, J=18.9 Hz), 5.40 (1H, d, J=17.1 Hz), 5.44 (1H, d, J=17.1 Hz), 5.54-5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.20-7.27 (5H, m), 7.30 (1H, s), 7.70 (1H, t, J=5.5 Hz), 7.80 (1H, d, J=11.0 Hz), 8.03 (1H, t, J=5.8 Hz), 8.08 (1H, t, J=5.5 Hz), 8.14 (1H, d, J=7.9 Hz), 8.25 (1H, t, J=6.1 Hz), 8.46 (1H, d, J=8.5 Hz).
MS (APCI) m/z: 1032 $(M+H)^+$

Process 4: Antibody-drug conjugate (1)

[0336] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mL mg^{-1} cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 $\pm$ 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating for 1 hour at 37°C.
[0337] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.109 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 3 (0.039 mL; 4.6 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 40 minutes. Next, an aqueous solution (0.008 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature to terminate the drug linker reaction for another 20 minutes.
[0338] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug

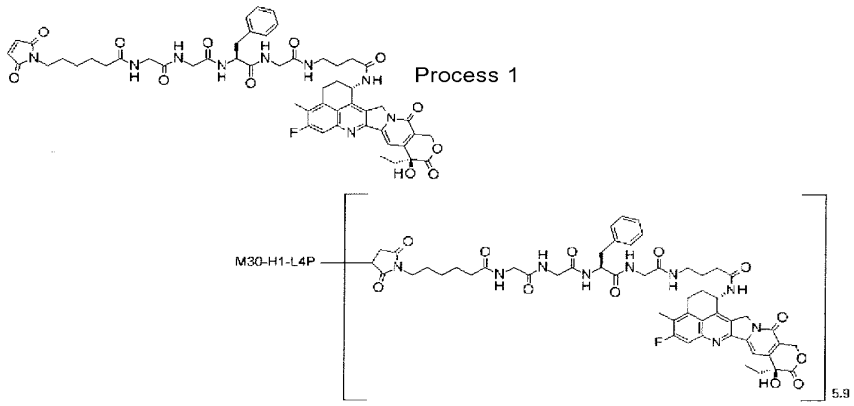
conjugate. After that, the solution was concentrated by the Common procedure A.

[0339] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 13.02 mg/mL, antibody yield: 9.1 mg (73%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 3 Antibody-drug conjugate (2)

[0340]

[Formula 72]



Process 1: Antibody-drug conjugate (2)

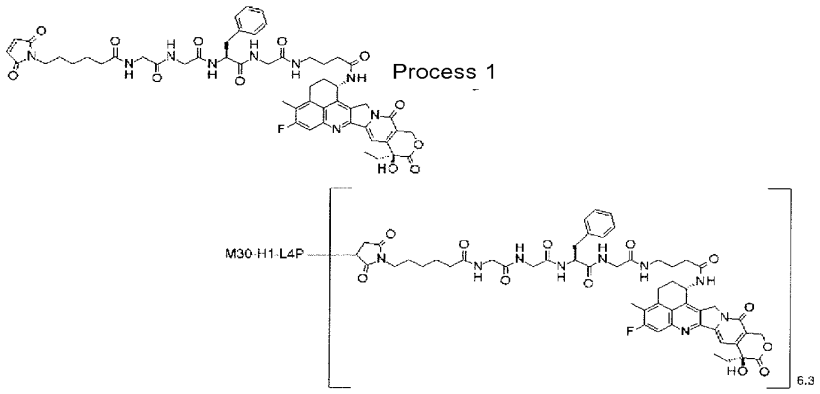
[0341] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (4.0 mL) was collected into a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.118 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.200 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubatingat 37°C for 1 hour . Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution (0.236 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.00471 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 17.5 mL of a solution containing the titled antibody-drug conjugate.

[0342] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.80 mg/mL, antibody yield: 26.1 mg (65%), and average number of conjugated drug molecules (n) per antibody molecule: 5.9.

Example 4 Antibody-drug conjugate (3)

[0343]

[Formula 73]



Process 1: Antibody-drug conjugate (3)

[0344] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0345] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.067 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 3 of Example 2 (0.085 mL; 10.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 60 minutes. Next, an aqueous solution (0.013 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.

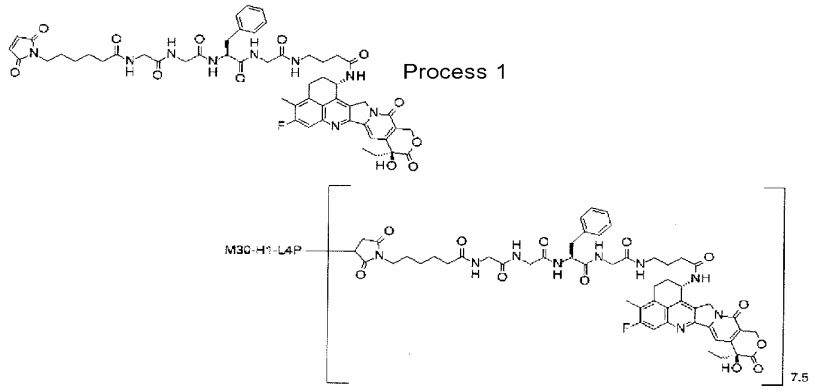
[0346] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0347] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.67 mg/mL, antibody yield: 10.02 mg (80%), and average number of conjugated drug molecules (n) per antibody molecule: 6.3.

Example 5 Antibody-drug conjugate (4)

[0348]

[Formula 74]



Process 1: Antibody-drug conjugate (4)

[0349] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0350] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.025 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 3 of Example 2 (0.127 mL; 15.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 60 minutes. Next, an aqueous solution (0.019 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.

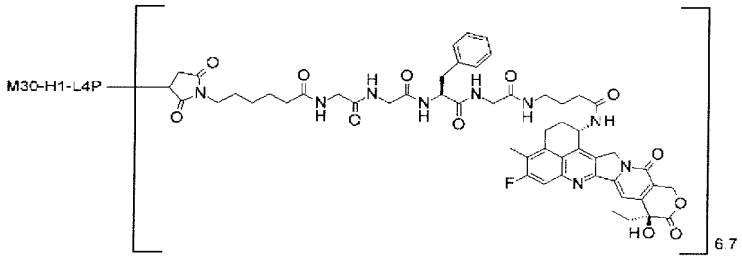
[0351] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

[0352] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.19 mg/mL, antibody yield: 7.14 mg (57%), and average number of conjugated drug molecules (n) per antibody molecule: 7.5.

Example 6 Antibody-drug conjugate (5)

[0353]

[Formula 75]

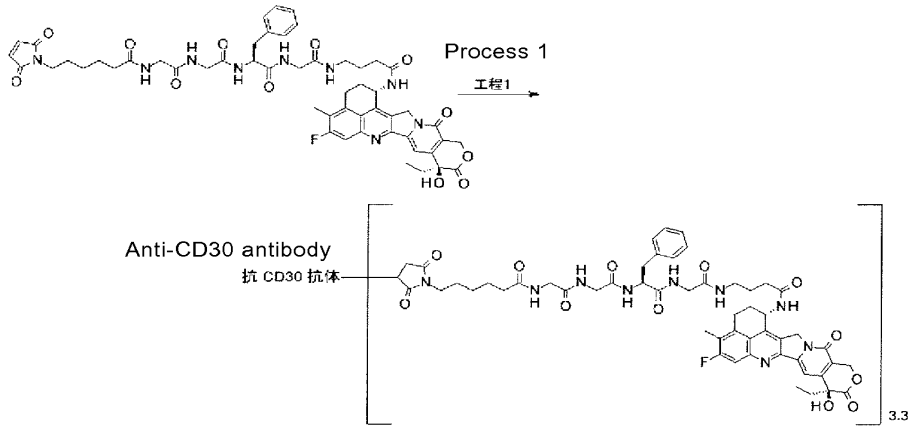


[0354] Almost the whole amounts of the antibody-drug conjugates of Examples 4 and 5 were mixed. The solution was concentrated by the Common procedure A to yield the titled antibody-drug conjugate.
Antibody concentration: 10.0 mg/mL, antibody yield: 15.37 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.7.

Example 7 Antibody-drug conjugate (6)

[0355]

[Formula 76]



Process 1: Antibody-drug conjugate (6)

[0356] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubatingat 37°C for 1 hour .

[0357] Conjugation between antibody and drug linker: After incubating the solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer

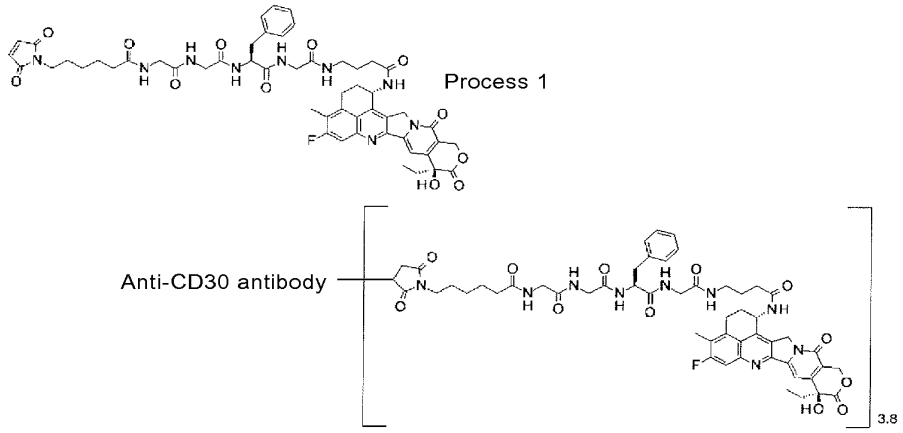
solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0358] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 270400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 0.99 mg/mL, antibody yield: 5.94 mg (59%), and average number of conjugated drug molecules (n) per antibody molecule: 3.3.

Example 8 Antibody-drug conjugate (7)

[0359]

[Formula 77]



Process 1: Antibody-drug conjugate (7)

[0360] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 30 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 6.9 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

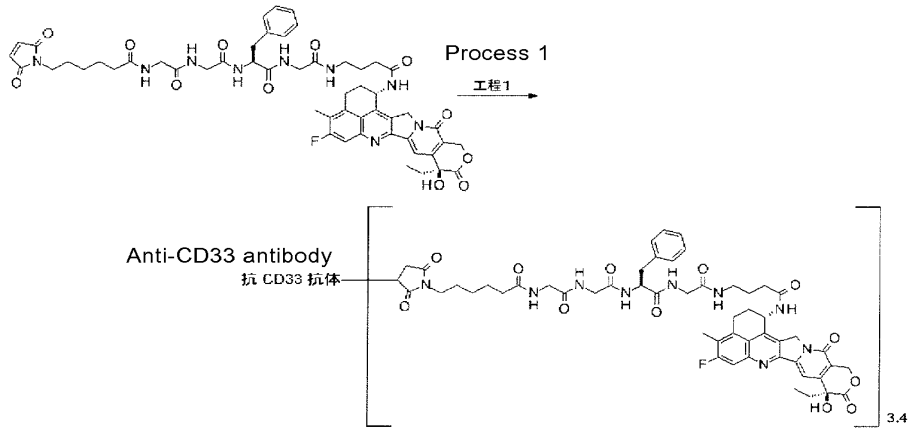
[0361] Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution (0.0297 mL; 13.8 equivalents per antibody molecule) containing 30 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for 40 minutes at 22°C for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0178 mL; 27.6 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0362] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 270400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 0.99 mg/mL, antibody yield: 5.94 mg (59%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 9 Antibody-drug conjugate (8)

[0363]

[Formula 78]



Process 1: Antibody-drug conjugate (8)

[0364] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

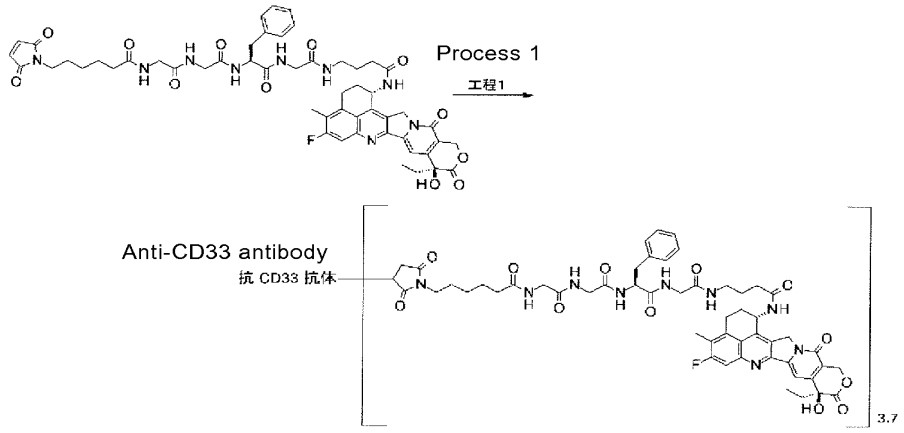
[0365] Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0366] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 256400 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.06 mg/mL, antibody yield: 6.36 mg (64%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 10 Antibody-drug conjugate (9)

[0367]

[Formula 79]



Process 1: Antibody-drug conjugate (9)

[0368] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 30 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 6.9 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

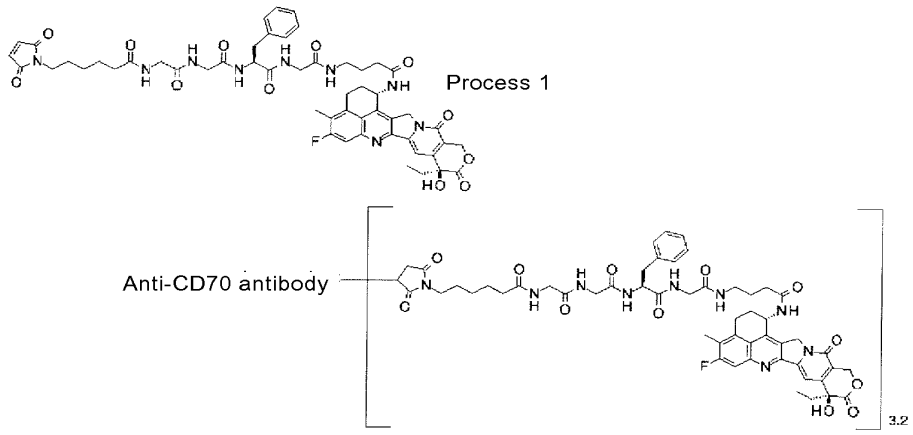
[0369] Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution (0.0297 mL; 13.8 equivalents per antibody molecule) containing 30 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0178 mL; 27.6 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0370] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 256400 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 0.95 mg/mL, antibody yield: 5.70 mg (57%), and average number of conjugated drug molecules (n) per antibody molecule: 3.7.

Example 11 Antibody-drug conjugate (10)

[0371]

[Formula 80]



Process 1: Antibody-drug conjugate (10)

[0372] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.69 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

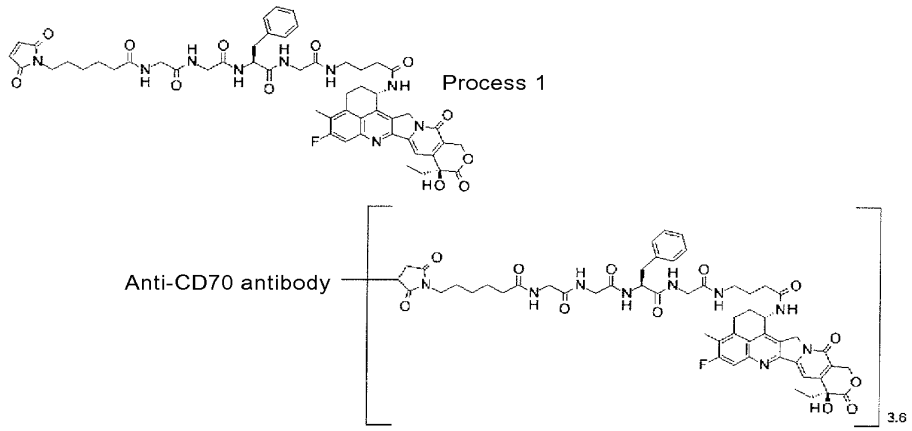
[0373] Conjugation between antibody and drug linker: After incubating the solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0374] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 262400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.00 mg/mL, antibody yield: 6.00 mg (60%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 12 Antibody-drug conjugate (11)

[0375]

[Formula 81]



Process 1: Antibody-drug conjugate (11)

[0376] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.69 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 30 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 6.9 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

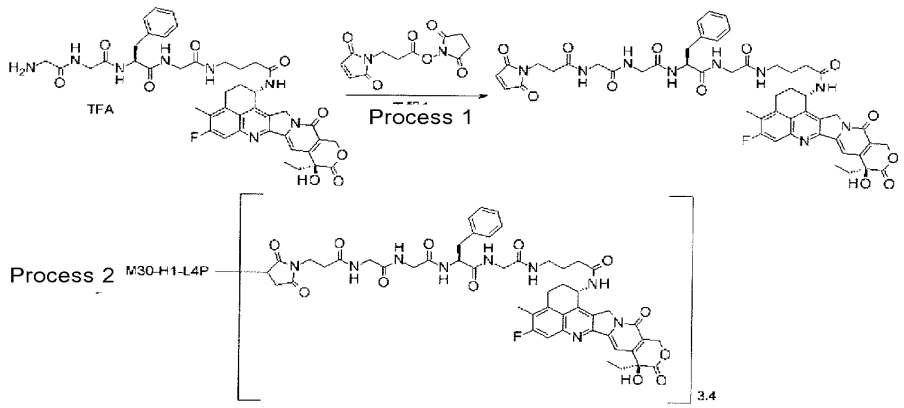
[0377] Conjugation between antibody and drug linker: After incubating the solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 13.8 equivalents per antibody molecule) containing 30 mM of the compound obtained in Process 3 of Example 2 was added thereto and incubated for 40 minutes at 22°C for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0178 mL; 27.6 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubatedto terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0378] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 262400 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 0.96 mg/mL, antibody yield: 5.76 mg (58%), and average number of conjugated drug molecules (n) per antibody molecule: 3.6.

Example 13 Antibody-drug conjugate (12)

[0379]

[Formula 82]



Process 1: N-[3-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoyl]glycylglycyl-L-phenylalanyl-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0380] The compound (80 mg, 0.084 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using N-succinimidyl 3-maleimide propioate (24.6 mg, 0.0924 mmol) instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (60.0 mg, 73%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.89 (3H, t, J=7.3 Hz), 1.70-1.78 (2H, m), 1.81-1.94 (2H, m), 2.12-2.23 (4H, m), 2.42 (3H, s), 2.81 (1H, dd, J=13.7, 9.8 Hz), 3.01-3.15 (3H, m), 3.16-3.23 (2H, m), 3.30-3.35 (1H, m), 3.58-3.71 (6H, m), 3.71-3.79 (1H, m), 4.44-4.51 (1H, m), 5.19 (1H, d, J=19.0 Hz), 5.27 (1H, d, J=19.0 Hz), 5.43 (1H, d, J=17.6 Hz), 5.47 (1H, d, J=17.6 Hz), 5.57-5.63 (1H, m), 6.56 (1H, s), 7.02 (2H, s), 7.17-7.22 (1H, m), 7.22-7.30 (5H, m), 7.34 (1H, s), 7.73 (1H, t, J=5.6 Hz), 7.83 (1H, d, J=10.7 Hz), 8.08 (1H, t, J=5.6 Hz), 8.15 (1H, d, J=7.8 Hz), 8.30 (2H, dt, J=18.7, 5.7 Hz), 8.49 (1H, d, J=8.8 Hz).
MS (APCI) m/z: 990 $(M+H)^+$

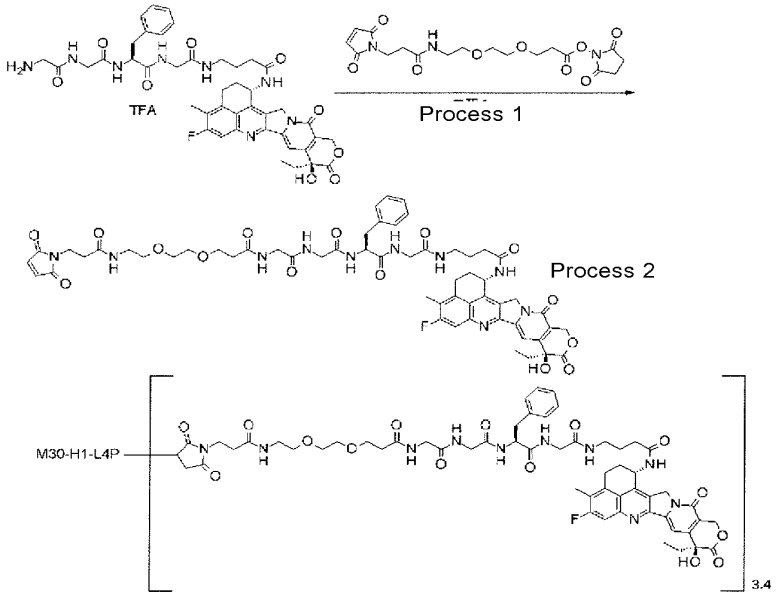
Process 2: Antibody-drug conjugate (12)

[0381] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1, the titled antibody-drug conjugate was obtained in the same manner as Process 4 of Example 2. Antibody concentration: 12.16 mg/mL, antibody yield: 8.5 mg (68%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 14 Antibody-drug conjugate (13)

[0382]

[Formula 83]



Process 1: N-{3-[2-(2-{[3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoyl]amino}ethoxy)ethoxy]propanoyl}glycylglycyl-L-phenylalanyl-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0383] The compound (100 mg, 0.119 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using diisopropylethylamine (20.8 μL, 0.119 mmol) instead of triethylamine and N-succinimidyl 3-(2-(2-(3-maleinimidepropanamide)ethoxy)ethoxy)propanoate (50.7 mg, 0.119 mmol) instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (66.5 mg, 48%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J=7.4 Hz), 1.65-1.74 (2H, m), 1.77-1.90 (2H, m), 2.07-2.19 (4H, m), 2.30 (2H, t, J=7.2 Hz), 2.33-2.36 (2H, m), 2.38 (3H, s), 2.76 (1H, dd, J=13.7, 9.8 Hz), 2.96-3.18 (9H, m), 3.42-3.44 (4H, m), 3.53-3.76 (10H, m), 4.43 (1H, td, J=8.6, 4.7 Hz), 5.14 (1H, d, J=18.8 Hz), 5.23 (1H, d, J=18.8 Hz), 5.38 (1H, d, J=17.2 Hz), 5.42 (1H, d, J=17.2 Hz), 5.52-5.58 (1H, m), 6.52 (1H, s), 6.98 (2H, s), 7.12-7.17 (1H, m), 7.18-7.25 (4H, m), 7.29 (1H, s), 7.69 (1H, t, J=5.5 Hz), 7.78 (1H, d, J=11.3 Hz), 7.98-8.03 (2H, m), 8.11 (1H, d, J=7.8 Hz), 8.16 (1H, t, J=5.7 Hz), 8.23 (1H, t, J=5.9 Hz), 8.44 (1H, d, J=9.0 Hz).
MS (APCI) m/z: 1149 $(M+H)^+$

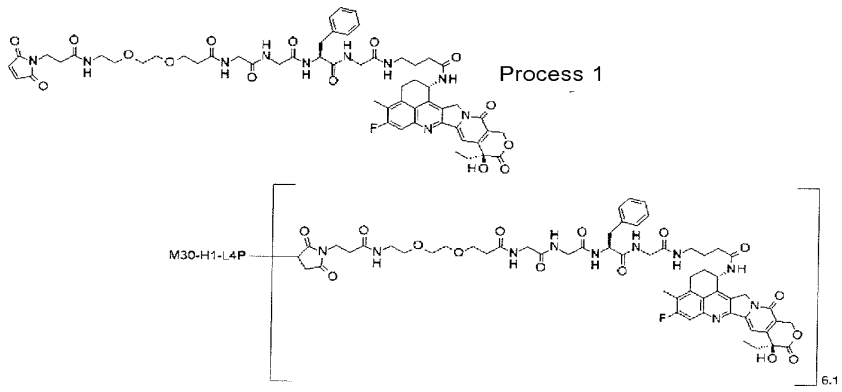
Process 2: Antibody-drug conjugate (13)

[0384] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1, the titled antibody-drug conjugate was obtained in the same manner as Process 4 of Example 2. Antibody concentration: 12.76 mg/mL, antibody yield: 8.9 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 15 Antibody-drug conjugate (14)

[0385]

[Formula 84]



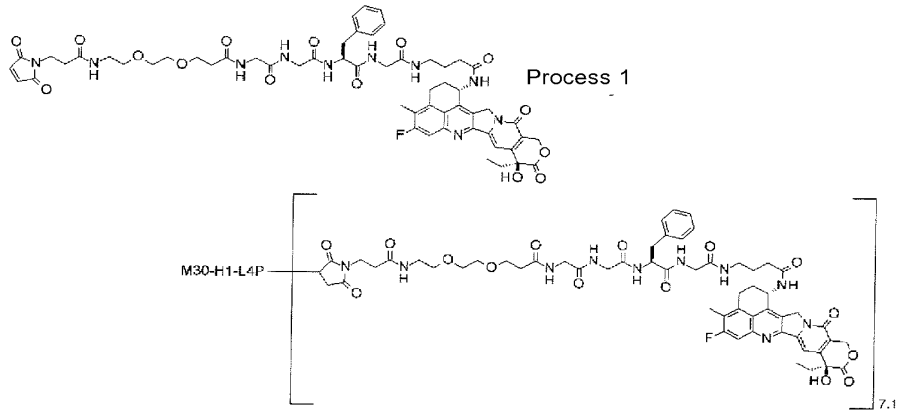
Process 1: Antibody-drug conjugate (14)

[0386] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1 of Example 14, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 4. Antibody concentration: 1.60 mg/mL, antibody yield: 9.60 mg (77%), and average number of conjugated drug molecules (n) per antibody molecule: 6.1.

Example 16 Antibody-drug conjugate (15)

[0387]

[Formula 85]



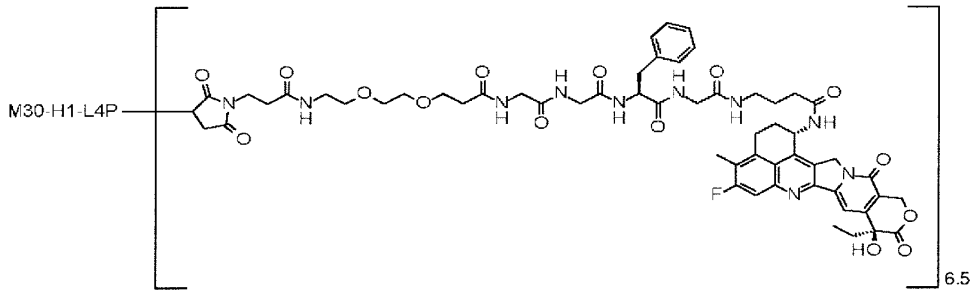
Process 1: Antibody-drug conjugate (15)

[0388] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1 of Example 14, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 5. Antibody concentration: 1.64 mg/mL, antibody yield: 9.84 mg (79%), and average number of conjugated drug molecules (n) per antibody molecule: 7.1.

Example 17 Antibody-drug conjugate (16)

[0389]

[Formula 86]

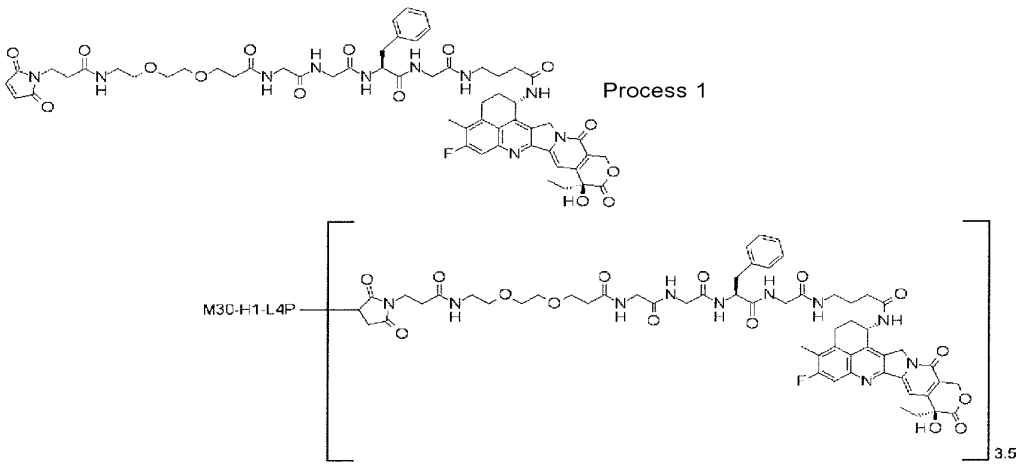


Almost the whole amounts of the antibody-drug conjugates of Examples 15 and 16 were mixed. The solution was concentrated by the Common procedure A to yield the titled antibody-drug conjugate.
Antibody concentration: 10.0 mg/mL, antibody yield: 17.30 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.5.

Example 18 Antibody-drug conjugate (17)

[0390]

[Formula 87]



Process 1: Antibody-drug conjugate (17)

[0391] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (100 mL, 1 g of the antibody) was placed in a 250 mL flask and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (2.43 mL; 3.6 equivalents per antibody molecule) and further with an aqueous solution of 1 M dipotassium hydrogen phosphate (5 mL). After confirming that the solution had pH near 7.4 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0392] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (2.14 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (3.51 mL; 5.2 equivalents per antibody molecule) to the above solution at room temperature, it was stirred with a stirrer for conjugating the drug linker to the antibody in a water bath at 15°C for 130 minutes. Next, an aqueous solution (0.547 mL) of 100 mM NAC was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes. Purification: The

above solution was subjected to ultrafiltration purification using an ultrafiltration apparatus composed of an ultrafiltration membrane (Merck Japan, Pellicon XL Cassette, Biomax 50 KDa), a tube pump (Cole-Parmer International, MasterFlex Pump model 77521-40, Pump Head model 7518-00), and a tube (Cole-Parmer International, MasterFlex Tube L/S16). Specifically, while ABS was added dropwise (a total of 800 mL) as a buffer solution for purification to the reaction solution, ultrafiltration purification was performed for removing unconjugated drug linkers and other low-molecular-weight reagents, also replacing the buffer solution with ABS, and further concentrating the solution, to yield about 70 mL of a solution containing the titled antibody-drug conjugate.

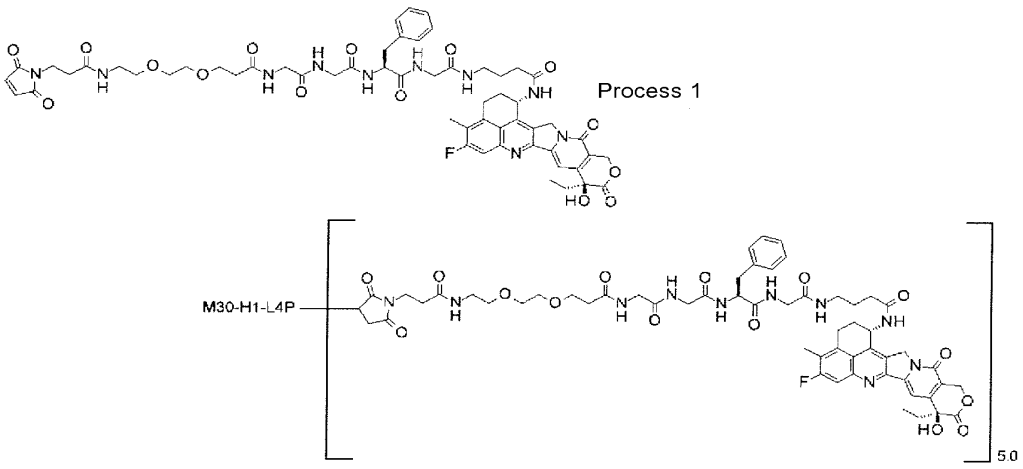
[0393] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0394] Antibody concentration: 14.5 mg/mL, antibody yield: 1.0 g (about 100%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

Example 19 Antibody-drug conjugate (18)

[0395]

[Formula 88]



Process 1: Antibody-drug conjugate (18)

[0396] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (5 mL, 50 mg of the antibody) was placed in a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.135 mL; 4 equivalents per antibody molecule). After confirming that the solution had pH near 7.4 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0397] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.064 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.219 mL; 6.5 equivalents per antibody molecule) to the above solution, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 90 minutes. Next, an aqueous solution (0.033 mL; 9.8 equivalents per antibody molecule) of 100 mM NAC was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0398] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 19 mL of a solution containing the titled antibody-drug conjugate.

[0399] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were

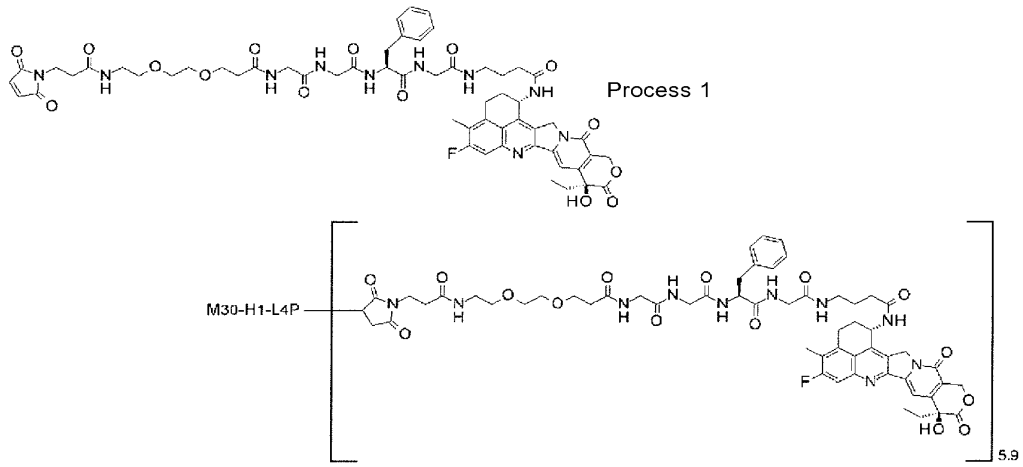
obtained.

[0400] Antibody concentration: 2.17 mg/mL, antibody yield: 41 mg (82%), and average number of conjugated drug molecules (n) per antibody molecule: 5.0.

Example 20 Antibody-drug conjugate (19)

[0401]

[Formula 89]



Process 1: Antibody-drug conjugate (19)

[0402] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (4 mL, 40 mg of the antibody) was placed in a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.140 mL; 5.2 equivalents per antibody molecule). After confirming that the solution had pH near 7.4 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour. Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.232 mL; 8.6 equivalents per antibody molecule) to the above solution, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 90 minutes. Next, an aqueous solution (0.035 mL; 12.9 equivalents per antibody molecule) of 100 mM NAC was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0403] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 13 mL of a solution containing the titled antibody-drug conjugate.

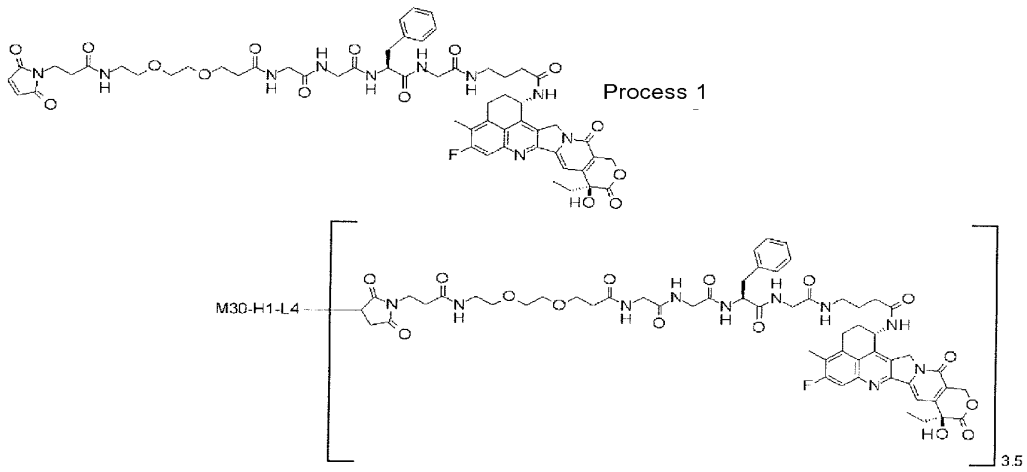
[0404] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0405] Antibody concentration: 2.36 mg/mL, antibody yield: 31 mg (77%), and average number of conjugated drug molecules (n) per antibody molecule: 5.9.

Example 21 Antibody-drug conjugate (20)

[0406]

[Formula 90]



Process 1: Antibody-drug conjugate (20)

[0407] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0408] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.0267 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0439 mL; 5.2 equivalents per antibody molecule) to the above solution at room temperature, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 1 hour. Next, an aqueous solution (0.0066 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0409] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

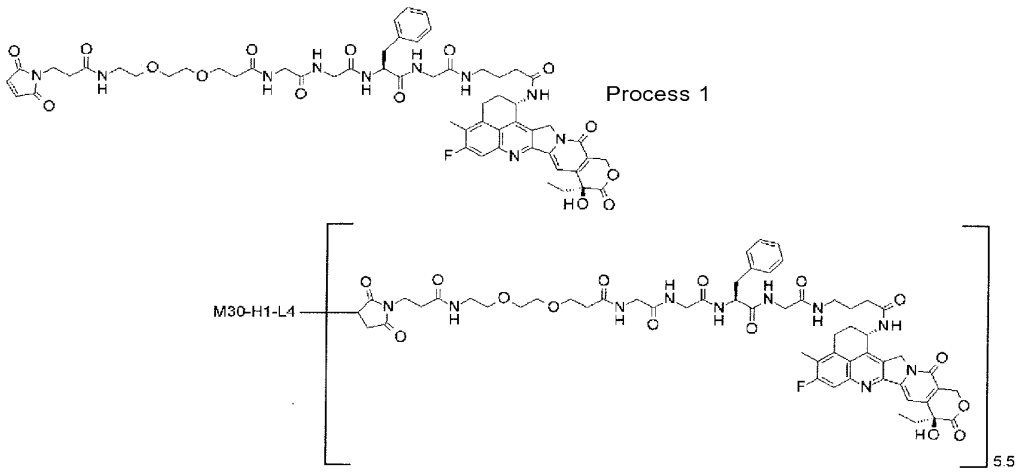
[0410] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0411] Antibody concentration: 10.0 mg/mL, antibody yield: 8.7 mg (70%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

Example 22 Antibody-drug conjugate (21)

[0412]

[Formula 91]



Process 1: Antibody-drug conjugate (21)

[0413] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0439 mL; 5.2 equivalents per antibody molecule) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0414] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0726 mL; 8.6 equivalents per antibody molecule) to the above solution at room temperature, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 1 hour. Next, an aqueous solution (0.011 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0415] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

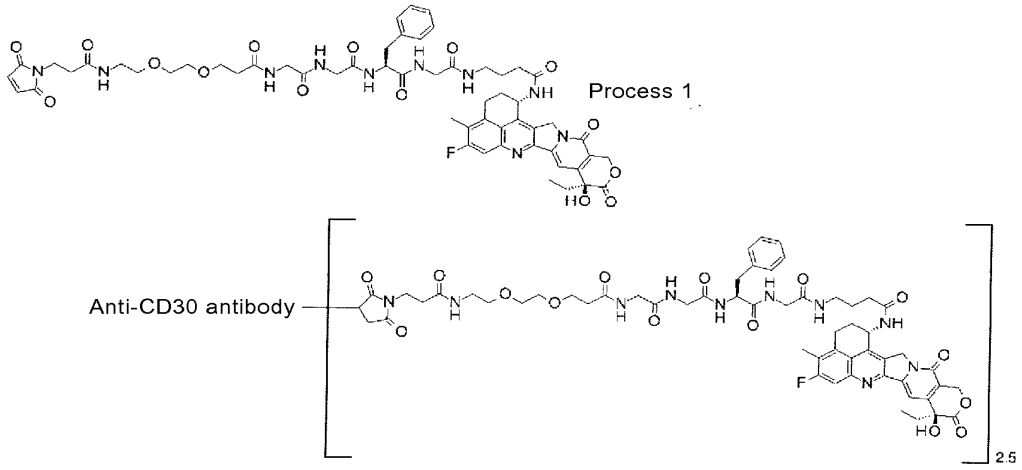
[0416] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0417] Antibody concentration: 10.0 mg/mL, antibody yield: 8.3 mg (66%), and average number of conjugated drug molecules (n) per antibody molecule: 5.5.

Example 23 Antibody-drug conjugate (22)

[0418]

[Formula 92]



Process 1: Antibody-drug conjugate (22)

[0419] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule). The disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0420] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.0098 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0116 mL; 4.5 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0421] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

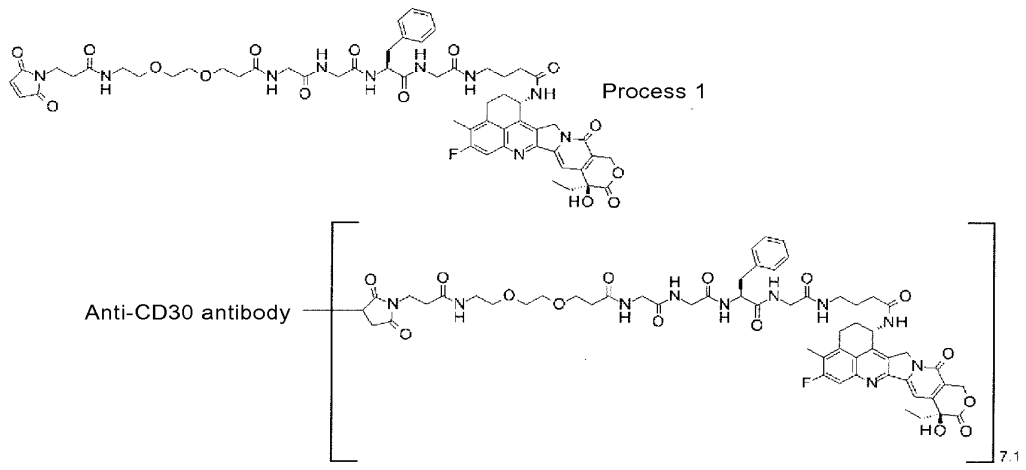
[0422] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 270400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0423] Antibody concentration: 0.86 mg/mL, antibody yield: 2.2 mg (54%), and average number of conjugated drug molecules (n) per antibody molecule: 2.5.

Example 24 Antibody-drug conjugate (23)

[0424]

[Formula 93]



Process 1: Antibody-drug conjugate (23)

[0425] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.35 mL, 3.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0113 mL; 5 equivalents per antibody molecule). The disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0426] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0204 mL; 9 equivalents per antibody molecule) and propylene glycol (Kanto Chemical Co., Inc., 0.18 mL) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0031 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0427] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

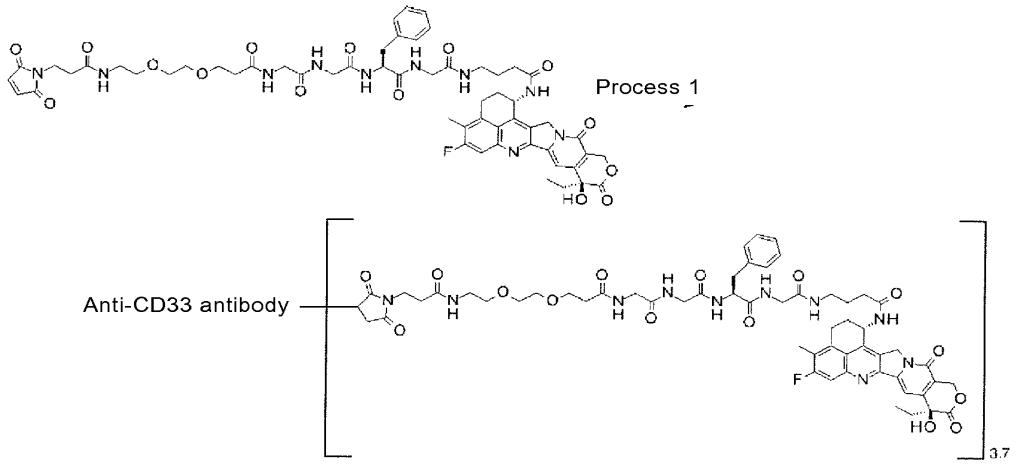
[0428] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 270400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0429] Antibody concentration: 0.41 mg/mL, antibody yield: 1.0 mg (29%), and average number of conjugated drug molecules (n) per antibody molecule: 7.1.

Example 25 Antibody-drug conjugate (24)

[0430]

[Formula 94]



Process 1: Antibody-drug conjugate (24)

[0431] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0058 mL). After confirming that the solution had pH of 7.0 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0432] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.0101 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0116 mL; 4.5 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0433] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

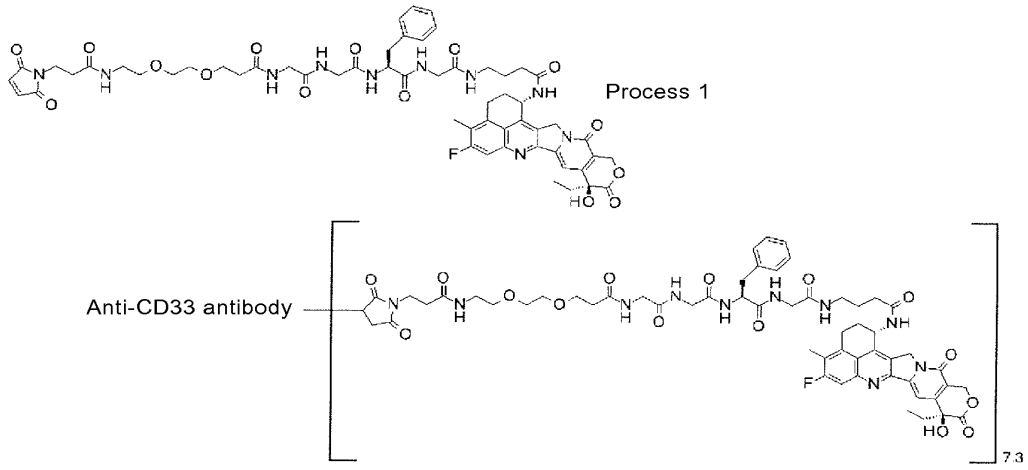
[0434] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 256400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0435] Antibody concentration: 1.25 mg/mL, antibody yield: 3.1 mg (78%), and average number of conjugated drug molecules (n) per antibody molecule: 3.7.

Example 26 Antibody-drug conjugate (25)

[0436]

[Formula 95]



Process 1: Antibody-drug conjugate (25)

[0437] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0129 mL; 5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.006 mL). After confirming that the solution had pH of 7.0 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0438] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0233 mL; 9 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0035 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0439] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

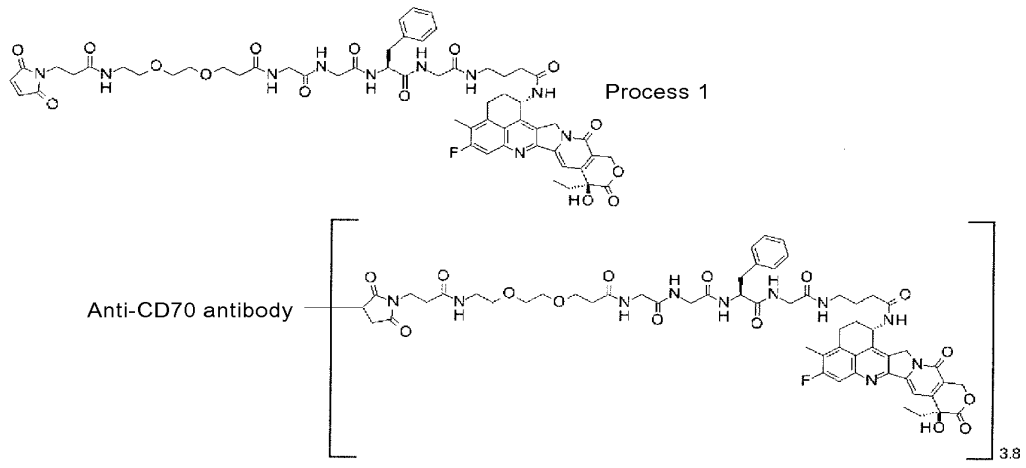
[0440] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 256400 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 4964 (measured value), and $\varepsilon_{D,\ 370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0441] Antibody concentration: 1.17 mg/mL, antibody yield: 2.9 mg (73%), and average number of conjugated drug molecules (n) per antibody molecule: 7.3.

Example 27 Antibody-drug conjugate (26)

[0442]

[Formula 96]



Process 1: Antibody-drug conjugate (26)

[0443] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.69 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0058 mL). After confirming that the solution had pH of 7.0 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0444] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.0101 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 of Example 14 (0.0116 mL; 4.5 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0445] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

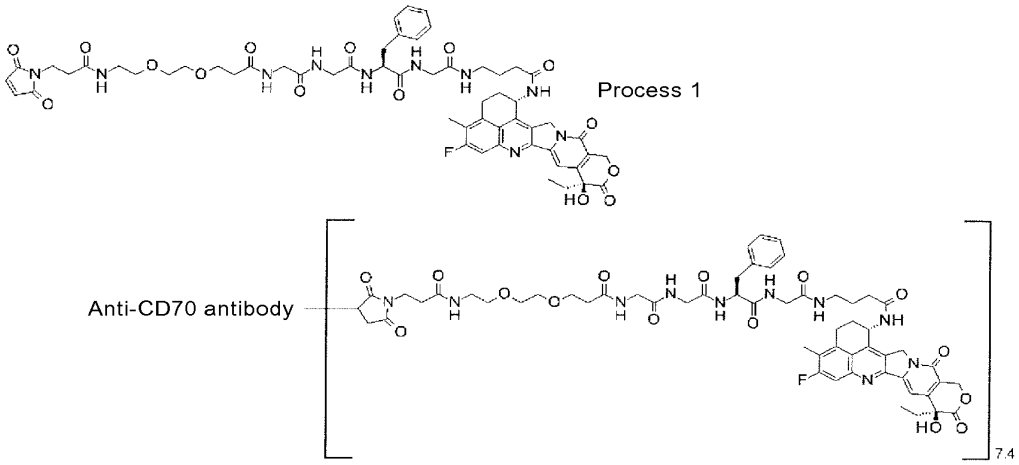
[0446] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 262400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0447] Antibody concentration: 1.14 mg/mL, antibody yield: 2.9 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 28 Antibody-drug conjugate (27)

[0448]

[Formula 97]



Process 1: Antibody-drug conjugate (27)

[0449] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.69 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0129 mL; 5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.006 mL). After confirming that the solution had pH of 7.0 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0450] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution (0.0233 mL; 9 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 1 of Example 14 to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0035 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0451] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

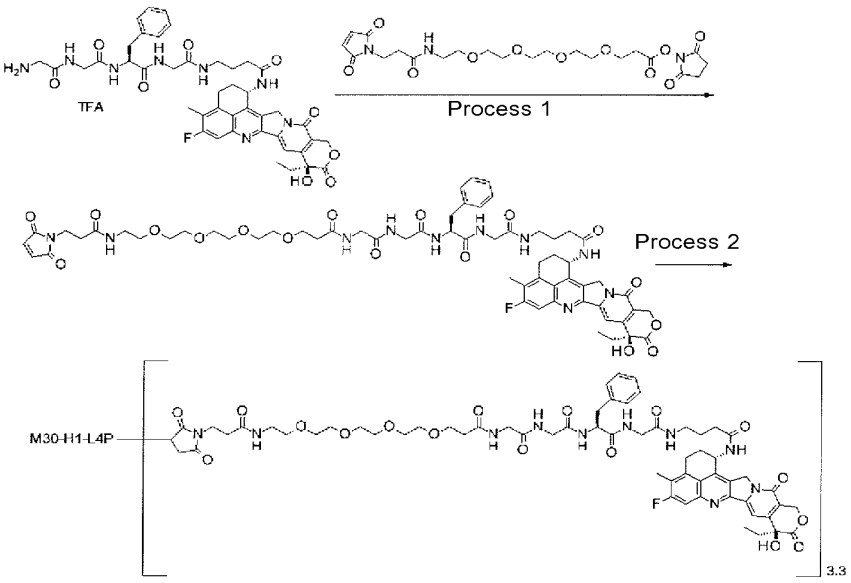
[0452] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\,280}$ = 262400 (estimated calculation value), $\varepsilon_{A,\,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 4964 (measured value), and $\varepsilon_{D,370}$ = 18982 (measured value) were used), the following characteristic values were obtained.

[0453] Antibody concentration: 1.13 mg/mL, antibody yield: 2.8 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 7.4.

Example 29 Antibody-drug conjugate (28)

[0454]

[Formula 98]



Process 1: N-[19-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-17-oxo-4,7,10,13-tetraoxo-16-azanonadecan-1-oyl]glycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0455] The compound (90 mg, 0.107 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using diisopropylethylamine (18.7 μL, 0.107 mmol) instead of triethylamine and N-succinimidyl 1-maleinimide-3-oxo-7,10,13,16-tetraoxa-4-azanonadecan-19-oate (55.1 mg, 0.107 mmol) instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (50 mg, 37%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J=7.2 Hz), 1.64-1.74 (2H, m), 1.77-1.90 (2H, m), 2.06-2.19 (4H, m), 2.27-2.32 (2H, m), 2.33-2.37 (2H, m), 2.38 (3H, s), 2.72-2.80 (3H, m), 2.96-3.19 (6H, m), 3.39-3.48 (10H, m), 3.52-3.75 (10H, m), 4.39-4.48 (1H, m), 5.14 (1H, d, J=18.8 Hz), 5.23 (1H, d, J=18.8 Hz), 5.38 (1H, d, J=17.0 Hz), 5.42 (1H, d, J=17.0 Hz), 5.52-5.58 (1H, m), 6.52 (1H, s), 6.98 (1H, s), 7.13-7.24 (5H, m), 7.29 (1H, s), 7.69 (1H, t, J=5.5 Hz), 7.78 (1H, d, J=10.9 Hz), 7.98-8.03 (2H, m), 8.10 (1H, d, J=7.8 Hz), 8.16 (1H, t, J=5.7 Hz), 8.23 (1H, t, J=5.7 Hz), 8.44 (1H, d, J=8.6 Hz).
MS (APCI) m/z: 1237 $(M+H)^+$

Process 2: Antibody-drug conjugate (28)

[0456] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mL mg^{-1} cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.
[0457] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.102 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 1 (0.047 mL; 5.5 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 40 minutes. Next, an aqueous solution (0.009 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.
[0458] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug

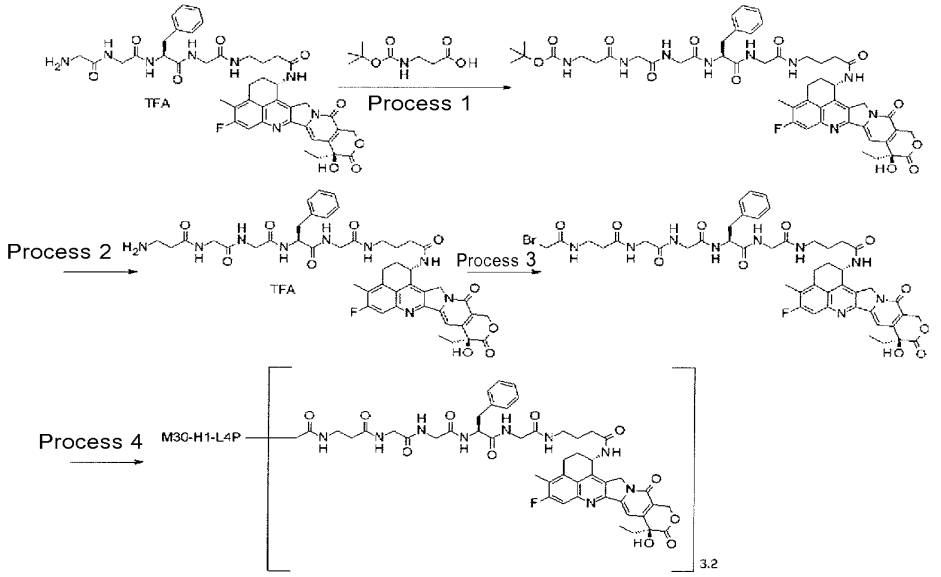
conjugate. After that, the solution was concentrated by the Common procedure A.

[0459] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\ 280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 13.60 mg/mL, antibody yield: 9.5 mg (76%), and average number of conjugated drug molecules (n) per antibody molecule: 3.3.

Example 30 Antibody-drug conjugate (29)

[0460]

[Formula 99]



Process 1: N-(tert-butoxycarbonyl)-β-alanylglycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0461] The compound (0.839 g, 1.00 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 1 of Example 1 by using N-(tert-butoxycarbonyl)-β-alanine instead of 4-(tert-butoxycarbonylamino)butanoic acid. The crude product obtained was used in the next process without performing purification.

Process 2: β-Alanylglycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0462] The crude product obtained in Process 1 was reacted in the same manner as Process 2 of Example 2 to yield the titled compound as a pale yellow solid (0.610 g, 67%).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.67-1.77 (2H, m), 1.79-1.92 (2H, m), 2.09-2.22 (4H, m), 2.40 (3H, s), 2.46-2.55 (2H, m), 2.82-2.73 (1H, m), 2.95-3.13 (5H, m), 3.14-3.21 (2H, m), 3.55-3.80 (6H, m), 4.44-4.52 (1H, m), 5.20 (2H, dd, J=35.0, 19.0 Hz), 5.42 (2H, s), 5.53-5.60 (1H, m), 6.54 (1H, s), 7.14-7.28 (5H, m), 7.31 (1H, s), 7.67 (2H, brs), 7.72-7.78 (1H, m), 7.80 (1H, d, J=11.0 Hz), 8.10-8.17 (2H, m), 8.29 (1H, t, J=5.9 Hz), 8.42 (1H, t, J=5.7 Hz), 8.47 (1H, d, J=8.6 Hz).

Process 3: N-(bromoacetyl)-β-alanylglycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0463] To a dichloromethane (4.5 mL) solution of 2-bromoacetic acid (96.3 mg, 0.693 mmol), N-hydroxysuccinimide (79.7 mg, 0.693 mmol) and 1,3-diisopropylcarbodiimide (0.107 mL, 0.693 mmol) were added and stirred at room temperature. The reaction solution was added to an N,N-dimethyl formamide (4.5 mL) solution of the compound (473 mg, 0.462 mmol) obtained in Process 2 and triethylamine (0.154 mL, 1.11 mmol) at 0°C and stirredat room temperature for 1 hour. The reaction solution was purified by silica gel column chromatography [elution solvent: chloroform - chloroform: methanol = 85 : 15 (v/v)]. The obtained solid was washed with chloroform: methanol: diethyl ether mixed solvent to yield the titled compound as a pale yellow solid (191 mg, 40%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.67-1.77 (2H, m), 1.79-1.92 (2H, m), 2.08-2.22 (4H, m), 2.33 (2H, t, J=7.0 Hz), 2.40 (3H, s), 2.74-2.83 (1H, m), 2.99-3.12 (3H, m), 3.14-3.21 (2H, m), 3.24-3.30 (2H, m), 3.56-3.77 (6H, m), 3.82 (2H, s), 4.41-4.51 (1H, m), 5.20 (2H, q, J=18.9 Hz), 5.42 (2H, s), 5.54-5.60 (1H, m), 6.54 (1H, s), 7.15-7.27 (5H, m), 7.31 (1H, s), 7.69-7.74 (1H, m), 7.80 (1H, d, J=10.9 Hz), 8.06 (1H, t, J=5.7 Hz), 8.13 (1H, d, J=7.8 Hz), 8.21-8.34 (3H, m), 8.46 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 1030, 1032 $(M+H)^+$

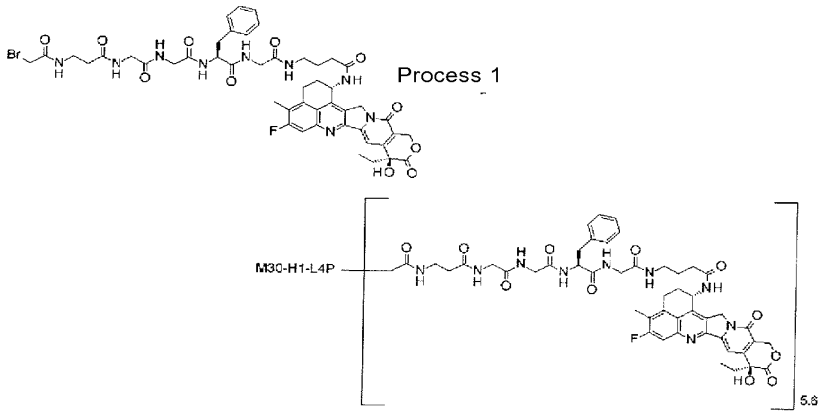
Process 4: Antibody-drug conjugate (29)

[0464] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.
[0465] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.09 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 3 (0.059 mL; 7.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 40 minutes. Next, an aqueous solution (0.009 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.
[0466] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.
[0467] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\ 280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 13.9 mg/mL, antibody yield: 9.7 mg (78%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 31 Antibody-drug conjugate (30)

[0468]

[Formula 100]

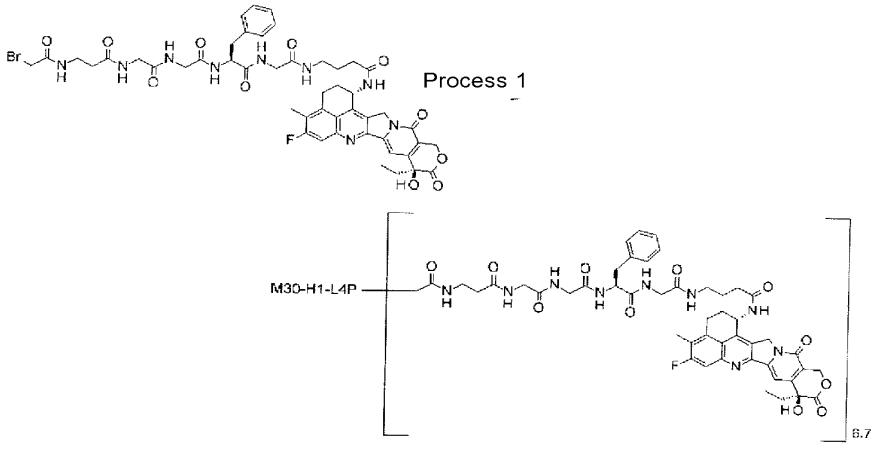


[0469] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3 of Example 30, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 4.
[0470] Antibody concentration: 1.94 mg/mL, antibody yield: 11.64 mg (93%), and average number of conjugated drug molecules (n) per antibody molecule: 5.6.

Example 32 Antibody-drug conjugate (31)

[0471]

[Formula 101]



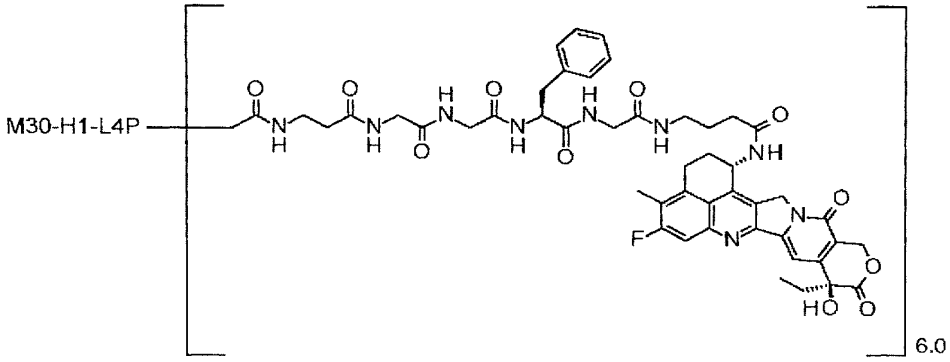
Process 1: Antibody-drug conjugate (31)

[0472] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3 of Example 30, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 5.
[0473] Antibody concentration: 1.90 mg/mL, antibody yield: 11.40 mg (91%), and average number of conjugated drug molecules (n) per antibody molecule: 6.7.

Example 33 Antibody-drug conjugate (32)

[0474]

[Formula 102]

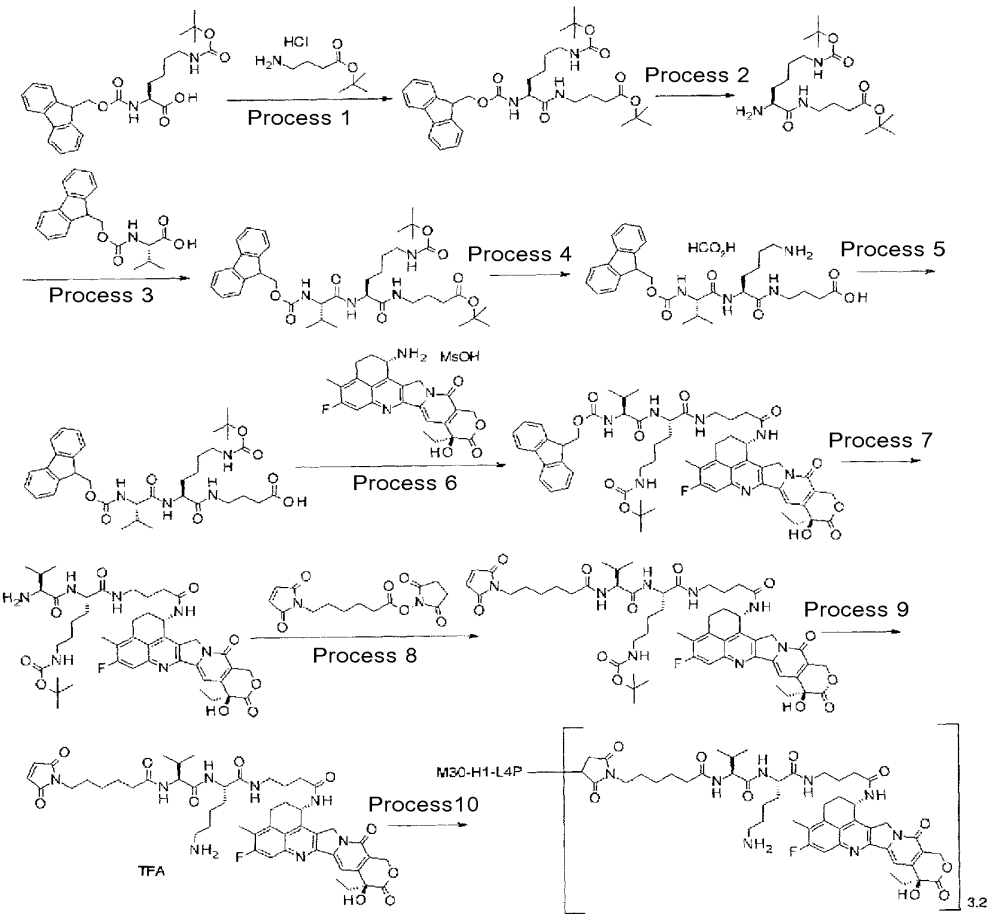


[0475] Almost the whole amounts of the antibody-drug conjugates of Examples 31 and 32 were mixed. The solution was concentrated by the Common procedure A to yield the titled antibody-drug conjugate.
Antibody concentration: 10.0 mg/mL, antibody yield: 21.06 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.0.

Example 34 Antibody-drug conjugate (33)

[0476]

[Formula 103]



Process 1: Tert-butyl 4-({N^6-(tert-butoxycarbonyl)-N^2-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysyl}amino)butanoate

[0477] To an N,N-dimethyl formamide (10.0 mL) solution of N^{ε}-(tert-butoxycarbonyl)-N^{α}-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysine (1.00 g, 2.14 mmol), N-hydroxysuccinimide (0.370 g, 3.20 mmol), and tert-butyl 4-aminobutanoic acid ester hydrochloride (0.830 g, 4.27 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.610 g, 3.20 mmol) and N,N-diisopropylethylamine (0.410 ml, 2.35 mmol) were added and stirred at room temperature for 3 days. The reaction solution was diluted with ethyl acetate and washed with an aqueous solution of 10% citric acid and a saturated aqueous solution of sodium hydrogen carbonate, and saturated brine, and then the organic layer was dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to yield the titled compound as a colorless solid (1.35 g, quantitative).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.14-1.42 (4H, m), 1.36 (9H, s), 1.37 (9H, s), 1.48-1.67 (4H, m), 2.18 (2H, t, J=7.6 Hz), 2.84-2.93 (2H, m), 2.99-3.11 (2H, m), 3.84-3.94 (1H, m), 4.18-4.30 (3H, m), 6.76 (1H, t, J=5.4 Hz), 7.33 (2H, t, J=7.3 Hz), 7.39-7.45 (3H, m), 7.73 (2H, dd, J=7.3, 2.7 Hz), 7.85-7.92 (3H, m).

Process 2: Tert-butyl 4-{[N^6-(tert-butoxycarbonyl)-L-lysyl]amino}butanoate

[0478] To an N,N-dimethyl formamide (8.00 mL) solution of the compound (1.35 g, 2.22 mmol) obtained in Process 1, piperidine (2.00 mL) was added and stirred for 1.5 hours at room temperature. The solvent was removed under reduced pressure to yield a mixture containing the titled compound. The mixture was used for the next reaction without performing further purification.

Process 3: N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-valyl-N^6-(tert-butoxycarbonyl)-N-(4-tert-butoxy-4-oxobutyl)-L-lysinamide

[0479] To an N,N-dimethyl formamide (30.0 mL) solution of the mixture (2.22 mmol) obtained in Process 2, N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-valine (1.13 g, 3.32 mmol), N-hydroxysuccinimide (0.310 g, 2.66 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.550 g, 2.88 mmol) were added and stirred at room temperature for 18 hours. The reaction solution was diluted with ethyl acetate and washed with a saturated aqueous solution of sodium hydrogen carbonate and saturated brine, and then the organic layer was dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform: methanol = 9 : 1 (v/v)] to yield the titled compound as a colorless solid (0.363 g, 23%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.84 (6H, t, J=6.0 Hz), 1.12-1.64 (8H, m), 1.34 (9H, s), 1.38 (9H, s), 1.90-2.04 (1H, m), 2.17 (2H, t, J=7.3 Hz), 2.79-2.90 (2H, m), 2.99-3.09 (2H, m), 3.83-3.91 (1H, m), 4.08-4.44 (4H, m), 6.71 (1H, t, J=5.4 Hz), 7.32 (2H, t, J=7.3 Hz), 7.42 (3H, t, J=7.3 Hz), 7.74 (2H, t, J=7.0 Hz), 7.85-7.91 (4H, m).
MS (ESI) m/z: 709 $(M+H)^+$

Process 4: N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-valyl-N-(3-carboxypropyl)-L-lysinamide formate

[0480] To the compound (0.363 mg, 0.512 mmol) obtained in Process 3, formic acid (10.0 ml) was added and stirred at room temperature for 4 hours. The solvent was removed under reduced pressure to yield the titled compound. The compound was used for the next reaction without performing further purification.

Process 5: N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-valyl-N^6-(tert-butoxycarbonyl)-N-(3-carboxypropyl)-L-lysinamide

[0481] To 1,4-dioxane (5.00 mL) suspension of the compound (0.512 mmol) obtained in Process 4, a saturated aqueous solution of sodium hydrogen carbonate (20.0 ml) and di-tert-butyl dicarbonate (0.178 ml, 0.769 mmol) were added and stirred at room temperature for 3 hours. The reaction solution was diluted with ethyl acetate and washed with an aqueous solution of 10% citric acid and saturated brine, and then the organic layer was dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to yield the titled compound as a colorless solid (0.295 g, 88%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.84 (6H, t, J=6.7 Hz), 1.13-1.39 (4H, m), 1.35 (9H, s), 1.48-1.62 (4H, m), 1.91-2.04 (1H, m), 2.20 (2H, t, J=7.3 Hz), 2.80-2.89 (2H, m), 2.99-3.11 (2H, m), 3.87 (1H, dd, J=8.5, 6.7 Hz), 4.06-4.35 (4H, m), 6.71 (1H, t, J=6.0 Hz), 7.32 (2H, t, J=7.6 Hz), 7.39-7.46 (3H, m), 7.74 (2H, t, J=7.6 Hz), 7.83-7.94 (4H, m).
MS (ESI) m/z: 653 $(M+H)^+$

Process 6: N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-valyl-N^6-(tert-butoxycarbonyl)-N-(4-{ [(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)-L-lysinamide

[0482] Mesylate of the compound (4) (0.240 g, 0.452 mmol) was reacted in the same manner as Process 1 of Example 1 by using the compound (0.295 g, 0.452 mmol) obtained in Process 5 instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a pale orange solid (0.208 g, 43%).
MS (ESI) m/z: 1071 $(M+H)^+$

Process 7: L-Valyl-N^6-(tert-butoxycarbonyl)-N-(4-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)-L-lysinamide

[0483] The compound (0.208 g, 0.194 mmol) obtained in Process 6 was reacted in the same manner as Process 2 to yield a mixture containing the titled compound. The mixture was used for the next reaction without performing further purification.

Process 8: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-valyl-N^6-(tert-butoxycarbonyl)-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]amino}-4-oxobutyl)-L-lysinamide

[0484] The mixture (0.194 mmol) obtained in Process 7 was reacted in the same manner as Process 3 of Example 2 to yield the titled compound as a pale yellow solid (0.133 g, 56%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.77 (6H, t, J=5.7 Hz), 0.87 (3H, t, J=7.3 Hz), 1.14-1.71 (10H, m), 1.35 (9H, s), 1.77-1.95

(3H, m), 2.02-2.23 (7H, m), 2.40 (3H, s), 2.84 (3H, q, J=6.4 Hz), 3.05 (2H, d, J=6.7 Hz), 3.17 (2H, s), 3.26-3.39 (3H, m), 4.01-4.16 (2H, m), 5.15 (1H, d, J=18.7 Hz), 5.24 (1H, d, J=18.7 Hz), 5.36-5.48 (2H, m), 5.51-5.60 (1H, m), 6.52 (1H, s), 6.72 (1H, t, J=6.0 Hz), 6.99 (2H, s), 7.31 (1H, s), 7.71-7.85 (5H, m), 8.41 (1H, d, J=9.1 Hz).
MS (ESI) m/z: 1041 $(M+H)^+$

Process 9: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-valyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]aminol-4-oxobutyl)-L-lysinamide trifluoroacetate

[0485] To dichloromethane (10.0 ml) solution of the compound (0.110 mg, 0.106 mmol) obtained in Process 8, trifluoroacetic acid (4.00 ml) was added and stirred for 5 hours at room temperature. The solvent was removed under reduced pressure to yield the titled compound as a pale yellow solid (70.0 mg, 64%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.76-0.81 (6H, m), 0.87 (3H, t, J=7.3 Hz), 1.12-1.31 (4H, m), 1.39-1.56 (8H, m), 1.57-1.74 (3H, m), 1.79-1.96 (3H, m), 2.06-2.18 (7H, m), 2.40 (3H, s), 2.70-2.80 (2H, m), 3.01-3.10 (2H, m), 3.13-3.22 (2H, m), 4.04 (1H, t, J=7.6 Hz), 4.10-4.20 (1H, m), 5.15 (1H, d, J=18.7 Hz), 5.24 (1H, d, J=18.7 Hz), 5.36-5.47 (2H, m), 5.52-5.60 (1H, m), 6.53 (1H, s), 7.00 (2H, s), 7.32 (1H, s), 7.61 (3H, brs), 7.75-7.88 (4H, m), 8.43 (1H, d, J=8.5 Hz).
MS (ESI) m/z: 941 $(M+H)^+$

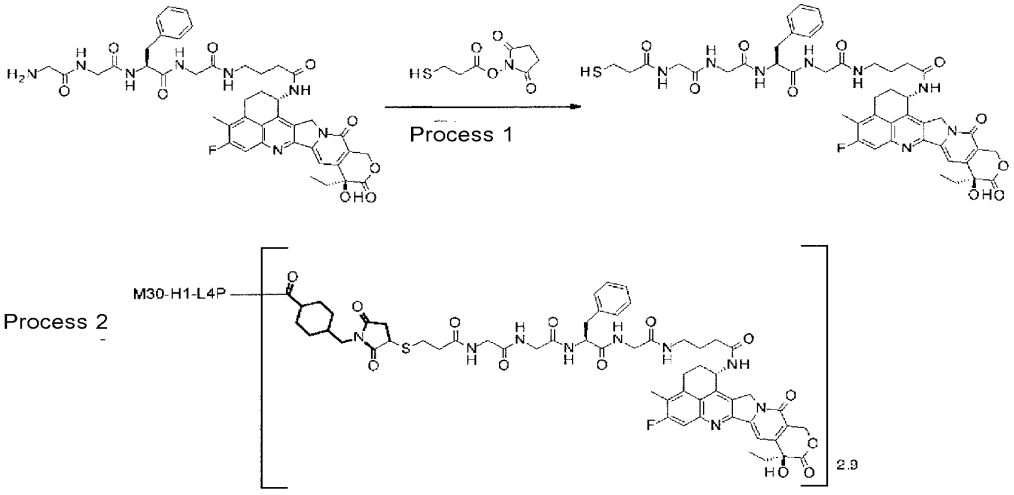
Process 10: Antibody-drug conjugate (33)

[0486] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 9, the titled antibody-drug conjugate was obtained in the same manner as Process 2 of Example 29. Antibody concentration: 12.0 mg/mL, antibody yield: 8.4 mg (67%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 35 Antibody-drug conjugate (34)

[0487]

[Formula 104]



Process 1: N-(3-sulfanylpropanoyl)glycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0488] The compound (84.0 mg, 0.100 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using N-succinimidyl 3-mercaptopropionate instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (61.2 mg, 66%).

^{1}H-NMR (DMSO-D_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.77-1.66 (2H, m), 1.79-1.92 (2H, m), 2.07-2.24 (4H, m), 2.31-2.47 (3H, m), 2.40 (3H, s), 2.59-2.69 (2H, m), 2.78 (1H, dd, J=13.7, 9.8 Hz), 2.98-3.13 (3H, m), 3.14-3.23 (2H, m), 3.54-3.79 (6H, m), 4.40-4.50 (1H, m), 5.20 (2H, dd, J=36.8, 19.2 Hz), 5.36-5.47 (2H, m), 5.52-5.63 (1H, m), 6.54 (1H, s), 7.14-7.28 (5H, m), 7.31 (1H, s), 7.68-7.74 (1H, m), 7.80 (1H, d, J=10.9 Hz), 8.03-8.09 (1H, m), 8.13 (1H, d, J=7.8 Hz), 8.19-8.29 (2H, m), 8.46 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 927 $(M+H)^+$

Process 2: Antibody-drug conjugate (34)

[0489] SMCC derivatization of antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 20 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used). The solution (0.25 mL) was placed in a 1.5 mL tube, charged with DMSO solution (0.0063 mL; which corresponds to about 2.55 equivalents per antibody molecule) containing 27.6 mM succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Thermo Fisher Scientific Inc.) at room temperature, and reacted at room temperature for 2 hours. This reaction solution was subjected to purification using the Common procedure D-2 to yield 0.7 mL of a solution containing about 5 mg of the SMCC-derivatized antibody.

[0490] Conjugation between antibody and drug linker: After adding DMSO (0.045 mL) and a DMSO solution containing 10 mM of the compound obtained in Process 1 (0.015 mL; which corresponds to about 2.4 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 16 hours.

[0491] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 3.5 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

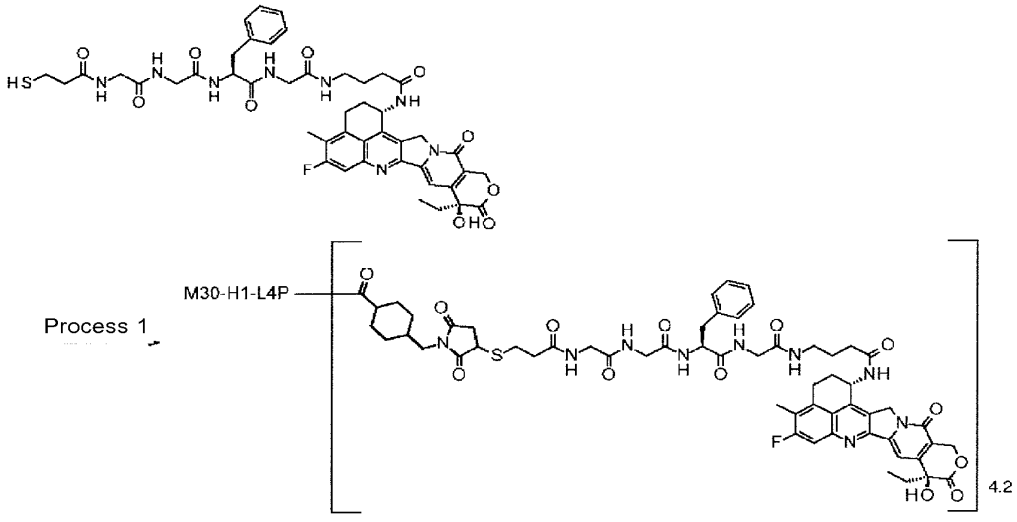
[0492] Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0493] Antibody concentration: 3.85 mg/mL, antibody yield: 0.8 mg (16%), and average number of conjugated drug molecules (n) per antibody molecule: 2.9.

Example 36 Antibody-drug conjugate (35)

[0494]

[Formula 105]



Process 1: Antibody-drug conjugate (35)

[0495] SMCC derivatization of antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 20 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used). The solution (0.25 mL) was placed in a 1.5 mL tube, charged with DMSO solution (0.0125 mL; which corresponds to about 5.1 equivalents per antibody molecule) containing 27.6 mM succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Thermo Fisher Scientific Inc.) at room temperature, and reacted at room temperature for 2 hours. This reaction solution was subjected to purification using the Common procedure D-2 to yield 0.7 mL of a solution containing about 5 mg of the SMCC-derivatized antibody.

[0496] Conjugation between antibody and drug linker: After adding DMSO (0.03 mL) and a DMSO solution containing 10 mM of the compound obtained in Process 1 of Example 35 (0.03 mL; which corresponds to about 4.8 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 16 hours.

[0497] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 3.5 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

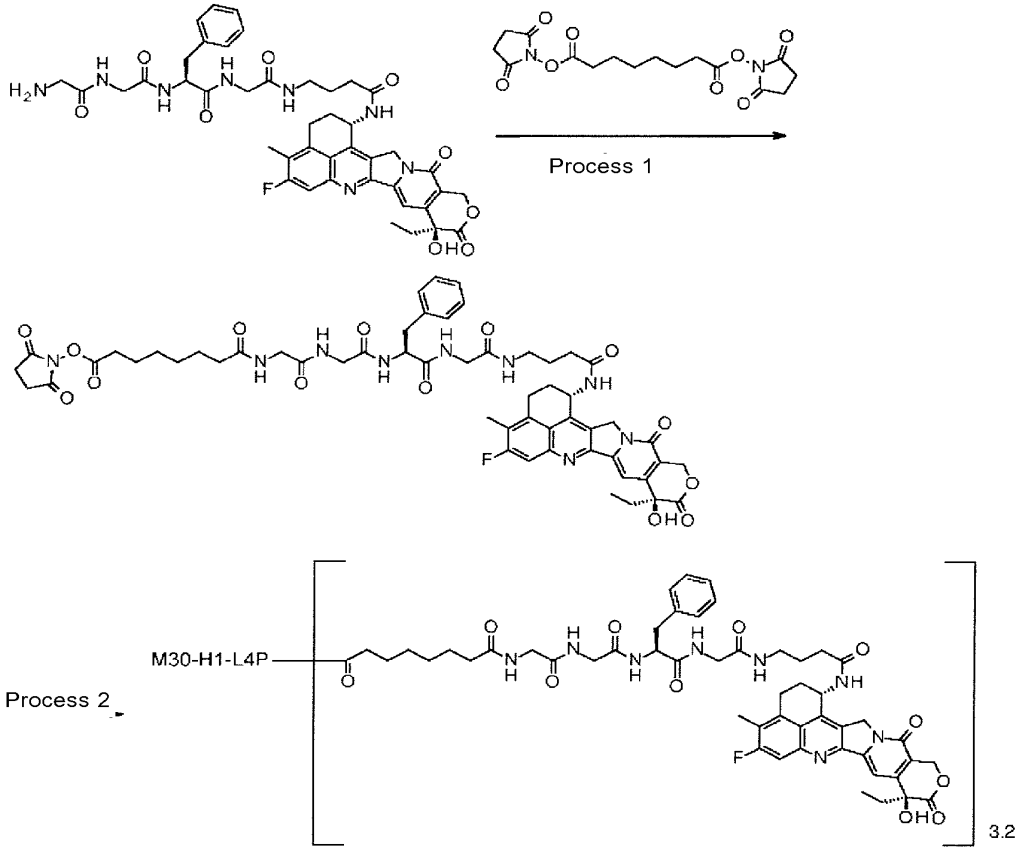
[0498] Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,\ 370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0499] Antibody concentration: 2.43 mg/mL, antibody yield: 0.5 mg (10%), and average number of conjugated drug molecules (n) per antibody molecule: 4.2.

Example 37 Antibody-drug conjugate (36)

[0500]

[Formula 106]



Process 1: N-{8-[(2,5-dioxopyrrolidin-1-yl)oxy]-8-oxooctanoyl}glycylglycyl-L-phenylalanyl-N-(4-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-4-oxobutyl)glycinamide

[0501] The compound (84.0 mg, 0.100 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using di(N-succinimidyl) suberate instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (77.1 mg, 71%).
^{1}H-NMR (DMSO-D_6) δ: 0.87 (3H, t, J=7.2 Hz), 1.21-1.38 (4H, m), 1.43-1.50 (2H, m), 1.55-1.63 (2H, m), 1.68-1.76 (2H, m), 1.80-1.91 (2H, m), 2.07-2.22 (6H, m), 2.40 (3H, s), 2.60-2.67 (2H, m), 2.76-2.84 (5H, m), 2.97-3.22 (5H, m), 3.56-3.76 (6H, m), 4.40-4.50 (1H, m), 5.20 (2H, q, J=18.8 Hz), 5.37-5.48 (2H, m), 5.53-5.62 (1H, m), 6.54 (1H, s), 7.15-7.28 (5H, m), 7.31 (1H, s), 7.71 (1H, t, J=5.5 Hz), 7.80 (1H, d, J=10.9 Hz), 8.04 (1H, t, J=5.9 Hz), 8.09 (1H, t, J=5.9 Hz), 8.14 (1H, d, J=7.8 Hz), 8.26 (1H, t, J=5.9 Hz), 8.47 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 1092 $(M+H)^+$

Process 2: Antibody-drug conjugate (36)

[0502] Conjugation between antibody and drug linker: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 20 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used). The solution (0.25 mL) was placed in a 1.5 mL tube, charged with DMSO solution containing 10 mM of the compound obtained in Process 1 (0.025 mL; which corresponds to about 3.7 equivalents per antibody molecule) at room temperature, and stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 16 hours. Purification: The above solution was subjected to purification using the

Common procedure D-1 (ABS was used as buffer solution) to yield 3.5 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

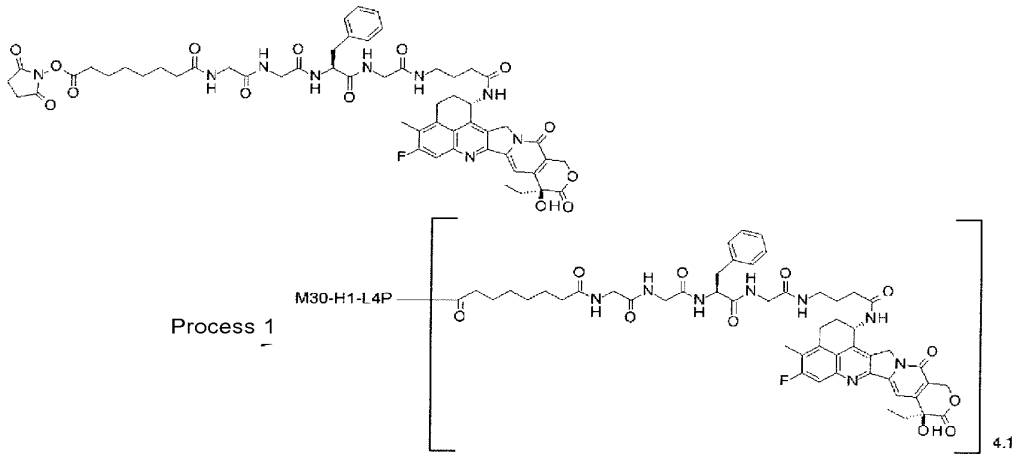
[0503] Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0504] Antibody concentration: 6.25 mg/mL, antibody yield: 1.3 mg (26%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 38 Antibody-drug conjugate (37)

[0505]

[Formula 107]



Process 1: Antibody-drug conjugate (37)

[0506] Conjugation between antibody and drug linker: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 20 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used). The solution (0.5 mL) was placed in a 1.5 mL tube, thereafter charged with DMSO solution containing DMSO (0.025 mL) and 10 mM of the compound obtained in Process 1 of Example 37 (0.025 mL; which corresponds to about 7.4 equivalents per antibody molecule) at room temperature, and stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 16 hours.

[0507] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 3.5 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

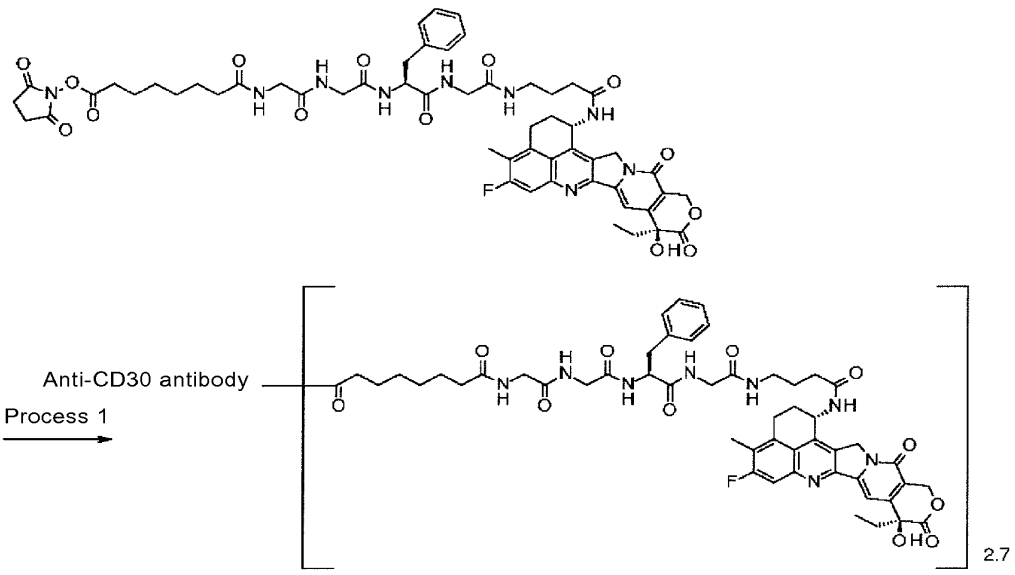
[0508] Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0509] Antibody concentration: 4.36 mg/mL, antibody yield: 0.9 mg (18%), and average number of conjugated drug molecules (n) per antibody molecule: 4.1.

Example 39 Antibody-drug conjugate (38)

[0510]

[Formula 108]



Process 1: Antibody-drug conjugate (38)

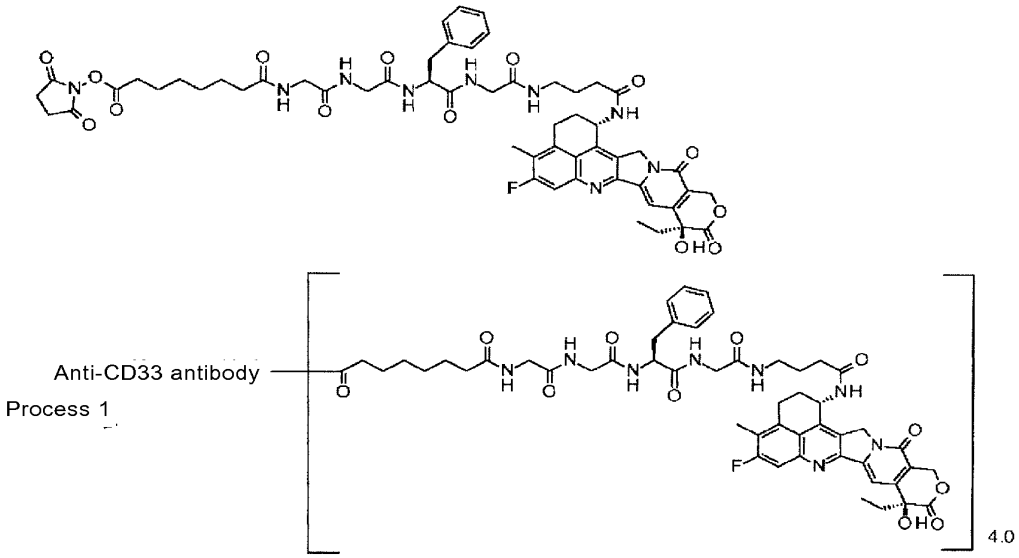
[0511] Conjugation between antibody and drug linker: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.75 $mLmg^{-1}cm^{-1}$ was used). The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube, thereafter charged with DMSO (0.017 mL) and a DMSO solution containing 10 mM of the compound obtained in Process 1 of Example 37 (0.023 mL; which corresponds to 9 equivalents per antibody molecule) at room temperature, and stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 4 hours.

[0512] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 2.5 mL of a solution containing the titled antibody-drug conjugate. Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 270400 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,\ 280}$ = 2670 (measured value), and $\varepsilon_{D,370}$ = 15820 (measured value) were used), the following characteristic values were obtained. Antibody concentration: 0.55 mg/mL, antibody yield: 1.4 mg (34%), and average number of conjugated drug molecules (n) per antibody molecule: 2.7.

Example 40 Antibody-drug conjugate (39)

[0513]

[Formula 109]



Process 1: Antibody-drug conjugate (39)

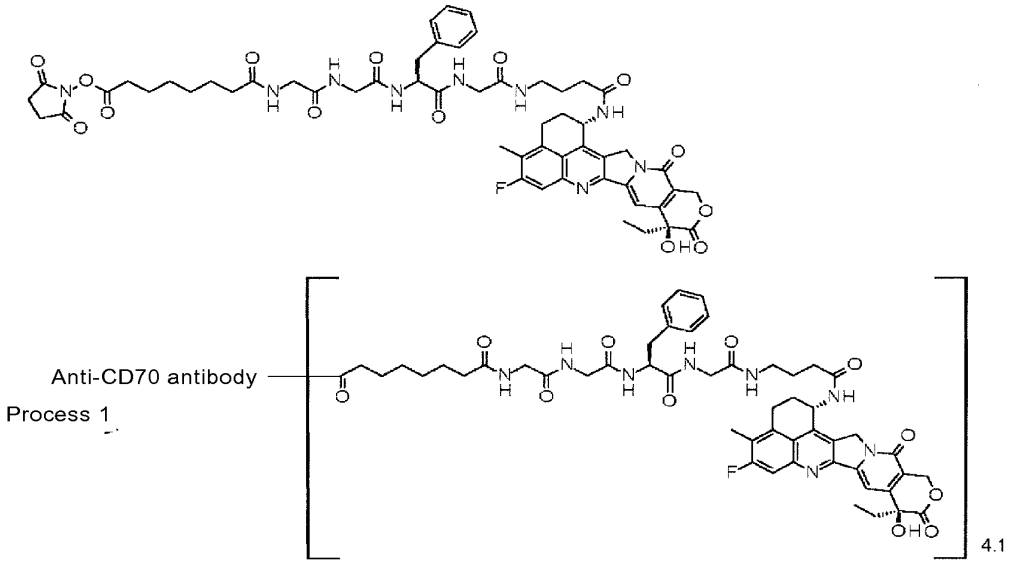
[0514] Conjugation between antibody and drug linker: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.66 $mLmg^{-1}cm^{-1}$ was used). The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube, thereafter charged with DMSO (0.017 mL) and a DMSO solution containing 10 mM of the compound obtained in Process 1 of Example 37 (0.023 mL; which corresponds to 9 equivalents per antibody molecule) at room temperature, and stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 4 hours.

[0515] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 2.5 mL of a solution containing the titled antibody-drug conjugate. Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 256400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 2670 (measured value), and $\varepsilon_{D,370}$ = 15820 (measured value) were used), the following characteristic values were obtained. Antibody concentration: 0.93 mg/mL, antibody yield: 2.3 mg (58%), and average number of conjugated drug molecules (n) per antibody molecule: 4.0.

Example 41 Antibody-drug conjugate (40)

[0516]

[Formula 110]



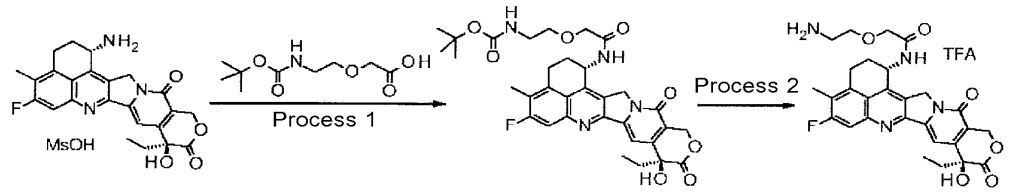
Process 1: Antibody-drug conjugate (40)

[0517] Conjugation between antibody and drug linker: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.5/EDTA by using the Common procedure C-2 and Common procedure B (as absorption coefficient at 280 nm, 1.69 $mLmg^{-1}cm^{-1}$ was used). The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube, thereafter charged with DMSO (0.017 mL) and a DMSO solution containing 10 mM of the compound obtained in Process 1 (0.023 mL; which corresponds to 9 equivalents per antibody molecule) of Example 37 at room temperature, and stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 4 hours.

[0518] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) to yield 2.5 mL of a solution containing the titled antibody-drug conjugate. Physicochemical characterization: By using the Common procedure E (as molar absorption coefficient, $\varepsilon_{A,280}$ = 262400 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 2670 (measured value), and $\varepsilon_{D,370}$ = 15820 (measured value) were used), the following characteristic values were obtained. Antibody concentration: 1.04 mg/mL, antibody yield: 2.6 mg (65%), and average number of conjugated drug molecules (n) per antibody molecule: 4.1.

[0519] Example 42 2-(2-Aminoethoxy)-N-[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]acetamide

[Formula 111]



[0520] Process 1: Tert-butyl [2-(2-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]amino}-2-oxoethoxy)ethyl]carbamate

[0521] Mesylate of the compound (4) (3.10 g, 5.47 mol) was reacted in the same manner as Process 1 of Example 1 by using {2-[(tert-butoxycarbonyl)amino]ethoxy}acetic acid (J. Med. Chem., 1992, vol. 35, pp. 2928) (1.55 g, 6.01 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a pale yellow solid (2.56 g, 73%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.3 Hz), 1.26 (9H, s), 1.81-1.91 (2H, m), 2.13-2.22 (2H, m), 2.40 (3H,

s), 3.08-3.26 (4H, m), 3.43-3.53 (2H, m), 4.00 (1H, d, J=15.1 Hz), 4.05 (1H, d, J=15.1 Hz), 5.14 (1H, d, J=18.7 Hz), 5.22 (1H, d, J=18.7 Hz), 5.40 (1H, d, J=16.6 Hz), 5.44 (1H, d, J=16.6 Hz), 5.59-5.66 (1H, m), 6.53 (1H, s), 6.86 (1H, t, J=5.4 Hz), 7.31 (1H, s), 7.79 (1H, d, J=10.9 Hz), 8.49 (1H, d, J=9.1 Hz).
MS (APCI) m/z: 637 $(M+H)^+$

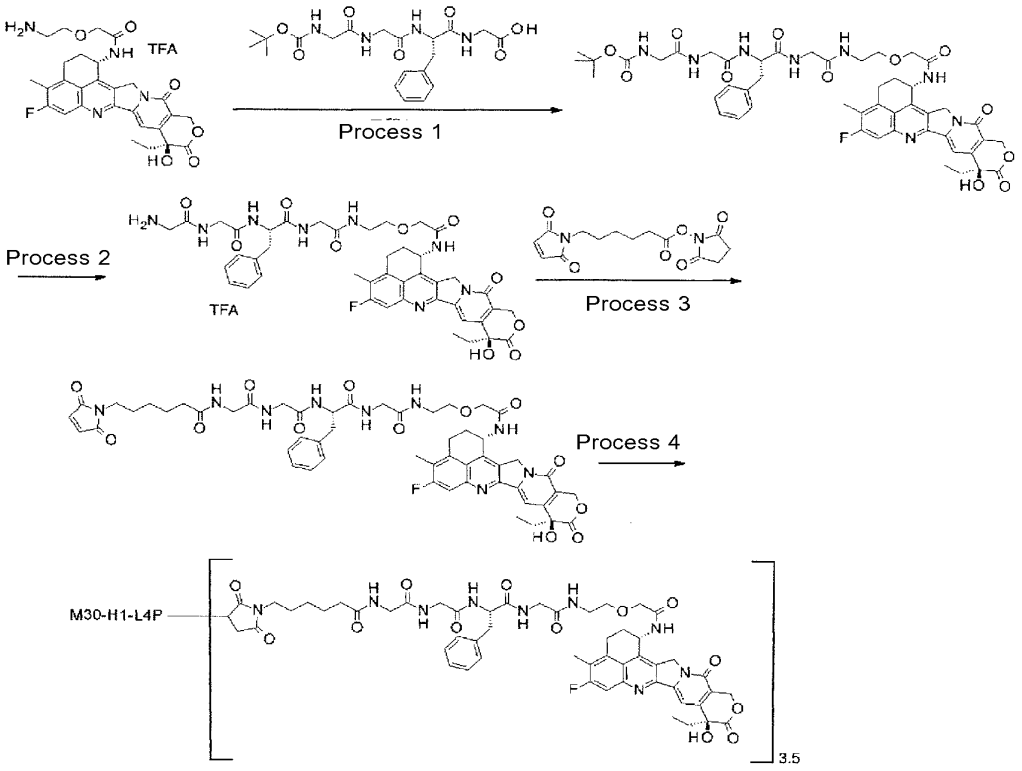
Process 2: 2-(2-Aminoethoxy)-N-[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]acetamide

[0522] The compound (1.50 g, 2.36 mol) obtained in Process 1 was reacted in the same manner as Process 2 of Example 1 to yield trifluorohydrochloride of the titled compound as a pale yellow solid (1.50 g, quantitative). This compound was confirmed in the tumor of a cancer-bearing mouse that received the antibody-drug conjugate (41).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.5 Hz), 1.81-1.92 (2H, m), 2.15-2.23 (2H, m), 2.41 (3H, s), 3.05 (2H, t, J=5.1 Hz), 3.15-3.23 (2H, m), 3.71 (2H, t, J=5.1 Hz), 4.10 (2H, s), 5.19 (1H, d, J=18.7 Hz), 5.24 (1H, d, J=18.7 Hz), 5.43 (2H, s), 5.58-5.66 (1H, m), 6.55 (1H, s), 7.33 (1H, s), 7.73-7.84 (4H, m), 8.55 (1H, d, J=9.1 Hz).
MS (APCI) m/z: 537 $(M+H)^+$

Example 43 Antibody-drug conjugate (41)

[0523]

[Formula 112]



[0524] Process 1: N-(Tert-butoxycarbonyl)glycylglycyl-L-phenylalanyl-N-[2-(2-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]aminol-2-oxoethoxy)ethyl]glycinamide The compound (554 mg, 0.85 mmol) of Example 42 was reacted in the same manner as Process 1 of Example 2 to yield the titled compound (775 mg, 95%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J=7.3 Hz), 1.36 (9H, s), 1.78-1.89 (2H, m), 2.13-2.22 (2H, m), 2.39 (3H, s), 2.71 (1H, dd, J=13.4, 9.8 Hz), 2.95 (1H, dd, J=13.4, 4.3 Hz), 3.09-3.23 (1H, m), 3.23-3.32 (2H, m), 3.40-3.62 (8H,

m), 3.73 (1H, dd, J=16.5, 5.5 Hz), 4.03 (2H, s), 4.39-4.47 (1H, m), 5.17 (1H, d, J=18.9 Hz), 5.25 (1H, d, J=18.9 Hz), 5.41 (1H, d, J=16.8 Hz), 5.45 (1H, d, J=16.8 Hz), 5.57-5.64 (1H, m), 6.54 (1H, s), 6.99 (1H, t, J=5.8 Hz), 7.13-7.26 (5H, m), 7.31 (1H, s), 7.76-7.82 (2H, m), 7.90 (1H, t, J=5.2 Hz), 8.13 (1H, d, J=7.9 Hz), 8.27 (1H, t, J=5.8 Hz), 8.49 (1H, d, J=8.5 Hz).
MS (APCI) m/z: 955 $(M+H)^+$

Process 2: Glycylglycyl-L-phenylalanyl-N-[2-(2-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]amino}-2-oxoethoxy)ethyl]glycinamide trifluoroacetate

[0525] The compound (630 mg, 0.659 mmol) obtained in Process 1 was reacted in the same manner as Process 2 of Example 2 to yield the titled compound (588 mg, 92%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.3 Hz), 1.79-1.90 (2H, m), 2.13-2.22 (2H, m), 2.39 (3H, s), 2.71 (1H, dd, J=13.4, 10.1 Hz), 2.99 (1H, dd, J=13.4, 4.3 Hz), 3.09-3.23 (1H, m), 3.24-3.32 (3H, m), 3.41-3.71 (7H, m), 3.86 (1H, dd, J=16.8, 5.8 Hz), 4.04 (2H, s), 4.52 (1H, td, J=9.0, 4.1 Hz), 5.17 (1H, d, J=18.9 Hz), 5.25 (1H, d, J=18.9 Hz), 5.41 (1H, d, J=16.5 Hz), 5.45 (1H, d, J=16.5 Hz), 5.56-5.65 (1H, m), 6.55 (1H, s), 7.13-7.26 (5H, m), 7.32 (1H, s), 7.80 (1H, d, J=11.0 Hz), 7.87-8.01 (4H, m), 8.29-8.36 (2H, m), 8.46-8.55 (2H, m).
MS (APCI) m/z: 855 $(M+H)^+$

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-[2-(2-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)ethyl]glycinamide

[0526] The compound (240 mg, 0.247 mmol) obtained in Process 2 was reacted in the same manner as Process 3 of Example 2 to yield the titled compound (162 mg, 62%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.6 Hz), 1.13-1.22 (2H, m), 1.40-1.51 (4H, m), 1.78-1.90 (2H, m), 2.09 (2H, t, J=7.6 Hz), 2.14-2.21 (2H, m), 2.39 (3H, s), 2.74 (1H, dd, J=13.6, 9.7 Hz), 2.96 (1H, dd, J=13.6, 4.5 Hz), 3.08-3.24 (1H, m), 3.24-3.30 (1H, m), 3.33-3.40 (4H, m), 3.47-3.68 (7H, m), 3.72 (1H, dd, J=16.6, 5.7 Hz), 4.03 (2H, s), 4.42 (1H, td, J=8.6, 4.2 Hz), 5.17 (1H, d, J=18.7 Hz), 5.25 (1H, d, J=18.7 Hz), 5.40 (1H, d, J=17.2 Hz), 5.44 (1H, d, J=17.2 Hz), 5.57-5.64 (1H, m), 6.52 (1H, s), 6.99 (2H, s), 7.13-7.25 (5H, m), 7.31 (1H, s), 7.74-7.81 (2H, m), 7.99 (1H, t, J=5.7 Hz), 8.03-8.11 (2H, m), 8.22 (1H, t, J=5.7 Hz), 8.47 (1H, d, J=9.1 Hz).
MS (APCI) m/z: 1048 $(M+H)^+$

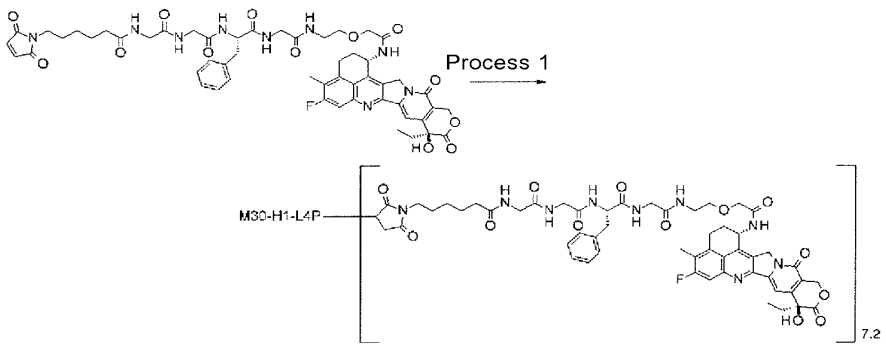
Process 4: Antibody-drug conjugate (41)

[0527] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3, the titled antibody-drug conjugate was obtained in the same manner as Process 2 of Example 29. Antibody concentration: 12.0 mg/mL, antibody yield: 8.4 mg (67%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

Example 44 Antibody-drug conjugate (42)

[0528]

[Formula 113]



Process 1: Antibody-drug conjugate (42)

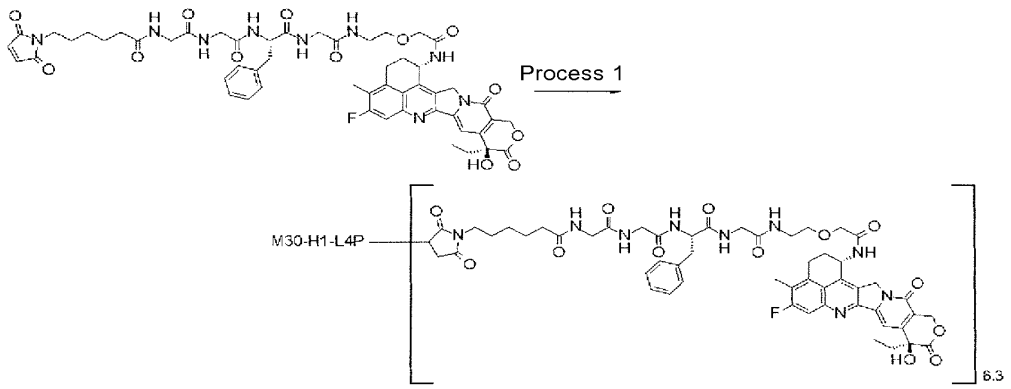
[0529] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3 of Example 43, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 5.

[0530] Antibody concentration: 0.83 mg/mL, antibody yield: 4.98 mg (40%), and average number of conjugated drug molecules (n) per antibody molecule: 7.2.

Example 45 Antibody-drug conjugate (43)

[0531]

[Formula 114]



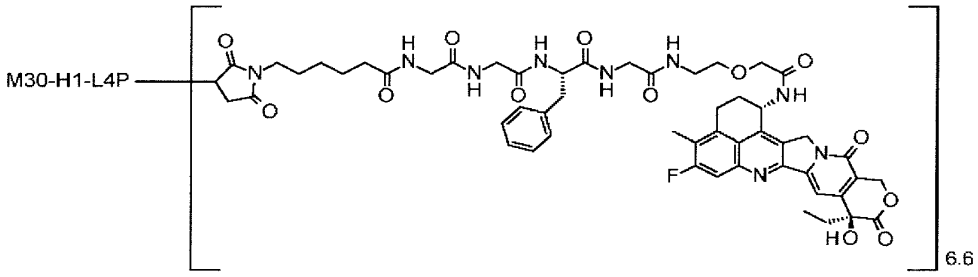
Process 1: Antibody-drug conjugate (43)

[0532] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3 of Example 43, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 4. Antibody concentration: 1.06 mg/mL, antibody yield: 6.36 mg (51%), and average number of conjugated drug molecules (n) per antibody molecule: 6.3.

Example 46 Antibody-drug conjugate (44)

[0533]

[Formula 115]

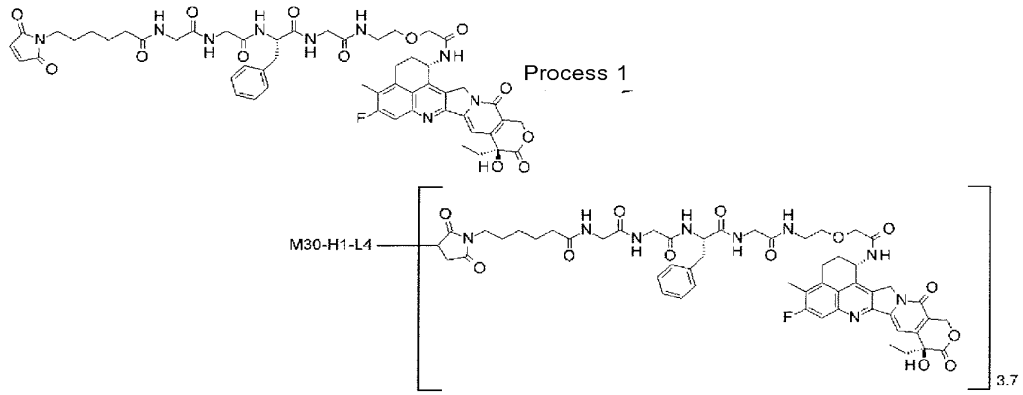


[0534] Almost the whole amounts of the antibody-drug conjugates of Examples 44 and 45 were mixed. The solution was concentrated by the Common procedure A to yield the titled antibody-drug conjugate.
Antibody concentration: 10.0 mg/mL, antibody yield: 10.21 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.6.

Example 47 Antibody-drug conjugate (45)

[0535]

[Formula 116]



[0536] Process 1: Antibody-drug conjugate (45)

[0537] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0538] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (0.0267 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 3 of Example 43 (0.0439 mL; 5.2 equivalents per antibody molecule) to the above solution at room temperature, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 1 hour. Next, an aqueous solution (0.0066 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0539] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

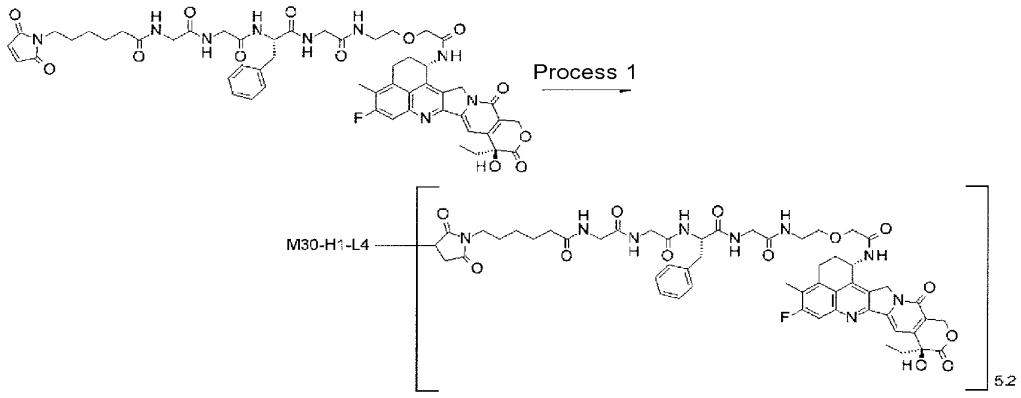
[0540] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\ 280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5193 (measured value), and $\varepsilon_{D,\ 370}$ = 20347 (measured value) were used), the following characteristic values were obtained.

[0541] Antibody concentration: 10.0 mg/mL, antibody yield: 9.3 mg (74%), and average number of conjugated drug molecules (n) per antibody molecule: 3.7.

Example 48 Antibody-drug conjugate (46)

[0542]

[Formula 117]



Process 1: Antibody-drug conjugate (46)

[0543] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0439 mL; 5.2 equivalents per antibody molecule) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0544] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution (0.0726 mL; 8.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 3 of Example 43 to the above solution at room temperature, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 1 hour. Next, an aqueous solution (0.011 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

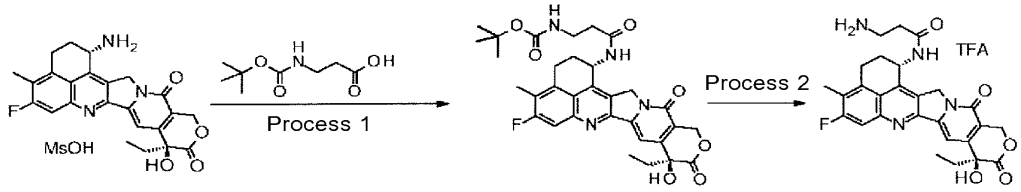
[0545] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

[0546] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\ 370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5193 (measured value), and $\varepsilon_{D,\ 370}$ = 20347 (measured value) were used), the following characteristic values were obtained.

[0547] Antibody concentration: 10.0 mg/mL, antibody yield: 7.8 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 5.2.

[0548] Example 49 N-[(1S, 9S)-9-Ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]-β-alaninamide

[Formula 118]



Process 1: Tert-butyl (3-{[(1S, 9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3', 4':6, 7]indolizino[1, 2-b]quinolin-1-yl]amino}-3-oxopropyl)carbamate

[0549] Mesylate of the compound (4) (500 mg, 0.941 mmol) was reacted in the same manner as Process 1 of Example 1 by using N-(tert-butoxycarbonyl)-β-alanine instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a yellow-brown solid (616 mg, quantitative).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.2 Hz), 1.29 (9H, s), 1.86 (2H, dt, J=15.1, 7.3 Hz), 2.04-2.22 (2H, m), 2.31 (2H, t, J=6.8 Hz), 2.40 (3H, s), 3.10-3.26 (4H, m), 5.15 (1H, d, J=18.8 Hz), 5.26 (1H, d, J=19.2 Hz), 5.42 (2H, dd, J=18.8, 16.4 Hz), 5.57 (1H, dt, J=8.5, 4.2 Hz), 6.53 (1H, s), 6.78 (1H, t, J=5.5 Hz), 7.30 (1H, s), 7.80 (1H, d, J=11.0 Hz), 8.46 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 607 $(M+H)^+$

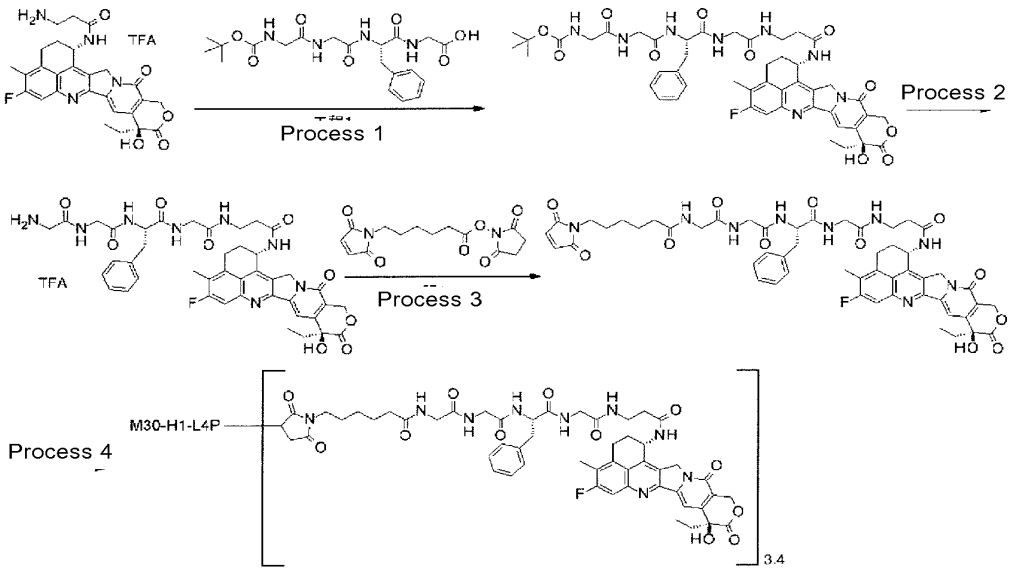
Process 2: N-[(1S, 9S)-9-Ethyl-5-fluoro-9-hydroxy-4-methyl-10, 13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0550] The compound obtained in Process 1 was reacted in the same manner as Process 2 of Example 1 to yield trifluoroacetate of the titled compound as a yellow solid (499 mg, 86%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.2 Hz), 1.86 (2H, dquin, J=14.6, 7.2, 7.2, 7.2, 7.2 Hz), 2.06-2.27 (1H, m), 2.41 (3H, s), 2.46-2.57 (2H, m), 3.08 (2H, t, J=6.8 Hz), 3.14-3.24 (2H, m), 5.22 (1H, d, J=18.8 Hz), 5.29 (1H, d, J=18.8 Hz), 5.43 (2H, s), 5.58 (1H, dt, J=8.5, 4.5 Hz), 6.55 (1H, s), 7.32 (1H, s), 7.74 (3H, brs), 7.82 (1H, d, J=11.0 Hz), 8.67 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 507 $(M+H)^+$

Example 50 Antibody-drug conjugate (47)

[0551]

[Formula 119]



Process 1: N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0552] The compound (484 mg, 0.780 mmol) of Example 49 was reacted in the same manner as Process 1 of Example 2 to yield the titled compound as a pale yellow solid (626 mg, 87%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.27-1.42 (9H, m), 1.77-1.93 (2H, m), 2.06-2.22 (2H, m), 2.36 (2H, t, J=7.2 Hz), 2.40 (3H, d, J=1.6 Hz), 2.44-2.54 (2H, m), 2.76 (1H, dd, J=14.5, 10.2 Hz), 3.02 (1H, dd, J=13.9, 4.5

Hz), 3.12-3.22 (2H, m), 3.52 (6H, d, J=6.3 Hz), 4.42-4.54 (1H, m), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.4 Hz), 5.42 (1H, dd, J=18.4, 16.4 Hz), 5.57 (1H, dt, J=8.7, 4.4 Hz), 6.53 (1H, s), 6.98 (1H, t, J=5.9 Hz), 7.14-7.28 (5H, m), 7.31 (1H, s), 7.77-7.84 (1H, m), 7.91 (1H, t, J=5.5 Hz), 8.16 (1H, d, J=7.8 Hz), 8.27 (1H, t, J=5.1 Hz), 8.52 (1H, d, J=9.0 Hz).

Process 2: Glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide trifluoroacetate

[0553] The compound (624 mg, 0.675 mmol) obtained in Process 1 was reacted in the same manner as Process 2 of Example 2 to yield the titled compound as a yellow solid (626 mg, 92%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, t, J=7.4 Hz), 1.86 (2H, tt, J=14.5, 7.2 Hz), 2.07-2.22 (2H, m), 2.36 (2H, t, J=7.2 Hz), 2.40 (3H, s), 2.44-2.54 (2H, m), 2.75 (1H, dd, J=13.7, 9.8 Hz), 3.04 (1H, dd, J=13.7, 4.3 Hz), 3.12-3.22 (2H, m), 3.58 (2H, d, J=4.7 Hz), 3.69 (3H, td, J=11.2, 5.7 Hz), 3.87 (1H, dd, J=17.0, 5.7 Hz), 4.54 (1H, m, J=17.8, 4.5 Hz), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.8 Hz), 5.43 (2H, s), 5.51-5.60 (1H, m), 6.55 (1H, s), 7.14-7.29 (5H, m), 7.32 (1H, s), 7.81 (1H, d, J=10.9 Hz), 7.88 (1H, t, J=5.7 Hz), 7.97 (3H, brs), 8.29-8.38 (2H, m), 8.50 (1H, t, J=5.7 Hz), 8.55 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 825 $(M+H)^+$

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0554] The compound (60.0 mg, 0.0646 mmol) obtained in Process 2 was reacted in the same manner as Process 3 of Example 2 to yield the titled compound as a solid (14.0 mg, 21%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.2 Hz), 1.12-1.22 (2H, m), 1.39-1.51 (4H, m), 1.79-1.91 (2H, m), 2.02-2.20 (2H, m), 2.07 (2H, t, J=7.4 Hz), 2.30-2.42 (4H, m), 2.40 (3H, s), 2.78 (1H, dd, J=14.1, 9.4 Hz), 3.02 (1H, dd, J=14.7, 4.9 Hz), 3.12-3.21 (2H, m), 3.26-3.42 (2H, m), 3.50-3.80 (6H, m), 4.40-4.51 (1H, m), 5.19 (1H, d, J=19.6 Hz), 5.26 (1H, d, J=19.2 Hz), 5.42 (2H, brs), 5.51-5.62 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.13-7.28 (5H, m), 7.31 (1H, s), 7.74-7.84 (2H, m), 8.01 (1H, t, J=5.3 Hz), 8.06 (1H, t, J=5.7 Hz), 8.14 (1H, d, J=8.2 Hz), 8.25 (1H, t, J=5.7 Hz), 8.53 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 1018 $(M+H)^+$

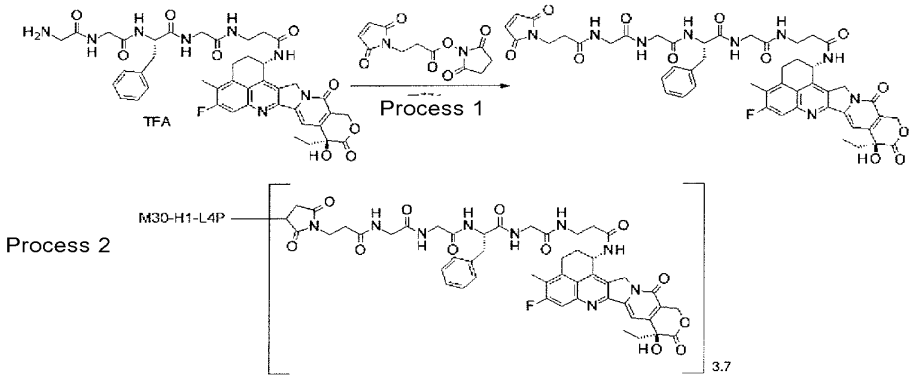
Process 4: Antibody-drug conjugate (47)

[0555] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 3, the titled antibody-drug conjugate was obtained in the same manner as Process 4 of Example 2. Antibody concentration: 12.27 mg/mL, antibody yield: 8.6 mg (69%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 51 Antibody-drug conjugate (48)

[0556]

[Formula 120]



Process 1: N-[3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoyl]glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0557] The compound (60.0 mg, 0.0646 mmol) obtained in Process 2 of Example 50 was reacted in the same manner as Process 3 of Example 2 by using N-succinimidyl 3-maleimide propionate instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a pale yellow solid (36.0 mg, 57%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.4 Hz), 1.85 (2H, dt, J=14.4, 7.5 Hz), 2.05-2.22 (2H, m), 2.40 (3H, s), 2.30-2.44 (5H, m), 2.73-2.84 (1H, m), 3.02 (1H, dd, J=13.9, 4.5 Hz), 3.17 (3H, d, J=5.1 Hz), 3.26-3.40 (2H, m), 3.41-3.81 (6H, m), 4.40-4.51 (1H, m), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.8 Hz), 5.42 (2H, brs), 5.52-5.61 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.13-7.28 (5H, m), 7.31 (1H, s), 7.80 (2H, d, J=10.2 Hz), 8.03 (1H, t, J=5.5 Hz), 8.12 (1H, d, J=8.2 Hz), 8.20-8.31 (2H, m), 8.52 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 976 $(M+H)^+$

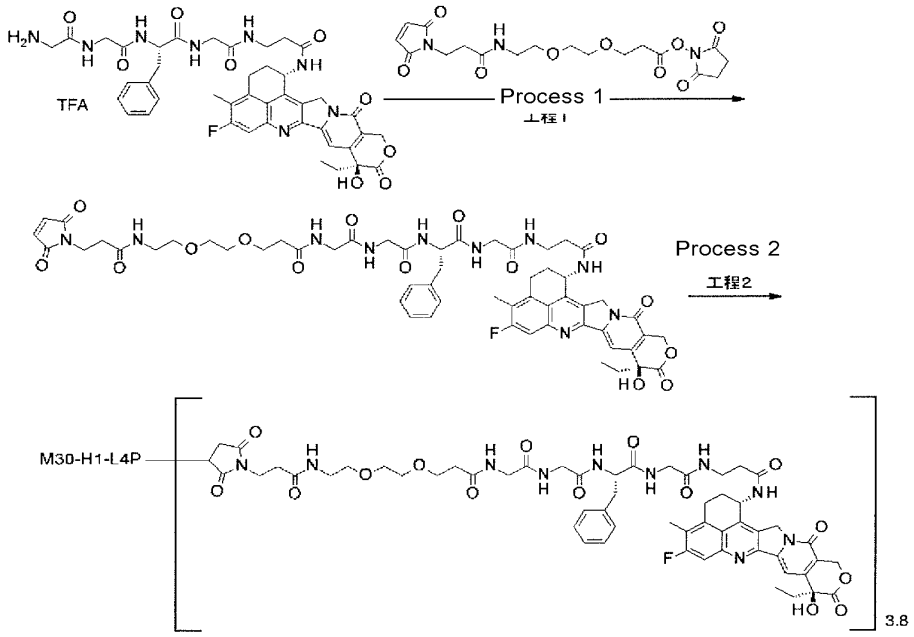
Process 2: Antibody-drug conjugate (48)

[0558] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1, the titled antibody-drug conjugate was obtained in the same manner as Process 4 of Example 2. Antibody concentration: 11.59 mg/mL, antibody yield: 8.1 mg (65%), and average number of conjugated drug molecules (n) per antibody molecule: 3.7.

Example 52 Antibody-drug conjugate (49)

[0559]

[Formula 121]



Process 1: N-{3-[2-(2-{[3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoyl]amino})ethoxy]propanoyl}glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-p-alaninamide

[0560] The compound (60.0 mg, 0.0646 mmol) obtained in Process 2 of Example 50 was reacted in the same manner as Process 3 of Example 2 by using N-succinimidyl 3-(2-(2-(3-maleinimidepropanamide)ethoxy)ethoxy)propanoate instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a solid (23.0 mg, 31%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.4 Hz), 1.77-1.92 (2H, m), 2.07-2.21 (2H, m), 2.27-2.42 (6H, m), 2.40 (3H, s), 2.74-2.84 (1H, m), 2.97-3.06 (1H, m), 3.09-3.21 (4H, m), 3.25-3.39 (6H, m), 3.45 (4H, s), 3.50-3.80 (8H, m), 4.41-4.51 (1H, m), 5.19 (1H, d, J=18.4 Hz), 5.26 (1H, m, J=18.4 Hz), 5.42 (2H, brs), 5.51-5.61 (1H, m), 6.54 (1H, s), 7.00 (2H, s), 7.13-7.28 (5H, m), 7.31 (1H, s), 7.74-7.87 (2H, m), 7.93-8.07 (2H, m), 8.09-8.21 (2H, m), 8.26 (1H, brs), 8.54 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 1135 $(M+H)^+$

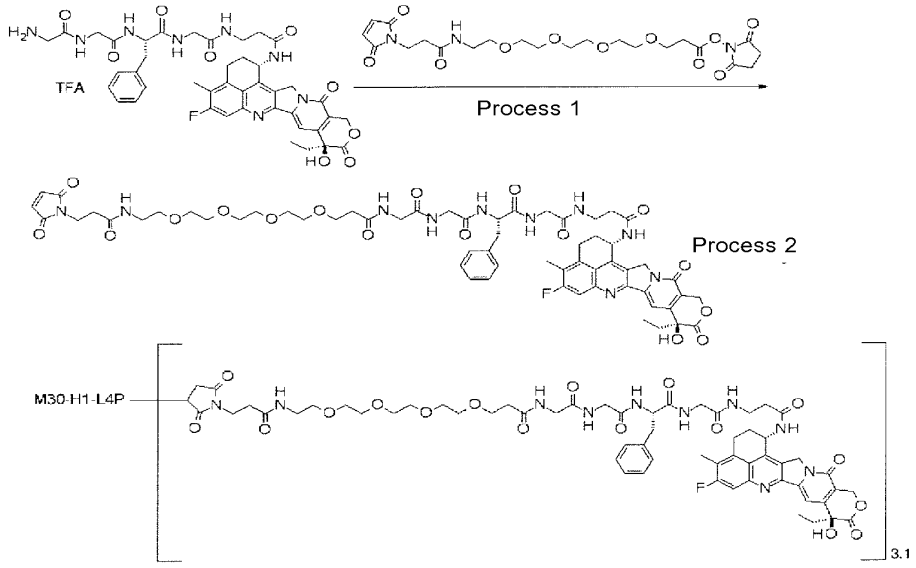
Process 2: Antibody-drug conjugate (49)

[0561] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1, the titled antibody-drug conjugate was obtained in the same manner as Process 2 of Example 29. Antibody concentration: 14.50 mg/mL, antibody yield: 10.2 mg (82%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 53 Antibody-drug conjugate (50)

[0562]

[Formula 122]



Process 1: N-[19-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)-17-oxo-4,7,10,13-tetraoxa-16-azanonadecan-1-oyl]glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0563] The compound (60.0 mg, 0.0646 mmol) obtained in Process 2 of Example 50 was reacted in the same manner as Process 3 of Example 2 by using N-succinimidyl 1-maleinimide-3-oxo-7,10,13,16-tetraoxa-4-azanonadecanoate instead of N-succinimidyl 6-maleimide hexanoate to yield the titled compound as a solid (23.0 mg, 29%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J=7.0 Hz), 1.85 (2H, tt, J=14.6, 7.1 Hz), 2.06-2.22 (2H, m), 2.40 (3H, s), 2.28-2.43 (6H, m), 2.78 (1H, dd, J=13.7, 9.4 Hz), 3.02 (1H, dd, J=14.1, 3.9 Hz), 3.09-3.22 (4H, m), 3.27-3.41 (4H, m), 3.47 (12H, d, J=8.6 Hz), 3.53-3.81 (10H, m), 4.41-4.51 (1H, m), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.8 Hz), 5.42 (2H, brs), 5.53-5.61 (1H, m), 6.54 (1H, s), 7.00 (2H, s), 7.12-7.29 (5H, m), 7.31 (1H, s), 7.74-7.85 (2H, m), 8.03 (2H, d, J=6.6 Hz), 8.11-8.21 (2H, m), 8.27 (1H, t, J=5.9 Hz), 8.54 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 1224 $(M+H)^+$

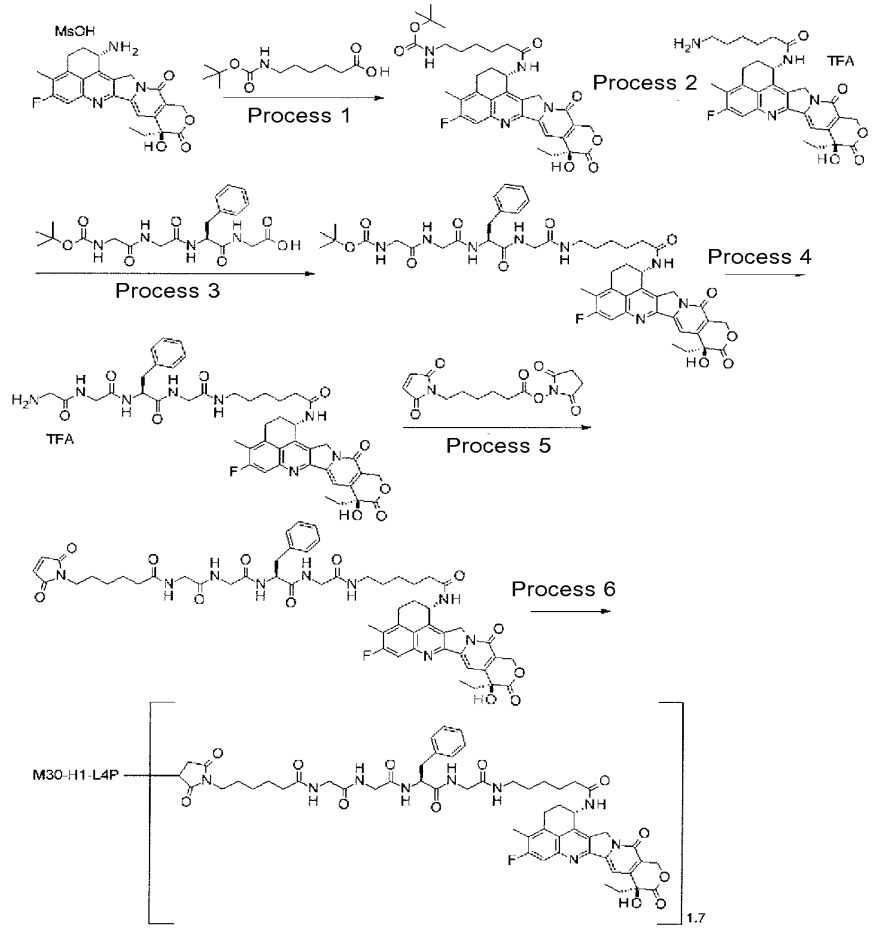
Process 2: Antibody-drug conjugate (50)

[0564] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 1, the titled antibody-drug conjugate was obtained in the same manner as Process 4 of Example 2. Antibody concentration: 13.47 mg/mL, antibody yield: 9.4 mg (75%), and average number of conjugated drug molecules (n) per antibody molecule: 3.1.

Example 54 Antibody-drug conjugate (51)

[0565]

[Formula 123]



Process 1: Tert-butyl (6-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]aminol-6-oxohexyl)carbamate

[0566] Mesylate of the compound (4) (0.500 g, 0.882 mmol) was reacted in the same manner as Process 1 of Example 1 by using 6-(tert-butoxycarbonylamino)hexanoic acid instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound (0.620 g, quantitative).

^{1}H-NMR (DMSO-d_6) δ: 0.83 (3H, t, J=7.8 Hz), 1.14-1.28 (2H, m), 1.31 (9H, s), 1.47-1.61 (2H, m), 1.75-1.89 (2H, m), 2.04-2.17 (4H, m), 2.35 (3H, s), 2.81-2.88 (2H, m), 3.09-3.16 (2H, m), 5.10 (1H, d, J=19.4 Hz), 5.16 (1H, d, J=19.4 Hz), 5.39 (2H, s), 5.48-5.55 (1H, m), 6.50 (1H, s), 6.73-6.78 (1H, m), 7.26 (1H, s), 7.74 (1H, d, J=10.9 Hz), 8.39 (1H, d, J=9.0 Hz).

Process 2: 6-Amino-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]hexanamide trifluoroacetate

[0567] The compound (0.397 g, 0.611 mmol) obtained in Process 1 was reacted in the same manner as Process 2 of Example 1 to yield the titled compound (0.342 g, 84%).

^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7.2 Hz), 1.31-1.41 (2H, m), 1.52-1.70 (4H, m), 1.80-1.94 (2H, m), 2.05-2.18 (2H, m), 2.21 (2H, t, J=7.4 Hz), 2.40 (3H, s), 2.81 (2H, t, J=7.4 Hz), 3.10-3.25 (2H, m), 3.33 (2H, brs), 5.18 (1H, d, J=19.8 Hz), 5.22 (1H, d, J=19.8 Hz), 5.41 (2H, d, J=16.6 Hz), 5.45 (2H, d, J=16.6 Hz), 5.53-5.60 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.80 (1H, d, J=10.9 Hz), 8.49 (1H, d, J=9.2 Hz).

Process 3: N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanyl-N-(6-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-6-oxohexyl)glycinamide

[0568] The compound (0.170 g, 0.516 mmol) obtained in Process 2 was reacted in the same manner as Process 1 of Example 2 to yield the titled compound (0.225 g, 91%).
^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7.4 Hz), 1.43-1.70 (6H, m), 1.87 (2H, td, J=15.0, 7.4 Hz), 2.10-2.22 (3H, m), 2.28-2.37 (1H, m), 2.42 (3H, s), 2.78-2.85 (1H, m), 3.01-3.10 (3H, m), 3.15-3.22 (2H, m), 3.54-3.61 (5H, m), 3.62-3.69 (1H, m), 4.44-4.53 (1H, m), 5.17 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=19.2 Hz), 5.45 (2H, s), 5.54-5.61 (1H, m), 6.55 (1H, s), 7.02 (1H, t, J=6.1 Hz), 7.11-7.28 (5H, m), 7.33 (1H, s), 7.63-7.69 (1H, m), 7.82 (1H, d, J=11.0 Hz), 7.90-7.96 (1H, m), 8.17 (1H, d, J=7.8 Hz), 8.28 (1H, t, J=5.5 Hz), 8.46 (1H, d, J=9.0 Hz).

Process 4: Glycylglycyl-L-phenylalanyl-N-(6-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-6-oxohexyl)glycinamide

[0569] The compound (0.105 g, 0.108 mmol) obtained in Process 3 was reacted in the same manner as Process 2 of Example 2 to yield the titled compound (0.068 mg, 65%).
^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7.4 Hz), 1.15-1.67 (6H, m), 1.79-1.97 (2H, m), 2.08-2.24 (4H, m), 2.42 (3H, s), 2.76-2.82 (1H, m), 3.00-3.10 (5H, m), 3.19 (1H, s), 3.50-3.63 (2H, m), 3.64-3.76 (3H, m), 3.84-3.92 (1H, m), 4.51-4.59 (1H, m), 5.17 (1H, d, J=19.4 Hz), 5.24 (1H, d, J=19.4 Hz), 5.44 (2H, s), 5.53-5.61 (1H, m), 6.55 (1H, brs), 7.15-7.29 (5H, m), 7.33 (1H, s), 7.72-7.78 (1H, m), 7.82 (1H, d, J=11.0 Hz), 7.96-8.08 (2H, m), 8.30-8.38 (2H, m), 8.46-8.56 (2H, m).

Process 5: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-(6-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-6-oxohexyl)glycinamide

[0570] The compound (58 mg, 0.060 mmol) obtained in Process 4 was reacted in the same manner as Process 3 of Example 2 to yield the titled compound (39 mg, 62%).
^{1}H-NMR (CD_3OD) δ: 0.99 (3H, t, J=7.4 Hz), 1.27 (2H, td, J=11.6, 6.1 Hz), 1.38-1.44 (2H, m), 1.50-1.63 (6H, m), 1.65-1.80 (2H, m), 1.89-1.98 (2H, m), 2.17-2.25 (3H, m), 2.26-2.36 (3H, m), 2.40 (3H, s), 2.95 (1H, dd, J=14.3, 9.2 Hz), 3.12 (1H, dd, J=13.7, 5.7 Hz), 3.15-3.25 (4H, m), 3.44 (2H, t, J=7.2 Hz), 3.65 (1H, d, J=17.2 Hz), 3.76 (1H, d, J=17.2 Hz), 3.79-3.86 (4H, m), 4.43 (1H, dd, J=8.9, 6.0 Hz), 5.10 (1H, d, J=18.9 Hz), 5.25 (1H, d, J=18.9 Hz), 5.35 (1H, d, J=16.6 Hz), 5.56 (1H, d, J=16.0 Hz), 5.60-5.64 (1H, m), 6.76 (2H, s), 7.12-7.24 (6H, m), 7.58 (1H, s), 7.60 (1H, d, J=10.9 Hz), 7.68 (1H, t, J=5.7 Hz).
MS (ESI) m/z: 1060 $(M+H)^+$

Process 6: Antibody-drug conjugate (51)

[0571] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0147 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0. 050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour. Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 (0.0295 mL; 4.6 equivalents per antibody molecule) was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.00590 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes.

[0572] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0573] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

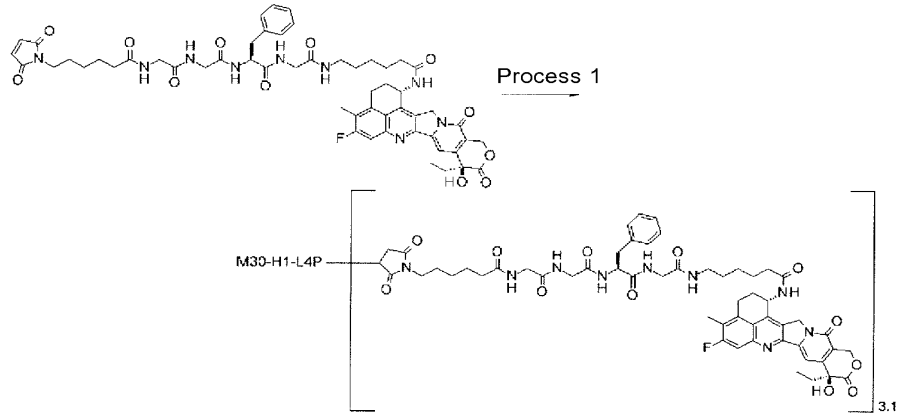
[0574] Antibody concentration: 0.97 mg/mL, antibody yield: 5.82 mg (58%), and average number of conjugated drug

molecules (n) per antibody molecule: 1.7.

Example 55 Antibody-drug conjugate (52)

[0575]

[Formula 124]



Process 1: Antibody-drug conjugate (52)

[0576] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0295 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0. 050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0577] Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 of Example 54 (0.0590 mL; 9.2 equivalents per antibody molecule) was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0118 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes.

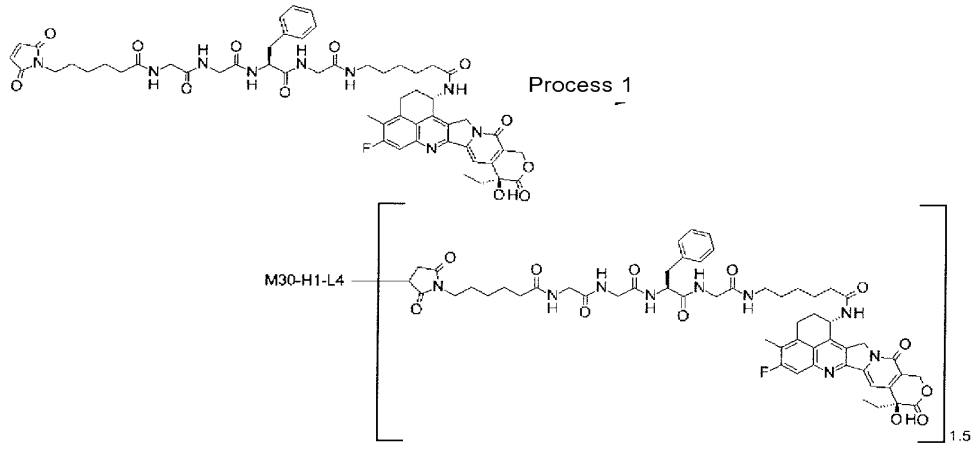
[0578] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0579] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\,370}$ = 0 (estimated calculation value), $\varepsilon_{D,\,280}$ = 5000 (measured average value), and $\varepsilon_{D,\,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 0.94 mg/mL, antibody yield: 5.64 mg (56%), and average number of conjugated drug molecules (n) per antibody molecule: 3.1.

Example 56 Antibody-drug conjugate (53)

[0580]

[Formula 125]



Process 1: Antibody-drug conjugate (53)

[0581] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0147 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0. 050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0582] Conjugation between antibody and drug linker: After incubating the solution at 22°C for 10 minutes, a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 of Example 54 (0.0295 mL; 4.6 equivalents per antibody molecule) was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.00590 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

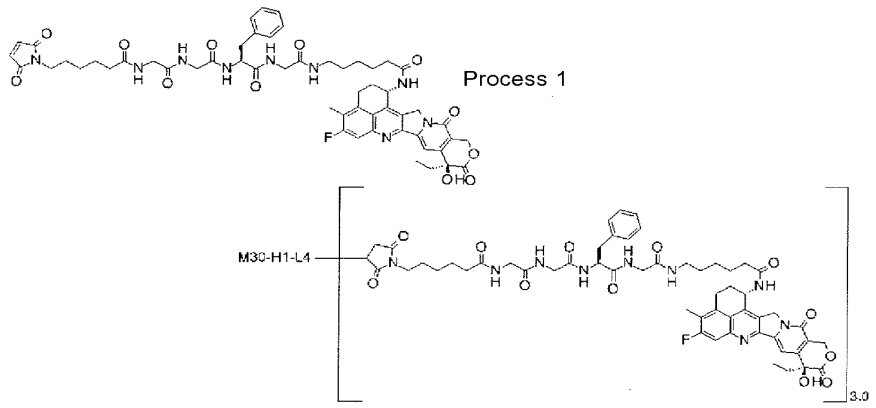
[0583] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,\,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,\,370}$ = 0 (estimated calculation value), $\varepsilon_{D,\,280}$ = 5000 (measured average value), and $\varepsilon_{D,\,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0584] Antibody concentration: 1.22 mg/mL, antibody yield: 7.32 mg (73%), and average number of conjugated drug molecules (n) per antibody molecule: 1.5.

Example 57 Antibody-drug conjugate (54)

[0585]

[Formula 126]



Process 1: Antibody-drug conjugate (54)

[0586] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0295 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0. 050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0587] Conjugation between antibody and drug linker: After incubating the solution for 10 minutes at 22°C, a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 of Example 54 (0.0590 mL; 9.2 equivalents per antibody molecule) was added thereto and incubated for conjugating the drug linker to the antibody at 22°C for 40 minutes. Next, an aqueous solution (0.0118 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at 22°C for another 20 minutes.

[0588] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

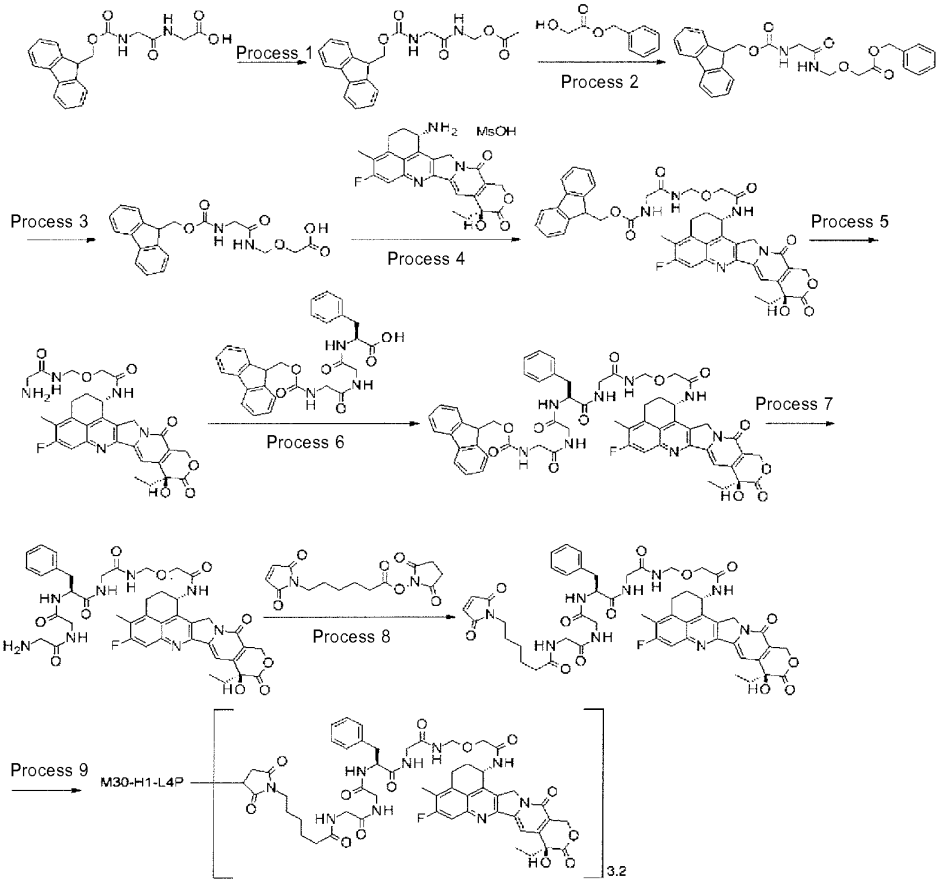
[0589] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0590] Antibody concentration: 1.06 mg/mL, antibody yield: 6.36 mg (64%), and average number of conjugated drug molecules (n) per antibody molecule: 3.0.

Example 58 Antibody-drug conjugate (55)

[0591]

[Formula 127]



Process 1: ({N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl}amino)methyl acetate

[0592] To a mixture containing N-9-fluorenylmethoxycarbonylglycylglycine (4.33 g, 12.2 mmol), tetrahydrofuran (120 ml), and toluene (40.0 ml), pyridine (1.16 ml, 14.7 mmol) and lead tetraacetate (6.84 g, 14.7 mmol) were added and refluxed under heating for 5 hours. After the reaction solution was cooled to room temperature, the insolubles were removed by filtration through Celite, and concentrated under reduced pressure. The residue obtained were dissolved in ethyl acetate and washed with water and saturated brine, and then the organic layer was dried over anhydrous magnesium sulfate. After the solvent was removed under reduced pressure, the residue obtained were purified by silica gel column chromatography [hexane : ethyl acetate = 9 : 1 (v/v) - ethyl acetate] to yield the titled compound as a colorless solid (3.00 g, 67%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.07 (3H, s), 3.90 (2H, d, J=5.1 Hz), 4.23 (1H, t, J=7.0 Hz), 4.46 (2H, d, J=6.6 Hz), 5.26 (2H, d, J=7.0 Hz), 5.32 (1H, brs), 6.96 (1H, brs), 7.32 (2H, t, J=7.3 Hz), 7.41 (2H, t, J=7.3 Hz), 7.59 (2H, d, J=7.3 Hz), 7.77 (2H, d, J=7.3 Hz).

Process 2: Benzyl [({N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl}amino)methoxy]acetate

[0593] To tetrahydrofuran (40.0 mL) solution of the compound (3.68 g, 10.0 mmol) obtained in Process 1 and benzyl glycolate (4.99 g, 30.0 mmol), potassium tert-butoxide (2.24 g, 20.0 mmol) was added at 0°C and stirred at room temperature for 15 minutes. The reaction solution was charged with ethyl acetate and water at 0°C and extracted with ethyl acetate and chloroform. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure. The residue obtained were dissolved in dioxane (40.0 mL) and water (10.0 mL), charged with sodium hydrogen carbonate (1.01 g, 12.0 mmol) and 9-fluorenylmethyl chloroformate (2.59 g, 10.0 mmol), and stirred at room temperature for 2 hours. The reaction solution was charged with water and extracted with ethyl acetate. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced

pressure and the residue obtained were purified by silica gel column chromatography [hexane : ethyl acetate = 100 : 0 (v/v) - 0 : 100] to yield the titled compound in colorless oily substance (1.88 g, 40%).
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.84 (2H, d, J=5.5 Hz), 4.24 (3H, t, J=6.5 Hz), 4.49 (2H, d, J=6.7 Hz), 4.88 (2H, d, J=6.7 Hz), 5.15-5.27 (1H, m), 5.19 (2H, s), 6.74 (1H, brs), 7.31-7.39 (7H, m), 7.43 (2H, t, J=7.4 Hz), 7.61 (2H, d, J=7.4 Hz), 7.79 (2H, d, J=7.4 Hz).

Process 3: [({N-[(9H-Fluoren-9-ylmethoxy)carbonyl]glycyl}amino)methoxy]acetic acid

[0594] The compound (1.88 g, 3.96 mmol) obtained in Process 2 was dissolved in ethanol (40.0 mL) and ethyl acetate (20.0 ml). After adding palladium carbon catalyst (376 mg), it was stirred under hydrogen atmosphere at room temperature for 2 hours. The insolubles were removed by filtration through Celite, and the solvent was removed under reduced pressure to yield the titled compound as a colorless solid (1.52 g, quantitative).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.62 (2H, d, J=6.3 Hz), 3.97 (2H, s), 4.18-4.32 (3H, m), 4.60 (2H, d, J=6.7 Hz), 7.29-7.46 (4H, m), 7.58 (1H, t, J=5.9 Hz), 7.72 (2H, d, J=7.4 Hz), 7.90 (2H, d, J=7.4 Hz), 8.71 (1H, t, J=6.5 Hz).

Process 4: 9H-Fluoren-9-ylmethyl(2-{[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]amino}-2-oxoethyl)carbamate

[0595] Under ice cooling, to an N,N-dimethyl formamide (10.0 mL) solution of mesylate of the compound (4) (0.283 g, 0.533 mmol), N-hydroxysuccinimide (61.4 mg, 0.533 mmol), and the compound (0.205 g, 0.533 mmol) obtained in Process 3, N,N-diisopropylethylamine (92.9 μL, 0.533 mmol) and N,N'-dicyclohexylcarbodiimide (0.143 g, 0.693 mmol) were added and stirred at room temperature for 3 days. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform-partitioned organic layer of chloroform: methanol: water = 7 : 3 : 1 (v/v/v)] to yield the titled compound as a pale brown solid (0.352 g, 82%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.81 (3H, t, J=7.4 Hz), 1.73-1.87 (2H, m), 2.06-2.20 (2H, m), 2.34 (3H, s), 3.01-3.23 (2H, m), 3.58 (2H, d, J=6.7 Hz), 3.98 (2H, s), 4.13-4.25 (3H, m), 4.60 (2H, d, J=6.7 Hz), 5.09-5.22 (2H, m), 5.32-5.42 (2H, m), 5.50-5.59 (1H, m), 6.49 (1H, s), 7.24-7.30 (3H, m), 7.36 (2H, t, J=7.4 Hz), 7.53 (1H, t, J=6.3 Hz), 7.66 (2H, d, J=7.4 Hz), 7.75 (1H, d, J=11.0 Hz), 7.84 (2H, d, J=7.4 Hz), 8.47 (1H, d, J=8.6 Hz), 8.77 (1H, t, J=6.7 Hz).
MS (ESI) m/z: 802 $(M+H)^+$

Process 5: N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide

[0596] To an N,N-dimethyl formamide (11.0 mL) solution of the compound (0.881 g, 1.10 mmol) obtained in Process 4, piperidine (1.1 mL) was added and stirred at room temperature for 2 hours. The solvent was removed under reduced pressure to yield a mixture containing the titled compound. The mixture was used for the next reaction without performing further purification.

Process 6: N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycylglycyl-L-phenylalanyl-N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide

[0597] Under ice cooling, to an N,N-dimethyl formamide (50.0 mL) solution of the mixture (0.439 mmol) obtained in Process 5, N-hydroxysuccinimide (0.101 g, 0.878 mmol), and N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycylglycyl-L-phenylalanine (that is, the compound described in Japanese Patent Laid-Open No. 2002-60351) (0.440 g, 0.878 mmol), N,N'-dicyclohexylcarbodiimide (0.181 g, 0.878 mmol) was added and stirred at room temperature for 4 days. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform: methanol = 9 : 1 (v/v)] to yield the titled compound as a pale orange solid (0.269 g, 58%).
MS (ESI) m/z: 1063 $(M+H)^+$

Process 7: Glycylglycyl-L-phenylalanyl-N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide

[0598] To an N,N-dimethyl formamide (4.00 mL) solution of the compound (0.269 g, 0.253 mmol) obtained in Process 6, piperidine (0.251 mL, 2.53 mmol) was added and stirred at room temperature for 2 hours. The solvent was removed under reduced pressure to yield a mixture containing the titled compound. The mixture was used for the next reaction

without performing further purification.

Process 8: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-[(2-{[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2, 3, 9, 10, 13, 15-hexahydro-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino}-2-oxoethoxy)methyl]glycinamide

[0599] To an N,N-dimethyl formamide (10.0 mL) solution of the compound (0.253 mmol) obtained in Process 7, N-succinimidyl 6-maleimide hexanoate (0.156 g, 0.506 mmol) was added and stirred at room temperature for 3 days. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform: methanol = 9 : 1 (v/v)] to yield the titled compound as a pale yellow solid (0.100 g, 38%).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.83 (3H, t, J=7.2 Hz), 1.09-1.21 (2H, m), 1.33-1.47 (4H, m), 1.75-1.90 (2H, m), 2.00-2.23 (4H, m), 2.36 (3H, s), 2.69-2.81 (1H, m), 2.94-3.03 (1H, m), 3.06-3.22 (2H, m), 3.23-3.74 (8H, m), 3.98 (2H, s), 4.39-4.50 (1H, m), 4.60 (2H, d, J=6.7 Hz), 5.17 (2H, s), 5.39 (2H, s), 5.53-5.61 (1H, m), 6.50 (1H, s), 6.96 (2H, s), 7.11-7.24 (5H, m), 7.28 (1H, s), 7.75 (1H, d, J=11.0 Hz), 7.97 (1H, t, J=5.7 Hz), 8.03 (1H, t, J=5.9 Hz), 8.09 (1H, d, J=7.8 Hz), 8.27 (1H, t, J=6.5 Hz), 8.48 (1H, d, J=9.0 Hz), 8.60 (1H, t, J=6.5 Hz).
MS (ESI) m/z: 1034 $(M+H)^+$

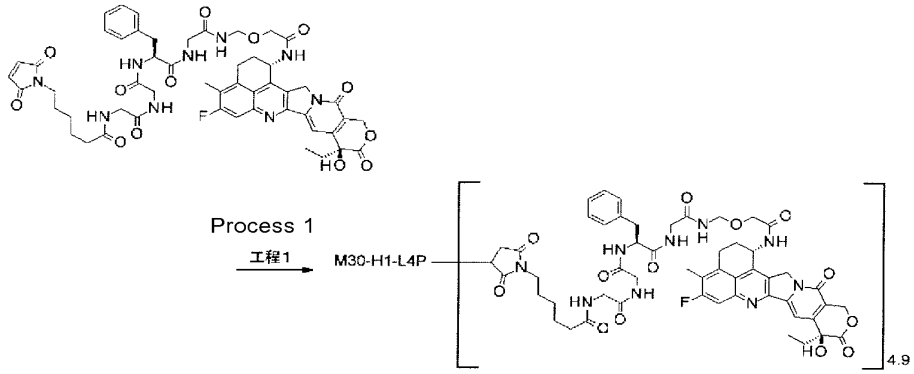
Process 9: Antibody-drug conjugate (55)

[0600] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mL mg^{-1} cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 $\pm$ 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.
[0601] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.109 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 (0.039 mL; 4.6 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 40 minutes. Next, an aqueous solution (0.008 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.
[0602] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A described in Production method 1.
[0603] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 12.57 mg/mL, antibody yield: 8.8 mg (70%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 59 Antibody-drug conjugate (56)

[0604]

[Formula 128]



Process 1: Antibody-drug conjugate (56)

[0605] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0606] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.067 mL) a dimethyl sulfoxide solution containing and 10 mM of the compound obtained in Process 8 of Example 58 (0.085 mL; 10.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 60 minutes. Next, an aqueous solution (0.013 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.

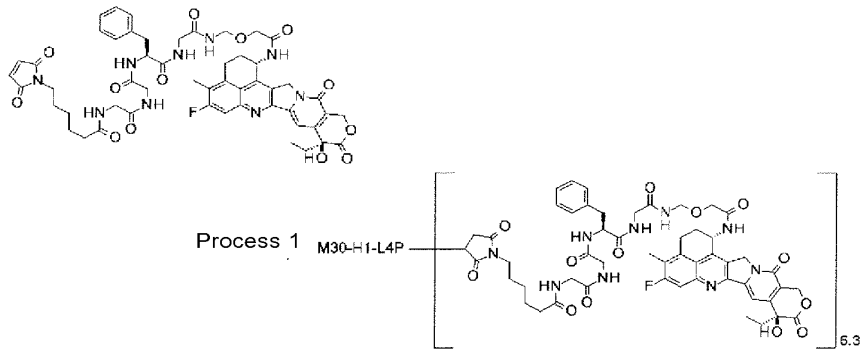
[0607] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0608] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained. Antibody concentration: 1.33 mg/mL, antibody yield: 7.98 mg (64%), and average number of conjugated drug molecules (n) per antibody molecule: 4.9.

Example 60 Antibody-drug conjugate (57)

[0609]

[Formula 129]



Process 1: Antibody-drug conjugate (57)

[0610] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was placed in a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0611] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.025 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.127 mL; 15.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 60 minutes. Next, an aqueous solution (0.019 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred to terminate the drug linker reaction at room temperature for another 20 minutes.

[0612] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A described in Production method 1.

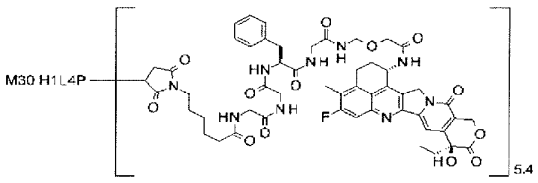
[0613] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5000 (measured average value), and $\varepsilon_{D,370}$ = 19000 (measured average value) were used), the following characteristic values were obtained.

[0614] Antibody concentration: 0.91 mg/mL, antibody yield: 5.46 mg (44%), and average number of conjugated drug molecules (n) per antibody molecule: 6.3.

Example 61 Antibody-drug conjugate (58)

[0615]

[Formula 130]



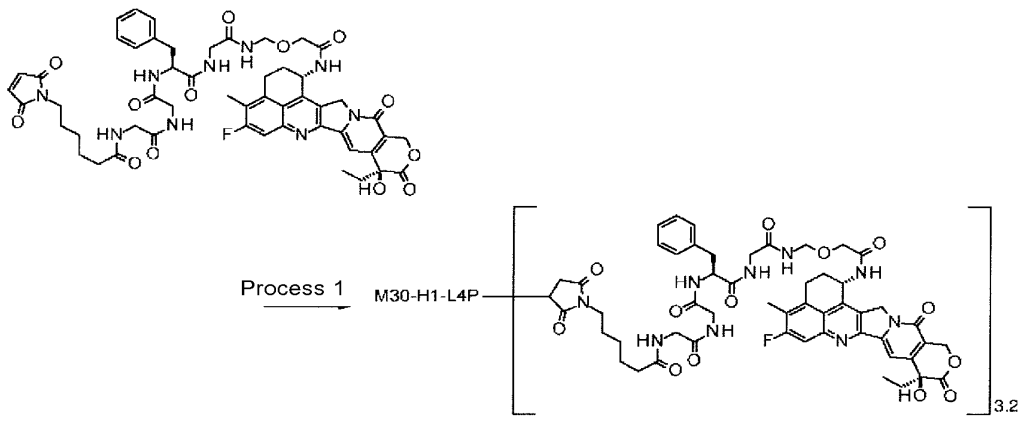
[0616] Almost the whole amounts of the antibody-drug conjugates of Examples 59 and 60 were mixed. The solution was concentrated by the Common procedure A described in Production method 1 to yield the titled antibody-drug conjugate.

Antibody concentration: 10.0 mg/mL, antibody yield: 12.30 mg, and average number of conjugated drug molecules (n) per antibody molecule: 5.4.

Example 62 Antibody-drug conjugate (59)

[0617]

[Formula 131]



Process 1: Antibody-drug conjugate (59)

[0618] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mL mg^{-1} cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (100 mL, 1 g of the antibody) was placed in a 250 mL flask and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (2.43 mL; 3.6 equivalents per antibody molecule) and further with an aqueous solution of 1 M dipotassium hydrogen phosphate (5 mL). After confirming that the solution had pH near 7.4 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0619] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (3.51 mL; 5.2 equivalents per antibody molecule) and dimethyl sulfoxide (2.14 mL) to the above solution at room temperature, it was stirred with a stirrer for conjugating the drug linker to the antibody in a water bath at 15°C for 130 minutes. Next, an aqueous solution (0.547 mL) of 100 mM NAC was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0620] Purification: The above solution was subjected to ultrafiltration purification using an ultrafiltration apparatus composed of an ultrafiltration membrane (Merck Japan, Pellicon XL Cassette, Biomax 50 KDa), a tube pump (Cole-Parmer International, MasterFlex Pump model 77521-40, Pump Head model 7518-00), and a tube (Cole-Parmer International, MasterFlex Tube L/S16). Specifically, while ABS was added dropwise (a total of 800 mL) as a buffer solution for purification to the reaction solution, ultrafiltration purification was performed for removing unconjugated drug linkers and other low-molecular-weight reagents, also replacing the buffer solution with ABS, and further concentrating the solution, to yield about 70 mL of a solution containing the titled antibody-drug conjugate.

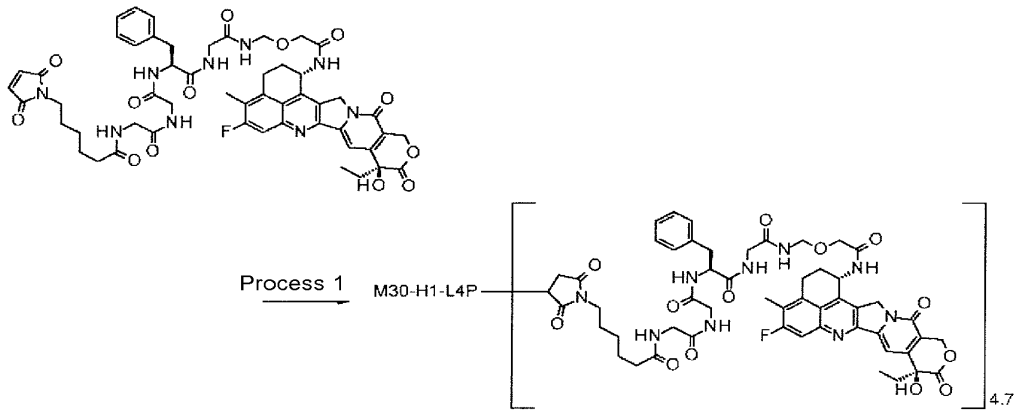
[0621] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5178 (measured value), and $\varepsilon_{D,370}$ = 20217 (measured value) were used), the following characteristic values were obtained.

[0622] Antibody concentration: 14.2 mg/mL, antibody yield: 1.0 g (about 100%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

Example 63 Antibody-drug conjugate (60)

[0623]

[Formula 132]



Process 1: Antibody-drug conjugate (60)

[0624] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 $mLmg^{-1}cm^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (5 mL, 50 mg of the antibody) was placed in a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.075 mL; 4 equivalents per antibody molecule). After confirming that the solution had pH near 7.0 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0625] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.219 mL; 6.5 equivalents per antibody molecule) and dimethyl sulfoxide (0.064 mL) to the above solution, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 90 minutes. Next, an aqueous solution (0.033 mL; 9.8 equivalents per antibody molecule) of 100 mM NAC was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0626] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 19 mL of a solution containing the titled antibody-drug conjugate.

[0627] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\varepsilon_{A,280}$ = 235300 (estimated calculation value), $\varepsilon_{A,370}$ = 0 (estimated calculation value), $\varepsilon_{D,280}$ = 5178 (measured value), and $\varepsilon_{D,370}$ = 20217 (measured value) were used), the following characteristic values were obtained.

[0628] Antibody concentration: 2.19 mg/mL, antibody yield: 42 mg (83%), and average number of conjugated drug molecules (n) per antibody molecule: 4.7.

Example 64 Antibody-drug conjugate (61)

[0629]