

CHAPTER 1

Dipeptidyl Peptidases: Substrates and Therapeutic Targeting in Human Health and Disease

CLAIRE H. WILSON AND CATHERINE A. ABBOTT

School of Biological Sciences, Flinders University, GPO BOX 2100,
Adelaide 5001, South Australia, Australia

1.1 Introduction

Dipeptidyl peptidase 4 (DP4), fibroblast activation protein (FAP), DP8, and DP9 are the enzymatic members of the serine protease S9b DP4-like gene family. One of the most important features of the DPs is their ability to preferentially cleave the N-terminal post-prolyl bond of regulatory peptides and small protein substrates. DP4 proteolysis results in the inactivation, activation, or alteration of its substrates function via changes in receptor selectivity; thus DP4 plays an important role in regulating biological function. Together, DP4 and FAP have been implicated in a number of diseases including liver disease, obesity, type II diabetes, arthritis, inflammatory bowel disease and cancer. Recently, evidence has emerged to implicate both DP8 and DP9 in innate immunity, and DP8/9 *in vitro* cleavage of well-known DP4 substrates, including neuropeptide Y (NPY), glucagon-like peptide (GLP)-1, and a number of chemokines, has been demonstrated. Despite this, the true pathophysiological roles of DP8/9 and their involvement within human biology and disease are still to be elucidated. Identification of the *in vivo* substrate repertoire of each DP will be an important step toward elucidating the biochemical pathways in which each protease is involved. This will allow us to unravel further the roles that the

RSC Drug Discovery Series No. 18

Proteinases as Drug Targets

Edited by Ben Dunn

© Royal Society of Chemistry 2012

Published by the Royal Society of Chemistry, www.rsc.org

DPs play in human biology and disease, and evaluate further their suitability as therapeutic targets.

In this chapter, we will provide an introduction to the significance of post-proline cleavage, the DP4-like gene family enzymatic members, and the related enzymes prolyl endopeptidase (PEP) and DP2. This will be followed by an in-depth examination of the biochemical characteristics of DP4, FAP, DP8, and DP9, their natural substrates, and biological relevance following DP cleavage. Lastly, the suitability of targeting the DP family for the development of therapeutics for the treatment of human health and disease will be discussed.

1.2 Post-Proline-Cleaving Enzymes

A number of biologically active proteins and regulatory peptides such as cytokines, chemokines, growth hormones, and neuropeptides are protected from general proteolysis due to an evolutionary conserved N-terminal proline residue.¹⁻³ Such protection results from the unique cyclic and imino structure of proline imposing conformational restrictions on the polypeptide backbone.³ Even proteases exhibiting a very broad substrate specificity are unable to attack peptide bonds where the prolyl residue is situated, and hence degradation of such peptides requires the use of proline specific peptidases.¹⁻³

Although a number of proline-cleaving enzymes have been identified, only a limited number of these proteases are capable of cleaving the N-terminal post-prolyl, X-Pro-, bond^{2,3} (Figure 1.1). The most notable of these enzymes are the N-terminal-specific, dipeptidyl peptidases of the serine protease SC clan, S9b sub-family such as DP4 (EC 3.4.15.5), the endopeptidase of the parent S9 family prolyl-endopeptidase (PEP; EC 3.4.21.26), and the S28 family member DP2 (EC 3.4.14.2). Lysosomal prolylcarboxypeptidase (PCP) (EC 3.4.16.2) also belongs to the S28 family, but in contrast to DP2, it functions as a C-terminal-specific protease cleaving the Pro-Xaa bond at the C-termini of proteins to release a single C-terminal amino acid. Carboxypeptidase P (EC 3.4.17.16) is a membrane-localized protease with similar cleavage specificity to lysosomal PCP; however, it is a metallo-, as opposed to serine, protease. Aminopeptidase P (EC 3.4.17.16), prolidase (EC 3.4.13.19) and prolinase are additional proline-cleaving metalloproteases. Aminopeptidase P is an N-terminal-specific protease, cleaving the pre-prolyl bond to release single amino acids at the N-termini of proteins. Importantly, aminopeptidase P is involved in cooperative activities with DP4 and related enzymes.¹ Prolidase and prolinase cleave dipeptides with pre- and post-proline respectively to release two amino acids.¹ The metalloprotease angiotensin-converting enzyme (ACE; EC 3.4.15.1) is also capable of cleaving prolyl bonds, although it is not renowned for its ability to do this. DP4, first discovered in 1966 by Hopsu-Havu and Glenner as the dipeptidyl cleaving glyclproline naphthylamidase,⁴ was the first N-terminal post-proline-cleaving enzyme identified and hence the most readily investigated. Since its discovery, related enzymes have been identified including fibroblast activation FAP,⁵ then DP8 and DP9.⁶

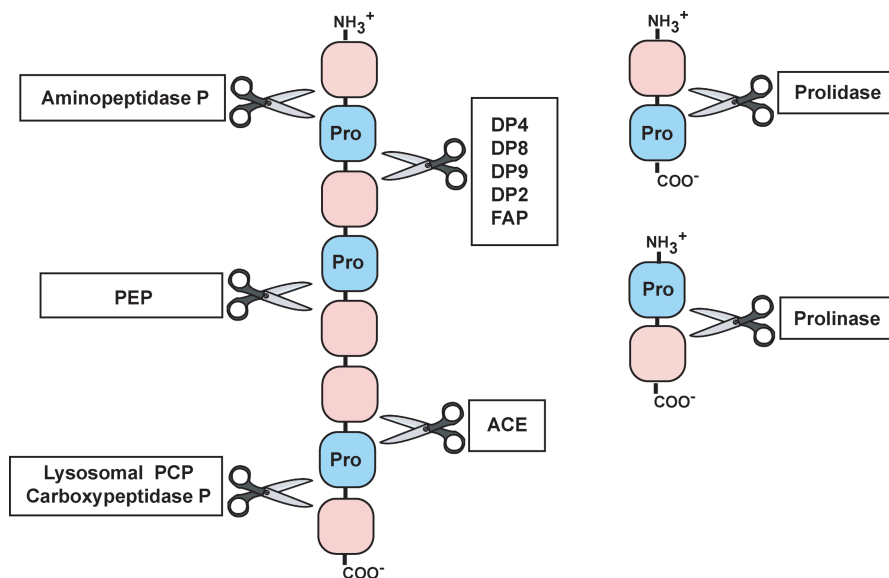


Figure 1.1 Proline-bond cleaving proteases. ACE, angiotensin-converting enzyme; DP, dipeptidyl peptidase; FAP, fibroblast activation protein; PCP, prolyl-carboxypeptidase; PEP, prolyl-endopeptidase.

1.3 DP4-Like Gene Family and Related Enzymes

Members of the DP4-like gene family, S9b, make up a sub-family of the prolyl-oligopeptidase (POP) S9 family within the serine protease clan SC.^{7,8} In total the S9b family consists of six homologous members; the four proteases, DP4, FAP, DP8 and DP9 and two inactive protease homologs, DP6 and DP10 (Figure 1.2). Sharing similar features, PEP from the parent S9 family is also capable of cleaving the N-terminal post-prolyl bond but with endopeptidase specificity.^{9,10} In contrast to DP4, PEP is limited in its ability to cleave unblocked, free N-terminal dipeptides from substrates;¹⁰ however, inhibitors designed specifically for DPs can still bind to, and block, the enzymatic function of PEP.^{11,12} FAP, in addition to its DP activity, also functions as an endopeptidase.^{13,14} Structurally, the three crystallized S9b family members DP4, FAP and DP6 contain an α/β hydrolase domain and eight-bladed β -propeller domain (Figure 1.2)^{13,15–18} distinguishing it from that of parent PEP which contains a seven-bladed β -propeller domain.¹⁹ Protease members of this family and PEP all contain the non-classical arrangement of the serine protease catalytic triad (Ser, Asp, and His), located within the C-terminal portion of the α/β hydrolase domain (Figure 1.2). The inactive protease homologs DP6 and DP10 contain a mutation of the catalytic serine residue to an aspartic acid²⁰ and glutamine residue²¹ respectively. All enzyme members contain the catalytic serine protease motif around the

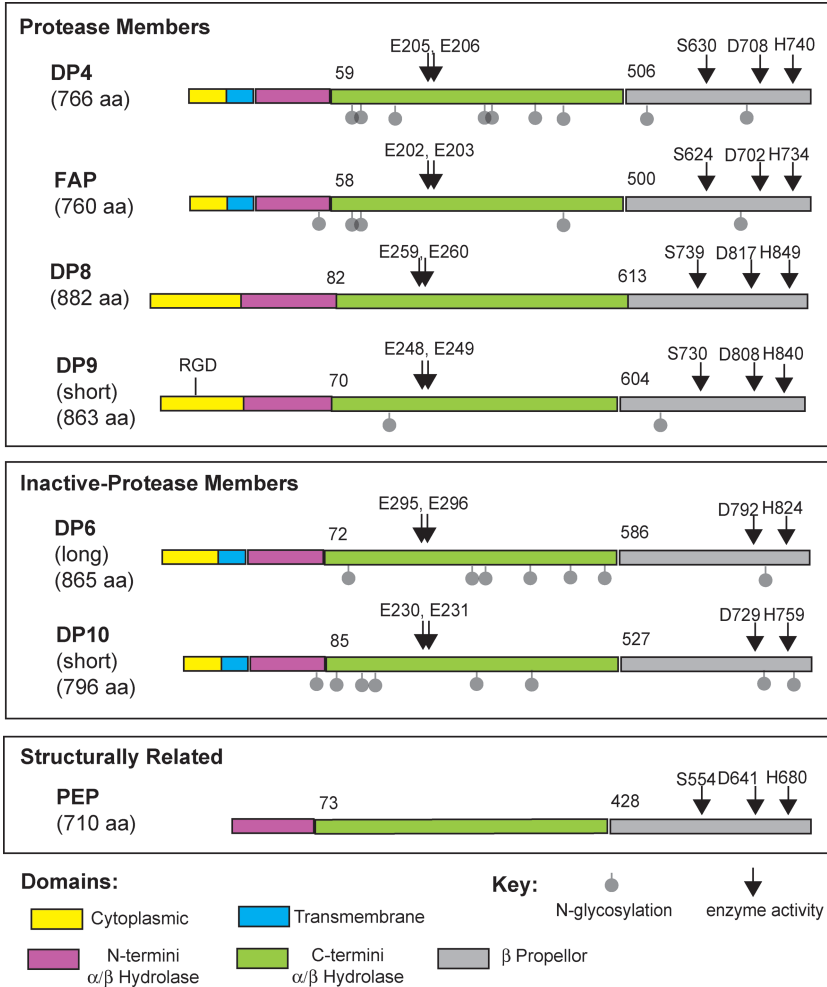


Figure 1.2 Schematic of the DP4-like gene family and related prolyl endopeptidase. Domains and residues of interest are not arranged to scale. Arrows indicate the catalytic residues required for enzyme activity. Only the main isoforms are represented. The majority of splice variants differ in regard to the length of their N-terminal cytoplasmic domain. Being intracellular proteases, DP8, DP9, and PEP do not contain a transmembrane domain. The integrin-binding domain of DP9 is also displayed within the N-terminal cytoplasmic domain. Figure adapted from Gorrell.⁶¹ Reproduced with permission, from Gorrell (2005), *Clinical Science*, **108**, 277–292. © The Biochemical Society. DP, dipeptidyl peptidase; FAP, fibroblast activation protein; PEP, prolyl-endopeptidase.

serine residue of GWSYG.⁷ Another distinguishing characteristic of the S9b sub-family that differentiates them from PEP is the pair of conserved glutamate residues situated within the β -propeller domain found to be essential for

catalytic activity of DP4²² and DP8²³ (Figure 1.2). DP2, belonging to the serine protease S28 family, is a non-structural homolog sharing a number of similarities with the DP4-like gene family including the conserved non-classical catalytic triad and similar substrate specificity with its ability to cleave N-terminal dipeptides from peptide substrates with proline in the penultimate position.²⁴ As for PEP, a number of inhibitors designed for targeting DP4 are also known to interact and inhibit DP2.^{11,24,25} Hence, when investigating the expression and roles of the S9b family, due consideration must also be given to the potential involvement (or targeting in the case of inhibition) of this related enzyme. The subcellular localization of each enzyme is diverse and sometimes overlapping; they are found membrane-bound (DP4, FAP, PEP), in lysosomes (DP2), in the cytoplasm (DP8/9, PEP, DP2), or in secretory vesicles (DP2), so it is likely that their substrate degradomes will mostly differ.

Containing a trans-membrane domain within their N-termini and a number of glycosylation sites, DP4, FAP, DP6, and DP10 are all type II-membrane glycoproteins localized to the cell surface.²⁶ Plasma-soluble forms of both DP4²⁷ and FAP^{28,29} have been identified with their presence in circulation believed to result from shedding events at the plasma membrane. DP8, DP9, and PEP lack a transmembrane domain and are cytosolic proteins.^{6,30,31} A number of membrane associated forms of PEP have been identified.^{31,32} Unique to DP9 is the presence of one of the best-known integrin-binding motifs the RGD (arginine-glutamine-aspartic acid; Arg-Gly-Asp) motif, within its N-termini³³ (Figure 1.2). DP9 also contains two potential N-linked glycosylation sites;^{23,34} however, there is no evidence at present of DP9 glycosylation,³⁵ so the significance of these features is yet to be revealed. DP4, FAP, DP8/9, and PEP all have neutral pH optimums of pH 7–8.^{6,21} In contrast, DP2 is a smaller, 492-amino-acid, lysosomal enzyme, active across a broad pH range with an acidic pH optimum of 5.5.²⁵ DP2 also localizes within intracellular vesicles distinct from lysosomes in resting quiescent cells.³⁶ It is likely that these vesicles contain a secretory component due to the release of fully functional DP2 in response to calcium stimulation.³⁶ All S9b family members and the S28 member DP2 form homodimers with dimerization being essential for the catalytic activity of DP4,^{37,38} FAP^{13,39} and DP2.⁴⁰ In contrast, PEP is a monomeric enzyme with SDS-PAGE mobility of 70–80 kDa.^{9,41} S9b proteins have a monomer mobility on SDS-PAGE gel of 90–110 kDa and dimer mobility ranging between 150 and 200 kDa.⁷ Being much smaller, DP2 migrates in its glycosylated form as a ~60 kDa monomer on SDS-PAGE and forms a ~120 kDa homodimer.^{40, 42–44}

A brief discussion of the homology and gene structure of the DP4-like gene family is provided below. Although both DP2 and PEP are of importance when discussing therapeutic targeting of DP4, FAP, and DP8/9, they will not be discussed here in detail; thus, the reader should refer to critical reviews on DP2 by Maes *et al.*²⁴ and on PEP by Garcia-Horsman *et al.*⁴⁵. The inactive protease homologs DP6 and DP10 will be discussed in brief, as they are not a focus of this review (recently reviewed in McNicholas *et al.*⁴⁶).

1.3.1 Homology and Gene Structure

A high degree of homology exists within the DP4-like gene family with all members sharing 25–60% amino acid sequence identity and 47–77% amino acid sequence similarity.⁷ Notably, FAP and DP4, located adjacent to each other on the long arm of chromosome 2 (2q24.2 and 2q24.3 respectively), share 52% amino acid identity. The two non-enzyme members, DP6 and DP10, located on chromosomes 7q36.2 and 2q14.1 respectively, share 53% amino acid sequence identity, and the most recently identified enzyme members, DP8 and DP9, located respectively on chromosomes 15q22.32 and 19q13.3, share 61% amino acid sequence identity. The close proximity and high level of similarity between the DP4 and FAP genes suggest that they have arisen from a recent gene duplication event.⁷ Likewise, it is possible that DP10, also located on the long arm of chromosome 2, was derived from either DP4 or FAP, followed by the divergence of DP6 on chromosome 7. Similarly, the high sequence identity and shared cytosolic localization suggest that DP8 and DP9 have arisen from a gene duplication event.⁷ Although of varying gene length, the *DP4* (82 kb), *FAP* (73 kb), *DP6* (935 kb), and *DP10* genes (1402 kb) all contain 26 exons,⁷ further supporting the likelihood of a common gene ancestor. In contrast, the *DP8* (72 kb) gene contains 20 exons,⁶ and the *DP9* gene contains 19 or 22 exons dependent on whether the gene is expressed in its short (863-amino-acid) or long (971 amino acid) forms.³⁴ Like DP9, short and long transcript variants with variable length N-termini arising from alternate first exon use exist for *DP6*^{47,48}, *DP10*,^{49,50} and *DP8*.^{6,51} Differing patterns of expression and tissue specificity are often associated with these alternative transcripts, but a comprehensive study investigating their significance to human biology is yet to be carried out.

1.4 Protease Members of the DP4-Like Gene Family

1.4.1 DP4 and FAP: Extracellular Proteins

DP4 is the best-characterized member of the DP enzyme family, followed to a lesser extent by FAP, which was identified almost 20 years after DP4. Both enzymes are implicated in a number of roles, including tissue remodeling/wound healing, inflammatory bowel disease, arthritis, type II diabetes, obesity, and cancer (reviewed in several previous studies^{52–55}). In cancer, both DP4 and FAP play conflicting roles acting as either a tumour suppressor or tumour promoter depending on the cancer type.⁵⁶ DP4 plays a key role in glucose homeostasis, regulating the activities of GLP-1 and glucose-dependent insulinotropic peptide (GIP) via its proteolytic activity, and has thus become a clinically validated target in the management of type II diabetes (reviewed in several previous studies^{57–59}). FAP is also of clinical relevance, particularly within a cancer and liver disease setting as discussed below. Here, the expression patterns and biological importance of DP4 and FAP will be introduced.

Human DP4 is ubiquitously expressed in a wide variety of tissues and cell types, exerting its functions on a broad range of physiological processes within the human body. DP4 is expressed at high levels on the surface of several endothelial, epithelial, and fibroblast cells of the lung, kidney, liver, intestine, bile duct, and other organs, thus affecting the gastrointestinal/digestive, cardiovascular, endocrine, neurological, and immunological systems.^{60,61} In kidney, DP4 is one of the major microvillus membrane/brush-border membrane proteins.⁶² Relatively high levels of soluble DP4 activity are observed in human bodily fluids,^{63–65} and thus the activity of soluble, circulating DP4 is likely to also contribute to effects observed in many of these systems. Variations in the level of serum DP4 activity have been associated with numerous diseases, and the effectiveness of measuring soluble DP4 activity levels as a biomarker for diagnosis, monitoring, and prognosis of diseases such as cancer, arthritis, and psychiatric disorders has been investigated.^{66–72} In the immune system, expression of DP4, known as the surface antigen CD26, is detectable at low levels on the surface of some resting T cells, B cells, and natural killer (NK) cells.^{73–75} DP4 functions as a co-stimulatory molecule of T-cell activation, displaying increased expression on the cell surface of activated T-cells.⁷³ On the surface of B cells, DP4 expression is upregulated following the immunogenic stimulation of B cells with pokeweed mitogen⁷⁶ or the *Staphylococcus aureus*-derived immunogen streptokinase.⁷⁷ Despite the widespread involvement of DP4 in animal biology, the absence of DP4 expression does not result in any known adverse defects, as demonstrated by the apparent healthy phenotype of knockout DP4 mice (DP4^{-/-})⁷⁸ and their favorable protection against diet-induced obesity and associated insulin resistance.⁷⁹ Recently, studies investigating the long-term chronic loss of DP4 expression in genetically induced DP4 deficient rats have identified some age-dependent alterations in immune system composition and stress-regulatory responses.^{80,81} Of interest to human pathological conditions, particularly cancer, is the apparent loss of DP4 expression during disease progression. Loss or alteration of DP4 surface expression has been observed in several malignant cells and carcinomas such as lung, breast and ovarian, prostate,^{82,83} and colon adenocarcinoma⁸⁴ and during the progression of melanocytes to melanoma.^{85,86} It should be made clear, however, that loss of DP4 expression is not a hallmark of all cancers with many studies examining the expression and roles of DP4 in cancer producing conflicting results. In fact, DP4 upregulation has been observed in differing cancer studies to contribute to the metastatic cancer phenotype, thus acting as a tumor promoter contributing to disease progression.

In contrast to DP4, FAP displays a more restricted and specific pattern of expression in adult humans. Detection of translated human FAP in normal adult tissue with the F19 monoclonal antibody is limited to the occasional fibroblasts and a subset of pancreatic islet cells.⁸⁷ Results of a master RNA blot provide evidence for there being a more ubiquitous pattern of FAP expression at the transcript level in tissues where protein has not been detected.⁵² FAP protein is most widely expressed in reactive stromal

fibroblasts of epithelial cancers including lung, colorectal, breast and ovarian carcinomas, subsets of bone and soft-tissue sarcomas, granulating tissue at sites of wound-healing, and foetal mesenchymal tissues.^{87,88} Additionally, FAP localizes to the advancing portion ('invadopodia') of cultured malignant LOX melanoma cells where it contributes to the invasive phenotype.⁸⁹ Like the DP4^{-/-} mice, FAP knockouts display a normal phenotype; thus a critical role for FAP in foetal development is unlikely.⁹⁰ FAP is expressed by hepatic stellate cells at the tissue remodeling interface in human cirrhosis⁹¹ where its expression is strongly and significantly correlated with the severity of liver fibrosis.⁹² Further studies in human liver have co-localized FAP with collagen fibers, fibronectin, and type I collagen.⁹³ Heterologous over-expression of FAP results in reduced adhesion and migration of human embryonic kidney (HEK)-293T cells.⁹³ In contrast, the same study found that over-expression of FAP in the human hepatic stellate line, LX-2, resulted in increased adhesion and migration of cells to extracellular matrix proteins and invasion across transwells, independent of transforming/tumour growth factor beta (TGF- β).⁹³ In both HEK-293T and LX-2 cell lines, FAP over expression resulted in enhanced staurosporine streptomycin-stimulated apoptosis.⁹³ It may be possible that, because of its roles in tissue remodeling and wound healing, FAP mRNA is kept at low levels in most cell/tissue types to initiate a quick response in the event of external damage. FAP expression can be upregulated by TGF- β , 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and retinoids (chemical compounds related to vitamin A).⁹⁴ TGF- β is a ubiquitously expressed cytokine shown to play central roles in numerous processes including FAP relevant wound healing, tissue repair, fibrosis, and cancer (reviewed in several previous studies⁹⁵⁻⁹⁸). FAP is also expressed at the remodeling interface of idiopathic pulmonary fibrosis (IPF),⁹⁹ a progressive and debilitating disease of the lung. TGF- β is likely to play a key role in the mediation of IPF.¹⁰⁰ Soluble FAP has been purified from bovine serum²⁹ and has been identified as the antiplasmin-cleaving enzyme (APCE) in humans.¹⁰¹ FAP is responsible for cleavage and increased activity of the human plasmin inhibitor, α_2 -antiplasmin (α_2 AP),²⁸ thus further supporting the pro-fibrotic role of FAP and its early involvement in wound healing.

Co-expression of FAP and DP4 results in heteromeric complex formation on the cell surface of COS-1 cells.¹⁰² DP4-FAP expression is found in reactive fibroblasts of healing wounds¹⁰² and has been localized to invadopodia of migratory cells.¹⁰³ *In vivo* DP4 and FAP colocalize on capillary endothelial cells, but not large blood vessels in invasive breast ductal carcinoma.¹⁰⁴ DP4-FAP complex formation is important in the development of the tissue-invasive phenotype in migratory cells during wound healing¹⁰³ and plays an important role in the migration and invasion of migratory fibroblasts and human endothelial cells in collagenous matrices.^{103,104} This is likely to involve localized DP4-FAP gelatin degradation. Blocking of the gelatin-binding domain of DP4 in the DP4-FAP complex blocks local gelatin degradation by endothelial and migratory fibroblasts and alters migration and invasion of cells on collagenous matrices.¹⁰⁴

1.4.1.1 Non-Enzymatic Roles of DP4 and FAP

In addition to their enzymatic roles, both DP4 and FAP exhibit a number of important functions independent of their enzyme activity, many of which involve binding with other proteins and indirect regulation of other proteases such as matrix metalloproteinase (MMPs) and plasmin. Of particular therapeutic interest are the non-enzymatic functions of DP4 and FAP that contribute to their involvement in cell adhesion and migration. Both DP4 and FAP interact with components of the extracellular matrix (ECM). The observed roles of DP4 in adhesion and migration of cancer cells may be attributable to its binding with cell surface fibronectin.¹⁰⁵ Notably, DP4 also functions as a binding partner and adhesion molecule for other proteins including adenosine-deaminase (DP4 is also known as ADA-binding protein),^{106,107} the kidney Na^+/H^+ exchanger isoform NHE3,¹⁰⁸ and the T-cell antigen CD45,¹⁰⁹ a protein tyrosine phosphatase. DP4 has also been identified as a cell surface receptor for plasminogen.¹¹⁰ DP4 binding with ADA and CD45 initiates a signal transduction pathway via tyrosine phosphorylation contributing to the co-stimulatory effect of DP4 on T-cells.^{73,111} Binding of the ADA–DP4 complex with plasminogen has been demonstrated in 1-LN human prostate-cancer cell lines¹¹² and is thought to contribute to FAP and DP4-mediated fibrinolysis.⁶¹ Despite homology between the two enzymes, no evidence exists for the binding of FAP with ADA.⁹¹ In renal brush-border membranes where the multimedia complex between DP4 and Na^+/H^+ exchanger isoform NHE3 is formed, inhibition of DP4 activity results in the downregulation of NHE3 activity via inhibition of a tyrosine kinase signaling pathway, thus suggesting that DP4 plays an important role in modulating Na^+/H^+ exchange mediated by NHE3.^{113,114} The exact mechanism by which DP4 inhibitors affect downstream tyrosine kinase signaling pathways is unknown, but it may be possible that inhibitor-binding results in a conformational change to DP4, altering downstream signaling pathways or that inhibition of DP4 activity prevents DP4 substrate-mediated signaling.¹¹³ In the human 1-LN prostate-tumor cell line, it has been shown that the association of plasminogen with DP4 and NHE3 is involved in regulating the invasive phenotype.¹¹⁵ Direct binding of plasminogen type II (Pg 2) with DP4 on the surface of prostate cancer cells results in the induction of an intracellular Ca^{2+} signaling cascade that in turn results in the increased expression of MMP9.¹¹⁶ DP4-plasminogen binding is thought to ‘lock’ plasminogen into a favorable configuration for its cleavage and activation by the urokinase plasminogen activator receptor (uPAR).¹¹⁰ It is this activation/interaction with uPAR that results in release of intracellular calcium stores and induction of MMP9 expression.¹¹⁰ Interestingly, FAP is thought to interact directly with uPAR via complex formation.¹¹⁷ uPAR is an important cell surface protease, converting plasminogen to plasmin, contributing to regulation of ECM proteolysis.¹¹⁸ Numerous studies have investigated physiological roles and therapeutic potential of uPAR in human diseases such as cancer (reviewed in several previous studies^{119–121}). Co localization of FAP and

uPAR in the plasma membrane of LOX malignant melanoma cells and FAP–uPAR complex formation has been demonstrated,¹¹⁷ likely increasing proteolytic progression of cells through the tumor microenvironment. Such non-enzymatic roles of FAP are thought to contribute to its roles in profibrinolysis.⁵² In 2005, Wang *et al.* found that an enzyme negative FAP mutant over-expressed in the LX-2 human hepatic stellate cell (HSC) resulted in enhanced cell adhesion, migration and staurosporine streptomyces-stimulated apoptosis, providing further evidence for the importance of FAPs non-enzymatic roles in the facilitation of tissue remodeling in chronic liver injury.⁹³ Similar to DP4, FAP expression has also been linked with changes in MMP expression. Over expression of either FAP or DP4 in 293T cells resulted in an increase in MMP-2 and CD44 expression accompanied by a reduction in integrin- β 1.⁹³ MMPs are well known for their roles in cell adhesion and migration via proteolytic degradation of the ECM, and the association of DP4 and FAP with altered MMP activities most likely contributes to their observed roles in cancer.

1.4.2 DP8 and DP9: Intracellular Proteins

DP8 was first identified and cloned by Abbott *et al.* in 2000,⁶ almost 40 years after the initial discovery of DP4. Within the Abbott *et al.* study, DP9 was first identified *in silico* by BLAST alignment with DP8.⁶ In 2002, Olsen and Wagtmann reported the first cloning of the short 863-amino-acid form of DP9³⁰ followed by repeated cloning of short DP9 by Qi *et al.* in 2003²¹ and the subsequent cloning of both short and long 892-amino-acid forms by Ajami *et al.* in 2004.³⁴ Due to the high structural homology of both DP8/9 with DP4 and FAP, investigations into their biochemical functions have rapidly increased since their discovery. At present, studies have led to the implication of both DP8/9 in immune functions, but clear physiological roles are yet to be defined for either of the enzymes. DP8 and DP9 are closely related molecules sharing 61% amino acid identity, intracellular localization, and ubiquitous tissue expression. Intuitively, it seems likely that DP8/9 play essential roles in human biology, and an overlap or redundancy in function may exist between the two. That said, it is also likely that each may have unique and independent functions that are yet to be revealed. An inherent problem that arises when investigating DP8 and DP9 is differentiating between the observed effects of these two enzymes using current research tools. Until knockout animals are generated and/or crystal structures resolved to enable the design of DP8 and DP9 selective inhibitors, this will continue to be an issue. If DP8 and DP9 are essential intracellular enzymes with redundant functions, it will be highly beneficial for a double DP8/9 knockout to be generated for an assessment of its viability. Likewise, if redundant/compensatory mechanisms exist between DP8 and DP9, then individual knockout animals should presumably result in viable offspring. At present, only speculations can be made.

Originally identified as monomeric enzymes, both DP8 and DP9 are now readily known to form catalytically active dimers.^{122–124} It has recently been shown that DP8 monomers still retain activity.¹²⁵ Recombinant human DP8 and DP9 display optimal enzyme activity between pH 7.0 and 8.5 with low levels of residual activity still observed at acidic pH 5.0.^{6,21,124} Recently, a natural form of DP9 was purified from bovine testes¹²⁶ and identified as being the short form of DP9 by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis of the N-terminal sequence.³⁵ This natural form of bovine DP9 displays a similar pH profile to the human DP9 expressed and purified from insect cells.¹²⁶ Enzymatic activities of both recombinant DP8 and DP9 can be inhibited in the presence of Zn^{2+} .^{6,126} DP9 activity is also reduced in the presence of Ni^+ and almost completely inhibited by the presence of Cu^{2+} and Hg^+ . These results suggest a physiological role for intracellular Zn^{2+} or other metal ions in the regulation of cytosolic DP8/9 activity. Recently, *in vitro* evidence has emerged suggesting that the activity of DP8/9 might also be regulated by intracellular redox events.^{126,127} Evidence of redox-state regulation of related PEP enzyme activity has been previously demonstrated.^{128,129}

Both DP8 and DP9 are ubiquitously expressed at the mRNA level in all human adult and foetal tissues as assayed by master RNA blot or Northern blot hybridization.^{6,34} High levels of DP8 transcript can be found in the testis, heart, liver, skeletal muscle, kidney, pancreas,⁶ and a number of endocrine-related tissues.⁵² In testis, a smaller alternative transcript of DP8 with high expression has been detected.^{6,51} Displaying a similar pattern of expression, the highest levels of DP9 transcript can be found in the heart, liver, skeletal muscle, spleen, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, and also a number of other endocrine related tissues.³⁴ In comparison with DP8, a much lower level of DP9 mRNA was detected in the testis;⁵² thus, it was surprising that natural DP9 was purified from bovine testis and not DP8.³⁵ The presence of different mRNA transcripts for DP8 and DP9 also suggests their importance in the male reproductive system. Expression of DP8/9 in this system has thus been further investigated.

Using individual DP specific inhibition profiles, Dubois *et al.* found that the majority of detectable DP activity in bovine and rat testis could be attributed to the activities of DP8/9, whereas in the epididymis, DP4 was the predominant contributor.¹³⁰ Immunohistochemistry revealed specific, and distinct staining of DP8 and DP9 in sperm cells from late stages of spermatogenesis; however, little to no DP8/9 activity was detectable in preparations of bovine sperm,¹³⁰ suggesting that DP8/9 play important roles in the late stages of development but play no role in mature male gametocytes. In support of these findings, a recent study by Yu *et al.* using microarray data analysis to determine the levels of DP8 and DP9 mRNA during the different stages of murine sperm cell development found a high abundance of DP9 in later stage spermatocytes.¹³¹ In addition, *in situ* hybridization of baboon testis found DP8/9 distributed in spermatogonia and spermatides but only weak staining of DP9 in

spermatocytes suggesting species-specific differences.¹³¹ If DP8 and DP9 play crucial roles in the development of sperm maturation, it can be speculated that loss of both DP8/9 expression within the male reproductive system could lead to sterility. Future studies analyzing human samples and using knockout animals will be useful to answer this question and to assess whether DP8 and/or DP9 are potential targets for the development of therapeutics in the treatment of infertility.

As above, individual DP specific inhibition profiles have also been used to assess the level of DP8/9 specific activity in human brain, rats and mice. Stremenova *et al.* identified DP8/9 as the major enzymes contributing to detectable DP activity in non-malignant human brain tissue.¹³² DP8/9 have also been identified as major enzymes contributing to DP activity in rat brains, with comparisons made between crude brain extracts isolated from wild-type and DP4 deficient rats.¹³³ Using DP selective inhibitors, Freker *et al.* found that in brain, the level of DP8/9 activity was higher than that of DP4 and lower than the activity attributable to the related DP2.¹³³ In a similar study using DP4 wild type and DP4^{-/-} mice, DP8/9 were identified as strong contenders for the observed DP activity in murine brain.¹³⁴ In addition, this Ansorge *et al.* study identified both DP8 and DP9 as the most probable enzymes responsible for the remaining observed DP4-like activity in skin and heart tissue of DP4^{-/-} mice.¹³⁴ DP8 and DP9 mRNA is detected at high levels in both these tissues as previously discussed. Yu *et al.* performed a similar study with their focus being on determining the *in vivo* expression and distribution of both DP8 and DP9 in human baboon and mouse tissues.¹³¹ This study found that both DP8/9 significantly contribute to the levels of DP activity observed in peripheral blood mononucleocytes (PBMcs), the thymus, spleen, colon, lung, liver brain, muscle, testis, pancreas, adipose and adrenal tissues.¹³¹

Both DP8/9 have been implicated in regulating the immune response. Initial reverse-transcriptase polymerase chain reaction (RT-PCR) suggested that full-length DP8 expression was upregulated in activated T cells.⁶ DP8/9 specific activity has since been detected in human peripheral blood leukocytes (lymphocytes and monocytes), Jurkat, and U937 (a human monocytic cell line) cells.¹³⁵ DP8/9 activity levels were similar in all leukocyte cell types tested¹³⁵ suggesting that DP8/9 have ubiquitous level of activity within the immune system. Increasing evidence has been mounting for the involvement of DP9/9 in regulating the immune response¹³⁴, thus making them potential targets for the development of anti-inflammatory based therapeutics which will be discussed later in this chapter.

1.4.2.1 *Non-Enzymatic Roles of DP8 and DP9: Protein-Binding Partners*

Potential roles for DP8 and DP9 in apoptosis, wound healing, and migration have been investigated.^{136,137} Using 293T cells, transient transfections were

performed to over express DP8- or DP9-green-fluorescent protein (GFP)-fusion proteins.¹³⁶ By this means, DP9 was found to be associated with impaired cell adhesion, migration, and monolayer wound healing.¹³⁶ DP8 was also associated with impaired migration and wound healing but not cell adhesion.¹³⁶ Both DP8 and DP9 were found to enhance staurosporine streptomyces (STS)-induced apoptosis, while over-expression of DP9 reportedly increased the rate of spontaneous apoptosis.¹³⁶ Interestingly, using enzyme negative mutants of each protein, the involvement of DP8 and DP9 in these processes was found to be independent of their enzymatic activity and of the RGD motif of DP9.¹³⁶ Over expression of DP9 was also found to decrease the mRNA level of proteins known to be associated with cell adhesion; discoidin domain receptor 1 (DDR1), E-cadherin, and tissue inhibitor of matrix metalloproteinases 2 (TIMP-2).¹³⁷ Thus, it is possible that the observed phenotypes arising from DP8- or DP9-over expression may result from direct protein–protein interactions of these molecules with as-yet unidentified ligands or via indirect means which alter the expression and function of additional proteins involved within cell adhesion and migration.

1.5 Protein Structure, Active Site Entry, and Substrate Specificity

Substrate binding and specificity inherently relies on the conformational structure of a protease. To date, numerous crystal structures of DP4, often in complex with substrates or inhibitors, have been resolved along with structures for FAP¹³ and DP6.¹⁸ A number of groups, including our own, have successfully expressed and purified recombinant DP8 and DP9, but the crystal structures of these enzymes are yet to be revealed. Structural information about DP8 and DP9 is inferred by building homology models using DP4, FAP, DP6, and PEP structures. In the absence of abundant knowledge regarding *in vivo* biological substrates, the substrate specificity of the DPs has been predominantly derived from *in vitro* screening of synthetic combinatorial substrate libraries with purified recombinant protein. Structural aspects relevant to catalytic activity, substrate entry, and substrate specificity of DP4, FAP, DP8, and DP9 are discussed below.

DP4 is catalytically active as a dimer with each monomer consisting of an α/β hydrolase domain and an eight-bladed β -propeller domain^{16,138} (Figure 1.3). Crystal-structure resolution has revealed that between these two domains lies a large cavity of > 2 nm (~ 3 – 4.5) nm, and within this cavity lies a smaller cavity, forming the catalytic cleft containing the active triad, Ser630, Asp708, and His 740.^{15,16} Entry of substrates to the active site located in this large cavity is hypothesized to occur via two different mechanisms/openings: (1) substrates may enter/pass through a funnel located in the top opening of the β -propeller domain or (2) substrates may enter the active site via the much larger, less restricted, side opening of the β -propeller domain.^{15–17,139} In favor of the second hypothesis, the entrance via the side opening appears to be the shortest and

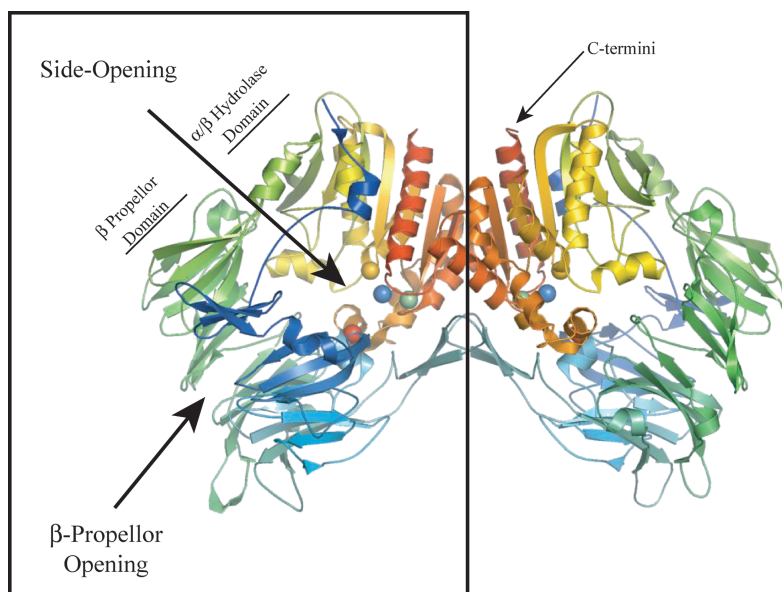


Figure 1.3 Dimeric structure of human DP4 demonstrating entry to the active site. The catalytic triad residues are displayed as spheres; Red = Ser630; Green = Asp708 and Blue = His740. The two Glu residues at 205 and 206 that interact with the substrate are displayed as yellow spheres. The structural representation was generated using *PyMOL* with structural coordinates (PDB code 1N1M) from Rasmussen *et al.*¹⁵

most easily accessible route.^{15–17} It is possible that entry and exit of substrates occur via different routes.¹⁷ Structural resolution of DP4 in complex with residues 1–10 of its substrate, NPY, provided evidence for preferred entry of substrates via the large side-opening.¹⁷ Furthermore, docking experiments of DP4 with aprotinin indicated that the aprotinin N terminus could enter the active site only via the side-opening.¹⁴⁰ The large cavity is negatively charged and would most likely attract the positively charged N-terminus of DP4 substrates into the active site.¹⁵ DP4 and FAP structures reveal that the pair of conserved glutamate residues that are required for enzymatic activity, (Glu²⁰⁵, Glu²⁰⁶) in DP4 and (Glu²⁰³, Glu²⁰⁴) in FAP, aid in catalysis due to their negative charge recognizing the positively charged N-termini of substrate peptides.^{13,139} Resolution of the FAP crystal structure revealed a similar proposed route of entry to the active site, via one of the two openings within the β -propeller domain.¹³ One of the main requirements for DP4 catalysis to occur is for a free, unblocked, protonated α -amino group to be present in the P1 position.^{141,142} In this regard, FAP differs from DP4 due to its ability to function also as an endopeptidase. Due to the restricted size of entry into the active site, it is thought that only elongated peptides or unfolded or partly unfolded protein fragments can reach the active site.¹⁵ Furthermore, only small

protein and peptide substrates for DP4 have been identified; hence it is speculated that substrates of all DPs will be limited to a maximum of 100–200 amino acids in length,^{1,143} thus limiting the potential substrate repertoire. However, FAP is known to cleave α_2 AP, which is 464 amino acids in length.²⁸ This suggests that it is also possible for larger proteins with a free, non-restricted N-termini to be *in vivo* substrates of DPs.

In comparison with DP4, the DP substrate specificity of FAP at the P2 position is much more restricted with FAP displaying a preference for isoleucine, proline, or arginine in the P2 position.¹⁴ Edosada *et al.* characterized the P4–P2' substrate specificity of FAP endopeptidase activity, revealing a strong requirement for the presence of glycine or small amino acids in the P2 position in order for the endopeptidase activity of FAP to occur.¹⁴ A reduction in specificity was observed at positions P3, P4, P1', and P2' with a wider range of residues being accepted.¹⁴ Considering that this specificity of FAP has been determined *in vitro*, it will be of interest in future to determine if a broader specificity is observed within the *in vivo* complex biological setting or against true endogenous substrates where other features interacting with the structure/conformation of FAP may come into play. Homology models reveal a high degree of similarity in structure of DP8 and DP9 compared with DP4 and FAP, suggesting that substrate access would be similar with slight variations in specificity.^{138,144} Differences in kinetics of purified recombinant DP8 to those of DP4 indicate the likelihood of there being subtle differences in substrate-binding pockets between these two enzymes.¹⁴⁵ A larger S1 pocket size would potentially allow for larger residues to be accommodated in the S1 position of DP8/9 compared to DP4. Elucidation of DP8/9 substrate specificities has been determined *in vitro* using combinatorial peptide libraries. *In vitro* analysis reveals a preference for DP8 and DP9 towards substrates containing small, basic, or large hydrophobic residues in the P2 position.^{123,124,145} Lee *et al.* confirmed DP8's strong preference for Pro in the P1 position and detected activity against substrates with alternate alanine, methionine, or threonine in the P1 position¹²³. DP4 is well known to cleave substrates with Ala, Ser, and Gly in the P1 position such as GLP-1. *In vitro*, DP8 and DP9 cleavage of GLP-1 has been demonstrated.¹²² Acidic residues in the P2 position were the least favorable for both DP8 and DP9 cleavage.^{123,124} In regards to these findings, consideration must be given to the fact that while substrate specificity is being determined *in vitro*, the specificity of DP8/9 may be considerably different in the complex biological setting *in vivo*. Interestingly, during our own research involving DP8/9 substrate discovery, utilizing stable cell lines and terminal affinity labeling of substrates (TAILS) technology¹⁴⁶ we have identified a potential biological substrate of DP8/9 with one of the least favored acidic residues in the P2 position using a peptide library (Wilson *et al.*, in preparation). While structural studies and determination of *in vitro* substrates specificity are useful, they tell us very little about the biological roles of a protease within the human body. Of more use is the identification of true biological substrates that allow for elucidation of the pathophysiological roles of a given protease.

1.6 DP Substrates: Biological Relevance to Human Health and Disease

Discovered over 40 years ago, a number of diverse biological substrates for DP4 have been identified. In contrast, only a limited number of potential FAP substrates have been found. Based on the high degree of sequence identity and structural similarity of DP8 and DP9 with DP4, it is thought that substrate preferences would be the same and potential substrates shared; however, the intracellular *vs.* extracellular localization makes this unlikely. Recently, DP8/9 cleavage of known DP4 substrates has been demonstrated *in vitro*, and a biological substrate of DP9 identified. In our own studies, we have identified two potential natural substrates of DP8/9 that may prove to be of interest in future, although they are not discussed here. DP4 is known to cleave numerous biologically active peptides, hormones, and chemokines sourced from a diverse range of families including: the pancreatic polypeptide family (NPY, PYY), pituitary adenylate cyclase-activating peptide (PACAP)/Glucagon family (GLP-1, glucagon, GLP-2, GIP, growth-hormone-releasing factor (GRF), vasoactive intestinal peptide (VIP), PACAP27/38, peptide histidine methionine (PHM)), chemokines (Mig, interferon- γ inducible protein (IP-10), interferon inducible T cell α -chemoattractant (I-TAC), stromal derived factor (SDF)1- α / β , human analog of macrophage inflammatory protein-1 α (LD78 β), RANTES (regulated on activation, normal T cell expressed and secreted), eotaxin, macrophage-derived chemokine (MDC)), and other molecules such as the neuropeptides; substance P, β -casomorphin, endomorphin, and morphiceptin (Table 1.1). Proteolytic cleavage by DP4 can lead to the inactivation, activation, or alteration in receptor specificity of a substrate, thus leading to changes in signaling pathways and numerous biological processes. Many DP4 substrates exert their effects on a number of physiological processes, but in the context of this review only the main ones will be discussed.

1.6.1 Glucose Homeostasis and Nutrient Metabolism

To date, *in vivo* cleavage has been demonstrated only for a few of the potential DP4 substrates with the most notable of these including GLP-1 and GIP. GLP-1 and GIP are highly insulinotropic proteins, functioning as potent stimulators of glucose-dependent insulin secretion. In addition, GLP-1 contributes to blood-glucose homeostasis by inhibiting the secretion of glucagon, gastric emptying and reducing food intake. Following the intake of food, the levels of GLP-1 will rise rapidly in response to the increasing blood glucose levels. Here DP4 plays an important role in maintaining glucose homeostasis by rapidly degrading the N-termini of GLP-1₍₇₋₃₆₎ and GIP₍₁₋₄₂₎ converting them into biologically inactive forms GIP₍₃₋₄₂₎ and GLP-1₍₉₋₃₆₎.¹⁴⁷⁻¹⁴⁹ Inhibition or loss of DP4 activity results in increased levels of stable GLP-1₍₇₋₃₆₎, leading to improved glucose tolerance via increases in the glucose-stimulated insulin secretion and decreased glucagon secretion (reviewed in several previous studies^{58,59,150,151}). DP4 plays an important role in regulating the activity of

Table 1.1 DP4 biological substrates.

<i>Physiological system</i>	<i>Biological substrate</i>	<i>Length (aa)</i>	<i>N-terminal sequence</i>	<i>Evidence</i>	<i>References</i>
Digestive and vascular system	GLP-1 _(7–37) , _(7–36)	30, 31	His-Ala ↓ Glu-Gly-Thr-Phe-	<i>In vitro/in vivo</i>	147–149,152
	GIP _(1–42)	42	Tyr-Ala ↓ Glu-Gly-Thr-Phe-	<i>In vitro/in vivo</i>	148,149
	glucagon _(1–29) , _(3–29)	29, 27	His-Ser ↓ Gln-Gly-Thr-Phe-	<i>In vitro</i>	153,154
	PHM _(1–27)	27	His-Ala ↓ Asp-Gly-Val-Phe-	<i>In vitro</i>	148
	VIP _(1–28) , _(3–28)	28, 26	His-Ser ↓ Asp-Ala ↓ Val-Phe-	<i>In vitro</i>	148,155
	GLP-2 _(1–33)	33	His-Ala ↓ Glu-Gly-Ser-Phe-	<i>In vitro/in vivo</i>	152,156,157
	GRP	27	Val-Pro ↓ Leu-Pro -Ala	<i>In vitro</i>	158
	PACAP _(1–27) , _(3–27) , _(1–38) , _(3–38)	27, 25, 38, 36	His-Ser ↓ Asp-Gly ↓ Ile-Phe-	<i>In vitro</i>	148,155
	enterostatin _(1–5) , _(3–5)	5, 3	Val-Pro ↓ Asp-Pro -Arg	<i>In vitro</i>	159
	bradykinin _(2–9)	9	Pro-Pro ↓ Gly-Phe-Ser-Pro	<i>In vitro</i>	160
	BNP _(1–32)	32	Ser-Pro ↓ Lys-Met-Val-Glu	<i>In vitro/in vivo</i>	161
	GRF _(1–44/40/32/29)	44, 40, 32, 29	Tyr-Ala ↓ Asp-Ala-Ile-Phe-	<i>In vitro</i>	148, 162–164
Nervous and endocrine system	vasostatin-1 _(1–76)	76	Leu-Pro ↓ Val-Asn-Ser-Pro	<i>In vitro</i>	165
	substance P _(1–11) , _(3–11)	11, 9	Arg-Pro ↓ Lys-Pro ↓ Gln-Gln-	<i>In vitro/in vivo</i>	166–168
	PYY _(1–36)	36	Tyr-Pro ↓ Ile-Lys-Pro-Glu-	<i>In vitro/in vivo</i>	169,170
	NPY _(1–36)	36	Tyr-Pro ↓ Ser-Lys-Pro-Asp-	<i>In vitro/in vivo</i>	169,171
	β-casomorphin _(1–7)	7	Tyr-Pro ↓ Phe-Val-Glu-Pro-	<i>In vitro</i>	172–176
	endomorphin 2 _(1–4)	4	Tyr-Pro ↓ Phe-Phe-NH ₂	<i>In vitro/in vivo</i>	177,178
	endomorphin-1 _(1–4)	4	Tyr-Pro ↓ Trp-Phe-NH ₂	<i>In vitro</i>	179,180
	morphiceptin _(1–4)	4	Tyr-Pro ↓ Phe-Pro-NH ₂	<i>In vitro/in vivo</i>	174
	hemorphin-7 _(2–9)	9	Tyr-Pro ↓ Tryp-Thre-Glut-Arg	<i>In vitro</i>	181
	RANTES (CCL5) _(1–68)	68	Ser-Pro ↓ Tyr-Ser-Ser-Asp	<i>In vitro</i>	182–184
	I-TAC (CXCL11) _(1–73)	73	Phe-Pro ↓ Met-Phe-Lys-Arg-	<i>In vitro</i>	184,185
Immune system	eotaxin (CCL11) _(1–74)	74	Gly-Pro ↓ Ala-Ser-Val-Pro-	<i>In vitro</i>	184,186
	GCP-2 (CXCL6) _(1–77)	77	Gly-Pro ↓ Val-Ser-Ala-Val	<i>In vitro</i>	183
	SDF-1α/β (CXCL12) _(1–68/72)	68/72	Lys-Pro ↓ Val-Ser-Leu-Ser-	<i>In vitro</i>	184,187–189
	MDC (CCL22) _(1–69) , _(3–69)	69, 67	Gly-Pro ↓ Tyr-Gly ↓ Ala-Asn-	<i>In vitro</i>	184,190
	IP-10 (CXCL10) _(1–77)	77	Val-Pro ↓ Leu-Ser-Arg-Thr-	<i>In vitro</i>	182,184
	LD78β (CCL3L1) _(1–70)	70	Ala-Pro ↓ Leu-Ala-Ala-Asp-	<i>In vitro</i>	184,191
	Groβ (CXCL2) _(1–73)	73	Ala-Pro ↓ Leu-Ala	<i>In vitro</i>	192
	Mig (CXCL9) _(1–104)	104	Thr-Pro ↓ Val-Val-Arg-Lys-	<i>In vitro</i>	184

Cleavage sites are indicated with an arrow, and DP4 cleaved dipeptides are indicated in bold.

BNP, B-type natriuretic peptide; GCP-2 (CXCL6), granulocyte chemotactic protein; GLP, glucagon like-peptide; GRF, growth-hormone-releasing factor; GRP, gastrin-releasing peptide; IP-10 (CXCL10), interferon-γ inducible protein; I-TAC (CXCL11), interferon inducible T cell α-chemoattractant; LD78β (CCL3L1) human analog of MIP-1α; MCP-1/2/3 (CCL2/7/8), monocyte chemotactic protein-1/2/3; MDC (CCL22), macrophage-derived chemokine; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine; PYY, peptide tyrosine tyrosine; RANTES (CCL5), regulated on activation, normal T cell expressed and secreted; SDF-1α (CXCL12), stromal derived factor-1α; VIP, vasoactive intestinal peptide.

additional members of the PACAP/glucagon families including glucagon, VIP, gastrin-releasing peptide (GRP), GRF, PACAP, and GLP-2 (Table 1.1); many of these substrates are involved in processes of nutrient metabolism and glucose homeostasis. GLP-2, derived from the same pro-glucagon molecule as GLP-1 and GIP, is of particular interest to our group. Expressed mainly in the gut, GLP-2 functions as a highly potent promoter of gastrointestinal growth and reduces the severity of intestinal injury that arises during inflammatory conditions of the bowel.¹⁹³ In a similar fashion to GLP-1 regulation, DP4 is responsible for the rapid inactivating degradation of GLP-2.¹⁵⁶ Research by our own group and others demonstrates the efficacy of DP4 inhibition in ameliorating the effects of inflammatory disease of the gastrointestinal tract such as inflammatory bowel disease, presumably partly due to the prolonged half-life of GLP-2 in circulation.¹⁹³ Interestingly, targeted deletion of NPY, an additional DP4 substrate, is also shown to be involved with inflammatory disorders with its ablation leading to attenuated inflammation in an experimentally induced dextran sulfate sodium (DSS) colitis model;¹⁹⁴ thus, it may prove to be an important avenue for future investigations into this work.

NPY is an additional *in vivo* substrate of DP4. Both NPY and PYY are 36-amino-acid members of the pancreatic polypeptide family. NPY is a widely distributed neurotransmitter, abundant in brain, while PYY is produced by L-type endocrine cells of the gastrointestinal tract, where it colocalizes with GLP-1.¹⁹⁵ NPY₍₁₋₃₆₎ acts through multiple Y receptor types, functioning as a potent vasoconstrictor through receptor Y1,¹⁹⁶ and playing an important role in the stimulation of feeding through receptors Y1 and/or Y5.^{197,198} Like NPY₍₁₋₃₆₎, PYY₍₁₋₃₆₎ acts through the same Y receptors and is involved in inhibiting gastrointestinal function, insulin secretion, blood pressure homeostasis and appetite and weight control (reviewed in¹⁹⁵). *In vitro*, NPY and PYY are rapidly cleaved to their truncated forms NPY₍₃₋₃₆₎ and PYY₍₃₋₃₆₎ by DP4.¹⁶⁹ DP4 proteolysis of NPY results in a shift in NPY receptor selectivity, changing its biological action from being a vasoconstrictor to a promoter of the angiogenic process.¹⁷¹ Ghersi *et al.* demonstrated that DP4 expression is upregulated in migrating cells at sites of wounding and that the increase in DP4 activity is required for the generation of NPY₍₃₋₃₆₎ and observed effects of NPY₍₃₋₃₆₎ in promoting cell migration and wound healing.²⁰⁰ Both PYY₍₁₋₃₆₎ and PYY₍₃₋₃₆₎ are abundant in circulation. Truncated PYY₍₃₋₃₆₎, generated by DP4 proteolysis, specifically acts through the Y2-receptor subtype,¹⁹⁹ again highlighting the resultant shift in receptor selectivity of a molecule upon DP4 cleavage. PYY₍₃₋₃₆₎ inhibits food intake and reduces weight gain,²⁰¹ the opposite effects to those observed for PYY₍₁₋₃₆₎. Thus, DP4 regulates effects in feeding, metabolism, and weight control via proteolytic cleavage of PYY, NPY, GLP-1, and also enterostatin, and this highlights the potential application of targeting DP4 for the treatment of obesity.

Recently, *in vitro* studies have demonstrated that both DP8 and DP9 are capable of cleaving GLP-1, GLP-2, NPY, and PYY (Table 1.2), but cleavage rates by DP8/9 are much slower than DP4.^{122,133} Interestingly, DP8/9 are the predominant enzymes responsible for DP activity in brain compared to DP4¹³³

Table 1.2 *In vitro* and biological substrates of DP8 and DP9.

<i>Demonstrated cleavage</i>	<i>Substrate</i>	<i>N-terminal sequence</i>	<i>References</i>
DP8, DP9	GLP-1 _(7–36)	His-Ala ↓ Glu-Gly-Thr-Phe-	122
	GLP-2 _(1–33)	His-Ala ↓ Glu-Gly-Ser-Phe-	122
	NPY _(1–36)	Tyr-Pro ↓ Ser-Lys-Pro-Asp-	122,133
	PYY _(1–36)	Tyr-Pro ↓ Ile-Lys-Pro-Glu-	122
DP8	SDF-1α (CXCL12) _(1–68)	Lys-Pro ↓ Val-Ser-Leu-Ser-	192
	SDF-1β (CXCL12) _(1–72)	Lys-Pro ↓ Val-Ser-Leu-Ser-	192
	IP-10 (CXCL10) _(1–77)	Val-Pro ↓ Leu-Ser-Arg-Thr-	192
	I-TAC (CXCL11) _(1–73)	Phe-Pro ↓ Met-Phe-Lys-Arg-	192
DP9	RU1 _(34–42)	Val-Pro ↓ Tyr-Gly-Ser-Phe-	202

Cleavage sites are indicated with an arrow, and DP8/9 cleaved dipeptides are indicated in bold.

where many of these substrates are found, but physiological relevance, if any, of NPY as a potential DP8/9 substrate is yet to be revealed, especially given that most substrates are soluble and secreted, while DP8/9 are localized in the cytoplasm.

1.6.2 Cardiovascular Effects

In addition to the implicated roles of DP4 in cardiovascular function via NPY truncation, a number of other substrates exerting cardiovascular effects are described. Bradykinin is an important vasoactive peptide involved in blood-pressure homeostasis that is primarily metabolized by ACE, but can also be degraded by the consecutive action of aminopeptidase P and DP4. Aminopeptidase P first removes an N-terminal arginine residue allowing DP4 to then cleave a Pro-Pro dipeptide from the N-terminus.¹⁶⁰ Substance P is also a substrate of ACE²⁰³ along with other proteases including DP4.²⁰⁴ Inhibitors of ACE are used widely for the treatment of hypertension, congestive heart failure and impaired renal function, but angioedema is a potentially life-threatening adverse side effect that can result from ACE inhibitors. The angioedema side-effect is thought to arise potentially from impaired degradation of ACE substrates, bradykinin, and substance P, by DP4 and aminopeptidase P due to decreased DP4 activity during ACE-inhibitor-associated angioedema.^{205,206} Co-administration of an ACE inhibitor with a DP4 inhibitor, vildagliptin (for treatment of type II diabetes), has been shown to increase significantly the risk of angioedema.²⁰⁷ It should be noted that administration of the DP4 inhibitor alone did not increase the risk of angioedema,²⁰⁷ thus highlighting the complex interplay between ACE inhibition, DP4 activity, and degradation of the vasoactive peptides, bradykinin and substance P in the development of ACE-inhibitor-associated angioedema. In addition to these findings, DP4 cleavage of vasostatin I, a chromogranin A-derived peptide implicated in the regulation of cardiovascular function,²⁰⁸ has been demonstrated *in vitro*.¹⁶⁵ However, to our knowledge, no further studies involving vasostatin I as a potential DP4

substrate have been reported. Further supporting a role for DP4 regulation in the cardiovascular system is the demonstrated cleavage of plasma B-type natriuretic peptide (BNP) by DP4, resulting in the release of its truncated form, BNP (3–32).¹⁶¹ BNP (3–32), known also as *des*-SerPro-BNP, has been detected in plasma of patients with heart failure.²⁰⁹ BNP is one of two natriuretic peptides secreted by the heart that are involved in cardiovascular homeostasis.²¹⁰

1.6.3 Nociception and Neuropsychiatric Disorders

Substance P is a widespread neuropeptide implicated in numerous roles, including nociception, mood and anxiety disorders, aggressive behavior, and immunity.^{211–214} It was demonstrated as early as 1978 that DP4 can cleave substance P,¹⁶⁶ and sequential DP4 cleavage results in the generation of substance P_(3–11), then substance P_(5–11).¹⁶⁷ Alterations in the level of serum DP4 activity have been associated with a number of psychiatric disorders such as depression, anxiety, schizophrenia, and anxiety.^{67,215} DP4 cleavage of substance P activity may be involved in these processes, but it is important to note that the levels of serum PEP activity, also capable of cleaving substance P, are also altered in these disorders.^{216,217} Recently, pre-incubation of substance P with a DP4-overexpressing glioma cell line was found to lead to a loss of substance P signaling via intracellular calcium mobilization.²¹⁸ DP4 cleavage modulation of pain is further discussed below.

Endomorphin-1 and endomorphin-2 are endogenous opioid peptides, expressed widely in the central nervous system, having key roles in nociception, and they participate in inflammatory disorders such as rheumatoid arthritis, osteoarthritis, and experimental arthritis (reviewed by Jessop *et al.*²¹⁹). Endomorphin-1 was identified as being a DP4 substrate based on the increased stability of endomorphin-1 in the presence of porcine buccal mucosa by Diprotin-A (Ile-Pro-Ile), a competitive DP inhibitor.¹⁷⁹ Diprotin-A is also capable of inhibiting PEP,²²⁰ aminopeptidase N,²²⁰ and DP8/DP9²¹ activity, making it possible that DP4 is not the enzyme responsible for the observed cleavage. DP2 is an unlikely candidate due its poor inhibition by Diprotin A with K_i of $>35\,000\text{ nM}$ compared to a K_i of $>250\text{ nM}$ for the inhibition of DP4.²⁵ Janecka *et al.* provide a detailed review on the degradation of endomorphins by DP4 and other proteases including aminopeptidase M, carboxypeptidase Y, and proteinase A.²²¹ DP4 has been implicated in the modulation of nociception via cleavage of endogenous substrates, endomorphin-1, endomorphin-2, and substance P.^{177,222} Inhibition of DP4 activity with Ala-Pyrrolidonyl-2-nitrile in mice has been found to lead to enhanced analgesic effects of exogenously administered endomorphin-2.¹⁷⁷ Morphiceptin, similar in sequence to the endomorphins and a reported analog of β -casomorphin, is another endogenous opioid peptide that acts as a highly potent and selective agonist for the μ -opioid receptor known for its roles in pain perception.²²³ *In situ* perfusion in rats genetically deficient in DP4 revealed a significant increase

in morphiceptin activity, administered into the intestinal lumen, compared to DP4^{+/+}, rats thus providing evidence for a role for DP4 as the major enzyme responsible for morphiceptin degradation¹⁷⁴ and further supporting a role for DP4 in the management of analgesia. β -Casomorphin is a milk-derived peptide with opioid activity with the ability to pass through the gut barrier and function on the immunological as well as neurological systems.¹⁷⁵ Proteolytic degradation of β -casomorphin by DP4 has been detected in a number of studies including a recent report by Iwan *et al.*, who demonstrated the transportation of β -casomorphin across Caco-2, a human intestinal cell line, monolayers known to express high levels of DP4.¹⁷⁵ More recently, DP4 has been shown to cleave an additional endogenous opioid peptide, hemorphin-7, that is derived from proteolytic degradation of hemoglobin.¹⁸¹ Thus, there is substantial evidence supporting roles for DP4 in the modulation of analgesia via its proteolytic activity.

1.6.4 Immune System and Disorders

Chemokines are a large family of small secretory proteins (5 to 10 kDa) involved in the immune system and in numerous inflammatory-related disorders including leukocyte maturation, migration, and chemoattractant activities. Chemokines interact with their cell-surface receptors via their N-terminal region, and so potential modulation of their receptor selectivity and regulation of their activity via proteolytic cleavage is of interest. DP4 cleavage of chemokines has been readily demonstrated, and kinetics indicate they could be physiological substrates of DP4.¹⁵⁵ N-terminal truncation by DP4 can result in a shift in receptor specificity and alter or abolish the chemoattractant activity of RANTES, SDF1- α/β , MDC, eotaxin, IP-10, I-TAC, and Mig towards lymphocytes, monocytes, CD34(+) progenitor cells, and eosinophils, and may reduce any anti-HIV-1 activities of these chemokines.^{182,183,186,189,190,224,225}

Increased chemotactic activity may also result, as occurs for LD78 $\beta_{(3-70)}$ after its derivation from LD78 $\alpha_{(1-70)}$ by DP4.¹⁹¹ LD78 $\beta_{(3-70)}$ displays enhanced chemotactic activity towards monocytes and maintains strong anti-HIV-1 activity. Both IP-10 and Mig can inhibit the angiogenic process, a feature that is retained following their truncation by DP4.²²⁵ Some of the altered migratory capabilities of DP4 expressing tumor cells may be associated with its chemokine substrates. In conjunction with the activity of aminopeptidase N, the involvement of DP4 in I-TAC proteolysis results in impaired migration of microvascular endothelial cells (HMVEC),²²⁶ while DP4 degradation of SDF-1 is suggested to be involved in the metastasis of prostate cancer cell lines.²²⁷ Sezary syndrome is a rare form of T-cell lymphoma affecting the skin in which a characteristic loss of DP4 from the cell surface of T lymphocytes occurs. This loss of DP4 is maintained upon T lymphocyte infiltration of the skin. Narducci *et al.* found that Sezary syndrome T-cells express the functionally active SDF-1 receptor, CXCR4, and that skin cells of patients produce a large amount of the SDF-1 ligand, thus making it likely that in the absence of DP4 expression,

intact SDF-1 is functioning as a potent skin-specific chemoattractant for infiltrating Sezary syndrome T cells.²²⁸ Targeted restoration of DP4 expression on the skin surface may help control this syndrome. A detailed review on the proteolytic processing of chemokines by DP4 and other proteases can be found in Wolf *et al.*²²⁹

DP8 cleavage of chemokines has also been demonstrated *in vitro* (Table 1.2). Screening of 27 chemokines for DP8 cleavage identified, in preference, SDF1- α/β > IP-10 >> I-TAC as *in vitro* DP8 substrates.¹⁹² DP8 cleavage had a relatively lower turnover than that for DP4.¹⁹² It can be hypothesized that DP9 will display a similar ability to cleave these chemokines. Future studies directly comparing the catalytic efficiencies of DP8/9 cleavage of chemokines will be of interest to reveal if subtle differences exist between these two. It should be noted that not all peptidic/protein-based molecules with a proline in P2 are substrates of DP4 or DP8/9. Seven out of 16 chemokines containing a proline residue in the penultimate position failed to be cleaved by either DP4 or DP8.¹⁹² Additionally, many natural cytokines including interleukin(IL)-1 β , IL-2, IL-3, and IL-6, tumor necrosis factor- β , granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF), have an evolutionary conserved proline residue within the second position of their N-termini. However, despite this feature making them ideal candidates as DP4 substrates, no cleavage by DP4 is observed against full-length intact forms of these molecules,²³⁰ suggesting that their N-terminus is inaccessible in the intact proteins.

Recently, the first natural substrate of DP9, the renal ubiquitous (RU)1₍₃₄₋₄₂₎ antigenic peptide, was identified by Geiss-Friedlander *et al.*²⁰² (Table 1.2). RU1₍₃₄₋₄₂₎ is produced in the cytoplasm by proteasome activity, then enters the ER, and then associates with MHC class I molecules for presentation on the cell surface where it is recognized by cytolytic T lymphocytes.^{231,232} Following the *in vitro* confirmation of both DP8 and DP9 cleavage of RU1₍₃₄₋₄₂₎, Geiss-Friedlander *et al.* used siRNA to silence DP8 and DP9, demonstrating the increased presentation of RU1₍₃₄₋₄₂₎ antigen on the cell surface following loss of active DP9 expression.²⁰² This finding points towards a possible role for DP9 in the regulation of MHC class I antigen presentation that may have important consequences in immune-related disorders. A possible role for DP8 in this process involving different antigenic peptides cannot be excluded at this stage.

1.6.5 Tissue Repair and Cancer

Both DP4 and FAP are known to play conflicting roles in cancer and appear to be of importance in the development of fibrinolytic disorders and fibrosis. Recent identification of α_2 AP as a likely physiological substrate for FAP is in keeping with the pro-fibronlytic role of this enzyme and its importance in the development of fibrogenic disorders. As previously discussed, soluble FAP cleaves the Pro12-Asn13 bond of the 464 residue human α_2 -AP with Met at its N-termini (Met- α_2 AP) (MEPLGRQLTSGP-NQEQVSPLT . . .) to yield the

452-residue version with Asn at the N-termini (Asn- α_2 AP) (NQEQVSPLT . . .) that exhibits increased cross-linking to fibrin making the fibrin resistant to plasmin degradation.²⁸ During a proof of concept proteomics study aimed at identifying candidate protease substrates in fractionated human plasma, the cleaved pro-peptide of Asn- α_2 AP with methionine removed (EPLGRQLTSGP) was identified as a potential DP4 substrate,²³³ but the biological relevance of this finding is as yet unknown. FAP has been previously demonstrated to cleave gelatin and potentially collagen type I but not collagen IV, fibronectin, or laminin.^{91,234} Evidence suggests that FAP cleaves only collagen type I and III that are partially degraded or denatured by the proteolytic activity of select MMPs known to have collagenase activity and participating in ECM remodeling.²³⁵ Thus, in the complex extracellular microenvironment of tumors or sites of tissue remodeling, FAP may be working in concert with other proteases that play important roles in ECM degradation, to participate in the degradation of ECM proteins and increase migratory abilities of tumors through stroma. In turn, the increased activation of α_2 -AP leading to decreased fibrinolysis of cross-linked fibrin might result in increased adhesion and migration of tumors. *In vitro* cleavage of collagen by DP4 has been suggested, but participation in ECM degradation is thought to occur via its non-enzymatic protein-binding capabilities. Recently, Jost *et al.* used Wistar rats for an *in vivo* protease inhibitor profiling approach coupled with endogenous blood peptidome analysis, demonstrating that DP4 inhibition affects collagen alpha 1 metabolism *in vivo* and identified BRI (1–23) amyloid peptide as a novel substrate of DP4.²³⁶ Because of the intracellular localization of DP8/9, their direct involvement in cleavage of ECM proteins is unlikely.

1.7 Therapeutic Targeting of the DPs

As illustrated, DP4 and FAP are involved in a wide range of pathophysiological processes, and the importance of DP8/9 in human biology is only now starting to emerge. This family is clearly of interest to Pharma with the development of DP4 inhibitors for the treatment of type II diabetes and past clinical investigations into the efficacy of targeting FAP in cancer or as a potential biomarker. Because of the high degree of homology among the DP4 gene family members and their shared specificity with PEP and DP2, the specificity and selectivity of DP inhibitors must be ensured. Additionally, the issue of inhibitor permeability must be addressed due to the intracellular localization of DP8 and DP9. Both DP4 and FAP have a number of non-enzymatic functions that appear to play important roles in cancer and fibrinolysis; hence, therapeutic targeting to prevent protein-binding interaction may also prove to be of use in future disease treatments. Here, we provide an overview of some work that has already been carried out with regard to designing therapeutics targeting the DPs with consideration given to enzymatic and non-enzymatic approaches.

1.7.1 Targeting Enzymatic Function

Numerous diseases have been targeted for therapeutic treatment via DP4 inhibition including rheumatoid arthritis, multiple sclerosis, psoriasis, wound healing, anxiety, type II diabetes and cancer. Successful inhibition of DP4 for the treatment of type II diabetes has been clearly clinically validated with several DP4 inhibitors developed by different pharmaceutical companies for the treatment of type II diabetes now available on the market or awaiting approval (Table 1.3). Targeted inhibition of DP4 as a therapeutic option is currently being investigated in a number of other disorders, particularly those of an inflammatory nature such as arthritis, skin inflammation, multiple sclerosis, and inflammatory bowel disease.²³⁷ Taking into account the overlap of substrates between DP4 and aminopeptidase N, a novel dual targeting therapy is also being investigated as a therapeutic concept in the treatment of inflammatory disease and acne pathogenesis.^{134,238} To attain clinical approval, the specificity, safety, and efficacy of these inhibitors need to be ensured so that off-target inhibition is not an issue. Lankas *et al.* also found that a number of DP4 inhibitors including L-*allo*-isoleucyl thiazolidide, Lys[Z(NO₂)]-pyrrolidide (LZNP) previously used in several studies implicating the roles of DP4 in immune function, were actually more potent inhibitors of DP8/9 and DP2 (IC₅₀(LZNP): DP8 < DP9 << DP2 < DP4 = 154 < 165 << 1210 < 1300).¹¹ Thus, it is highly likely that some of the reported effects of these inhibitors on immune function are due to off-target inhibition of DP8/9 or DP2 rather than the reported DP4. Lankas *et al.* also demonstrated that Val-boroPro, used in clinical trials as discussed below, is a highly non-selective DP inhibitor with IC₅₀ values for DP4 < DP8 < DP9 << DP2 ≈ PEP < FAP (expressed in nmol l⁻¹) being <4, 4, 11, 310, 390, and 560 respectively.¹¹

Table 1.3 DP4 inhibitors for the treatment of type II diabetes.

<i>Company</i>	<i>Oral inhibitor</i>	<i>Trade name</i>	<i>Current status</i>
Merck	Sitagliptin ^{242–244}	Januvia®	Used in >40 countries FDA approved in 2006 EMA approved in 2007 FDA approved in 2009
Bristol-Myers Squibb & Astra Zeneca	Saxagliptin ^{245–247}	Onglyza®	
Novartis	Vildagliptin ^{248–250}	Galvus®	EMA approved in 2007
Takeda Pharmaceutical company	Alogliptin ²⁵¹		FDA application sus- pended as of 2009, insufficient data
Boehringer Ingelheim Pharma GmbH and Co. KG	Linagliptin ^{252,253}	Ondero®	Phase III clinical trials ^a
Phenomix Corporation	Dutogliptin ^{254,255}		Phase III clinical trials ^a
LG Life Sciences	Gemigliptin		Phase III clinical trials ^a

^aAt time of publication.

FDA, Food and Drug Administration (USA); EMA, European Medicines Agency; LG, Life's Good.

1.7.1.1 Targeting DP8 and DP9

Selective inhibition of DP8/9 results in attenuation of T-cell proliferation and release of the cytokine IL-2.¹¹ In human T-cells, the synthesis of DNA and thus cell proliferation can be suppressed upon treatment of cells with the DP8/9 selective inhibitor, UAMC00132.¹³⁴ Similar evidence provided by Reinhold *et al.* demonstrates that selective inhibition of DP8/9 activity with UAMC00132 results in suppression of DNA synthesis in mitogen-stimulated splenocytes isolated from both wild-type and DP4 knockout mice.²³⁹ Recently, DP8/9 have been implicated in the allergic response of the lung in rat models of experimental induced asthma.²⁴⁰ Asthma is a well-known chronic inflammatory disease of the airways characterized by airway obstruction and resulting in breathing difficulties, typically wheezing, coughing, and breathlessness. Thus, future studies investigating short-term suppression of the immune response during asthma attacks via selective DP8/9 inhibition may prove to be of interest. In research performed by our own group, the expression of DP8 at the transcript level was found to be significantly upregulated in C57BL/6J mice with DSS-induced colitis compared to untreated controls,²⁴¹ thus providing additional evidence for a role of DP8 in acute inflammatory responses.

Controversy has surrounded the potential safety in targeting DP8/9 activity making their suitability as therapeutic targets questionable. Using the DP8/9 selective inhibitor, UAMC00132, over a 2-week period at doses of 10, 30, and 100 mg/kg⁻¹, rats reportedly developed toxic effects including alopecia, thrombocytopenia, reticulocytopenia, enlarged spleen, inflammatory cell infiltration of multiple organs, including the gastrointestinal tract, and mortality.¹¹ In contrast a rebuttal study by Burkey *et al.* using Vildagliptin[®] at high doses of 1500 mg/kg/day for 12 weeks in mice and 900 mg/kg for 12 weeks in rats observed no toxicity or mortality of animals.²⁵⁶ Vildagliptin[®] was designed to selectively inhibit DP4, but a modest level of DP8/9 inhibition remains at these high doses. Wu *et al.* used an alternative DP8/9 selective inhibitor, 1G244, displaying a 15- and eightfold more potent inhibition against DP8/9 than the inhibitor UAMC00132, to assess toxicity, reporting that although toxic effects were not observed, some mild symptoms were identified.²⁵⁷ One of the more concerning mild symptoms observed in animals receiving higher doses of 10 or 30 mg kg⁻¹ day⁻¹ was the development of severe cyanosis which resulted in animals being killed on days 4 and 5 of the 2-week study.²⁵⁷ Cyanosis also developed at the lower dose of 1 mg kg⁻¹ day⁻¹. Development of cyanosis suggests that the inhibitor 1G244 may be causing a hypoxic effect in animals, allowing us to speculate on the involvement of DP8/9 in this process. Without further evidence from a more complete study it cannot be definitively concluded that 1G244, and thus the inhibition of DP8/9, does not cause toxicity. Although progress has been made towards the development of potent and selective inhibitors of DP8 and DP9,^{144,258–269} an inhibitor that can selectively differentiate between these two is still lacking. Such an inhibitor would help to further elucidate the enzyme, if any, responsible for the apparent adverse affects following DP8/9 inhibition.

1.7.1.2 Targeting FAP

Due to the highly restricted and specific expression of FAP, particularly within tumor stroma, FAP is clinically interesting as a target for immunotherapy in the treatment of cancer and non-invasive bio-imaging. Clinical trials aimed at directly targeting the enzymatic activity of FAP with Val-boroPro (Talabostat; PT-100), an amino boronic dipeptide inhibitor of DPs, for the treatment of solid tumors managed to reach phase II studies but failed to demonstrate clinical efficacy.²⁶¹ Val-boroPro had previously shown promise in the *in vivo* reduction in tumour growth in mice,²⁶² but the direct inhibition of FAP by this compound is unlikely. Due to the strict specificity of FAPs endopeptidase activity, FAP selective inhibitors have been developed²⁶³ but are yet to be used in clinical studies. Clinical trials involving immunotherapy targeting of FAP for the treatment of colon cancer and non-small cell lung cancers have also reached phase II studies but failed due to a lack of efficacy.^{264–267} Immunotherapy targeting FAP may inhibit both enzymatic activities and extracellular matrix interactions of FAP. It appears that FAP shows more promise as a biomarker and target for non-invasive imaging of tumours or for use as site-directed targeted cancer radiotherapy as being developed by Molecular Insight Pharmaceuticals.²⁶⁸ Currently immunoconjugate therapy utilizing a novel FAP antibody-maytansinoid conjugate, monoclonal antibody (mAb) FAP5-DM1, is being explored as a treatment option in epithelial cancers.²⁶⁹ Maytansinoid is an immunotoxic substance that enters into cancer cells, blocking their growth upon binding of the antibody to the cell surface. Preliminary studies in mouse xenograft models of lung, pancreas, head, and neck cancers demonstrated long-lasting inhibition of tumor growth and tumor rejection upon immunoconjugate treatment with mAb FAP5-DM1 with no adverse side-effects.²⁶⁹ Loeffler *et al.* constructed an oral DNA vaccine targeting FAP, suppressing tumor growth and metastasis in FAP-vaccinated mice and increasing their uptake of chemotherapeutics.²⁷⁰ Mice vaccinated against FAP also displayed decreased collagen type I expression, a physiological substrate of FAP that can contribute to decreased chemotherapeutic drug uptake in tumors and play a role in regulating tumor sensitivity.²⁷⁰ Effects of this treatment were reported to be from a CD8⁺ T cell-mediated anti-FAP immune response.

1.7.1.3 Targeting DP4 and FAP for Prodrug Development

The ubiquitous distribution of DP4 in the human body coupled with its rare ability to selectively cleave the post-proline bond of N-terminal dipeptides makes it a suitable target for the development of prodrugs where systemic drug delivery is required, such as blood disorders including human immunodeficiency virus (HIV). In contrast, the strict specificity of FAP endopeptidase activity and highly specific expression in cancer and at sites of tissue remodeling make it a valuable pro drug target for the delivery of site-specific treatment. Practicality in utilizing the enzymatic activity of DP4 or FAP in the development of prodrugs has been demonstrated. Using a specific di- or tetra-peptide linked to

therapeutic anti-HIV TSO (non-nucleoside reverse transcriptase inhibitors) molecules via an amide bond specifically cleaved by DP4, Garcia-Apricio *et al.* demonstrated the efficiency of DP4 at releasing a pharmacological active compound upon proteolytic cleavage and suggested the possibility of altering the half-life of the drug/prodrug by modifying the di- or tetra-peptide moiety.^{271,272} Aggarwal *et al.* identified a consensus sequence for FAP endopeptidase cleavage for use in the development of cytotoxic prodrugs.²⁷³ This led to the development of FAP selective peptide ‘protoxins’ in which the FAP selective peptide is incorporated into the activation domain of a cytotoxic protein or peptide rather than being directly linked to the activated toxin itself.²⁷⁴ LeBeau *et al.* demonstrated the feasibility of this approach with the generation of a FAP selective ‘promelittin’ toxin that was efficiently cleaved by FAP, releasing the active cytotoxic melittin resulting in site directed toxicity to FAP expressing cells.²⁷⁴ The anti-tumor effect of these protoxins was also demonstrated by direct injection of the promelittin into human-prostate and breast-cancer xenografts resulting in an increase in tumor necrosis and reduced tumor size.²⁷⁴ These exciting results show promise for the future development of clinically validated prodrugs targeting drugs to sites where DP4 or FAP are expressed for treatment of systemic diseases such as HIV or FAP selective tumors.

1.7.2 Targeting Non-Enzymatic Function

Non-enzymatic functions of DP4 and FAP contribute to their involvement in cell adhesion and migration, thus providing an additional therapeutic pathway in the treatment of cancer progression. Targeting non-enzymatic functions for treatment of disease by blocking protein-binding interactions has its difficulties. In the case of immunotherapy targeting of proteases, there is the added benefit of directly blocking the enzymatic activity of the enzyme or altering its conformation so that substrate entry is blocked. However, mAbs are not guaranteed to block the numerous protein-binding sites of a protein and hence have to be developed with the specific aim in mind of blocking a single protein–protein interaction. There are also a number of issues regarding administering an antibody and its stability within the human body once exposed to the milieu of proteases that are present *in vivo*. Antibodies targeting FAP for immunotherapy have been discussed above, but note should be given as to their potential to disrupt binding/interactions of FAP to extracellular matrix proteins, uPAR, and even DP4. It may be possible that DP4 forms tetrameric structures between adjacent cells that might participate in the adhesion and growth of tumors within certain cancers. Future work may demonstrate the use of antibodies to block such interactions. Studies demonstrating the efficacy of immunotargeting DP4 have been demonstrated.

Binding of soluble anti-DP4 mAb has been shown to inhibit the growth of human renal carcinoma cells (RCC) *in vitro* and *in vivo*, demonstrating its potential clinical use for the treatment of DP4-positive RCC.²⁷⁵ In a similar study, Inamoto *et al.* demonstrated the effectiveness of humanized anti-DP4 in

the growth inhibition of human malignant mesothelioma, thus indicating the potential use of anti-DP4 mAb as a therapeutic in the treatment of DP4-positive malignant mesothelioma as well as RCC.²⁷⁶ In the 2006 study by Inamoto *et al.*, growth inhibition of RCC resulted from anti-DP4 mAb induction of G₁-S cell cycle arrest.²⁷⁵ *In vitro* anti-DP4 mAb also resulted in blocked adhesion of RCC to the ECM presumably due to a increased internalization of cell surface DP4 leading to a decreased binding capacity of RCC to collagen and fibronectin.²⁷⁵ Using a mouse xenograft model of human RCC *in vivo*, evidence was provided for the inhibition of tumor growth and enhanced survival of mice upon anti-DP4 mAb treatment.²⁷⁵ Research by the same group had previously shown a similar potential for anti-DP4 mAb in the growth inhibition of DP4-positive T-cell malignancies via G₁/S cell cycle arrest.^{277,278}

The development of protein-binding inhibitors is another therapeutic option. Gonzalez-Gronow *et al.* demonstrated that the binding of plasminogen to DP4 expressed on the cell surface of L-LN prostate cancer cells could be blocked with angiostatin 2e, a peptide fragment of plasminogen.²⁷⁹ This in turn resulted in inhibition of the induced intracellular calcium pathway and suppression of MMP9 expression, and thus invasive capabilities of the cells, suggesting that the fragment angiostatin 2e may directly block tumor metastasis.²⁷⁹ For the development of therapeutics targeting non-enzymatic activities of DP8 and DP9, further research will need to be performed first to identify any potential protein binding partners of these intracellular enzymes and whether targeting these would be a viable option. In the context of the DPs, targeting of the enzymatic site, or development of DP-resistant substrate analogs, still remains the most viable option for therapeutic treatment.

1.8 Conclusion

In this review, we have introduced the DPs and placed their rare ability to cleave the post-prolyl bond in context with other proteases in the human body. We have provided a thorough overview of the S9b, DP4 gene family, discussing both homology and gene structure in context with the related PEP and non-related DP2. An overview of the biochemical features, biological substrates and the importance of DP4, FAP, DP8, and DP9 to human biology, and disease has also been discussed followed by issues concerning therapeutic targeting of these enzymes. While numerous studies of the multifunctional DP4 have, and still are, being investigated, less is known about the importance of FAP, and even less still about the intracellular DP8 and DP9. Future studies focusing on elucidating crystal structures for understanding of DP8/9 catalytic mechanism and development of highly selective inhibitors, along with the generation of DP8 and DP9 knockout animals, will provide us with some of the most valuable tools for future investigations into the potential physiological and pathological roles of these proteases. In turn, these may lead to the development of further therapeutic strategies for the treatment of disease such as cancer, type II diabetes, and inflammatory disorders targeting this important enzyme family.

References

1. R. Mentlein, *FEBS Lett.*, 1988, **234**, 251–256.
2. D. F. Cunningham and B. O'Connor, *Biochim. Biophys. Acta*, 1997, **1343**, 160–186.
3. G. Vanhoof, F. Goossens, I. De Meester, D. Hendriks and S. Scharpé, *FASEB J.*, 1995, **9**, 736–744.
4. V. K. Hopsu-Havu and G. G. Glenner, *Histochemie*, 1966, **7**, 197–201.
5. W. J. Rettig, P. G. Chesa, H. R. Beresford, H. J. Feickert, M. T. Jennings, J. Cohen, H. F. Oettgen and L. J. Old, *Cancer Res.*, 1986, **46**, 6406–6412.
6. C. A. Abbott, D. M. Yu, E. Woollatt, G. R. Sutherland, G. W. McCaughan and M. D. Gorrell, *Eur. J. Biochem.*, 2000, **267**, 6140–6150.
7. C. A. Abbott and M. D. Gorrell, in *Ectopeptidases: CD13/Aminopeptidase N and CD26/Dipeptidylpeptidase IV in Medicine and Biology*, eds. J. Langner and S. Ansorge, Kluwer/Plenum, New York, 2002. vol. ISBN 0-306-46788-7, pp. 171–195.
8. T. Chen, K. Ajami, G. W. McCaughan, M. D. Gorrell and C. A. Abbott, *Adv. Exp. Med. Biol.*, 2003, **524**, 79–86.
9. F. Goossens, I. De Meester, G. Vanhoof, D. Hendriks, G. Vriend and S. Scharpé, *Eur. J. Biochem.*, 1995, **233**, 432–441.
10. T. Yoshimoto, M. Fischl, R. C. Orlowski and R. Walter, *J. Biol. Chem.*, 1978, **253**, 3708–3716.
11. G. R. Lankas, B. Leiting, R. S. Roy, G. J. Eiermann, M. G. Beconi, T. Biftu, C. C. Chan, S. Edmondson, W. P. Feeney, H. He, D. E. Ippolito, D. Kim, K. A. Lyons, H. O. Ok, R. A. Patel, A. N. Petrov, K. A. Pryor, X. Qian, L. Reigle, A. Woods, J. K. Wu, D. Zaller, X. Zhang, L. Zhu, A. E. Weber and N. A. Thornberry, *Diabetes*, 2005, **54**, 2988–2994.
12. A. Stockel-Maschek, C. Mrestani-Klaus, B. Stiebitz, H. U. Demuth and K. Neubert, *Biochim. Biophys. Acta*, 2000, **1479**, 15–31.
13. K. Aertgeerts, I. Levin, L. Shi, G. P. Snell, A. Jennings, G. S. Prasad, Y. Zhang, M. L. Kraus, S. Salakian, V. Sridhar, R. Wijnands and M. G. Tennant, *J. Biol. Chem.*, 2005, **280**, 19441–19444.
14. C. Y. Edosada, C. Quan, T. Tran, V. Pham, C. Wiesmann, W. Fairbrother and B. B. Wolf, *FEBS Lett.*, 2006, **580**, 1581–1586.
15. H. B. Rasmussen, S. Branner, F. C. Wiberg and N. Wagtman, *Nat. Struct. Biol.*, 2003, **10**, 19–25.
16. H. Hiramatsu, K. Kyono, Y. Higashiyama, C. Fukushima, H. Shima, S. Sugiyama, K. Inaka, A. Yamamoto and R. Shimizu, *Biochem. Biophys. Res. Commun.*, 2003, **302**, 849–854.
17. M. Engel, T. Hoffmann, L. Wagner, M. Wermann, U. Heiser, R. Kiefersauer, R. Huber, W. Bode, H. U. Demuth and H. Brandstetter, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 5063–5068.
18. P. Strop, A. J. Bankovich, K. C. Hansen, K. C. Garcia and A. T. Brunger, *J. Mol. Biol.*, 2004, **343**, 1055–1065.
19. V. Fülöp, Z. Bocskei and L. Polgar, *Cell*, 1998, **94**, 161–170.

20. N. Yokotani, K. Doi, R. J. Wenthold and K. Wada, *Hum. Mol. Genet.*, 1993, **2**, 1037–1039.
21. S. Y. Qi, P. J. Riviere, J. Trojnar, J. L. Junien and K. O. Akinsanya, *Biochem. J.*, 2003, **373**, 179–189.
22. C. A. Abbott, G. W. McCaughan and M. D. Gorrell, *FEBS Lett.*, 1999, **458**, 278–284.
23. K. Ajami, C. A. Abbott, M. Obradovic, V. Gysbers, T. Kahne, G. W. McCaughan and M. D. Gorrell, *Biochemistry*, 2003, **42**, 694–701.
24. M. B. Maes, S. Scharpe and I. De Meester, *Clin. Chim. Acta*, 2007, **380**, 31–49.
25. B. Leiting, K. D. Pryor, J. K. Wu, F. Marsilio, R. A. Patel, C. S. Craik, J. A. Ellman, R. T. Cummings and N. A. Thornberry, *Biochem. J.*, 2003, **371**, 525–532.
26. M. D. Gorrell, X. M. Wang, J. Park, K. Ajami, D. M. Yu, H. Knott, D. Seth and G. W. McCaughan, *Adv. Exp. Med. Biol.*, 2006, **575**, 45–54.
27. C. Durinx, A. M. Lambeir, E. Bosmans, J. B. Falmagne, R. Berghmans, A. Haemers, S. Scharpe and I. De Meester, *Eur. J. Biochem.*, 2000, **267**, 5608–5613.
28. K. N. Lee, K. W. Jackson, V. J. Christiansen, K. H. Chung and P. A. McKee, *Blood*, 2004, **103**, 3783–3788.
29. P. J. Collins, G. McMahon, P. O'Brien and B. O'Connor, *Int. J. Biochem. Cell Biol.*, 2004, **36**, 2320–2333.
30. C. Olsen and N. Wagtmann, *Gene*, 2002, **299**, 185–193.
31. R. M. O'Leary, S. P. Gallagher and B. O'Connor, *Int. J. Biochem. Cell Biol.*, 1996, **28**, 441–449.
32. J. Tenorio-Laranga, J. I. Venalainen, P. T. Mannisto and J. A. Garcia-Horsman, *FEBS J.*, 2008, **275**, 4415–4427.
33. S. E. D'Souza, M. H. Ginsberg and E. F. Plow, *Trends Biochem. Sci.*, 1991, **16**, 246–250.
34. K. Ajami, C. A. Abbott, G. W. McCaughan and M. D. Gorrell, *Biochim. Biophys. Acta*, 2004, **1679**, 18–28.
35. V. Dubois, A. M. Lambeir, S. Vandamme, V. Matheeussen, Y. Guisez, S. Scharpe and I. De Meester, *Biochim. Biophys. Acta*, 2010, **1804**, 781–788.
36. M. Chiravuri, F. Agarraberes, S. L. Mathieu, H. Lee and B. T. Huber, *J. Immunol.*, 2000, **165**, 5695–5702.
37. C. H. Chien, L. H. Huang, C. Y. Chou, Y. S. Chen, Y. S. Han, G. G. Chang, P. H. Liang and X. Chen, *J. Biol. Chem.*, 2004, **279**, 52338–52345.
38. C. H. Chien, C. H. Tsai, C. H. Lin, C. Y. Chou and X. Chen, *Biochemistry*, 2006, **45**, 7006–7012.
39. M. L. Pineiro-Sanchez, L. A. Goldstein, J. Dodt, L. Howard, Y. Yeh, H. Tran, W. S. Argraves and W. T. Chen, *J. Biol. Chem.*, 1997, **272**, 7595–7601.
40. M. Chiravuri, H. Lee, S. L. Mathieu and B. T. Huber, *J. Biol. Chem.*, 2000, **275**, 26994–26999.

41. S. Kalwant and A. G. Porter, *Biochem. J.*, 1991, **276**(Pt 1), 237–244.
42. M. B. Maes, A. M. Lambeir, K. Gilany, K. Senten, P. Van der Veken, B. Leiting, K. Augustyns, S. Scharpe and I. De Meester, *Biochem. J.*, 2005, **386**, 315–324.
43. R. Underwood, M. Chiravuri, T. Schmitz, K. Yardley, H. Lee and B. T. Huber, *J. Biol. Chem.*, 1999, **274**, 34053–34058.
44. T. Sakai, K. Kojima and T. Nagatsu, *J. Chromatogr.*, 1987, **416**, 131–137.
45. J. A. Garcia-Horsman, P. T. Mannisto and J. I. Venalainen, *Neuropeptides*, 2007, **41**, 1–24.
46. K. McNicholas, T. Chen and C. A. Abbott, *Clin. Chem. Lab. Med.*, 2009, **47**, 262–267.
47. K. Wada, N. Yokotani, C. Hunter, K. Doi, R. J. Wenthold and S. Shimasaki, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 197–201.
48. M. S. Nadal, Y. Amarillo, E. Vega-Saenz de Miera and B. Rudy, *Brain Res.*, 2006, **1094**, 1–12.
49. T. Chen, K. Ajami, G. W. McCaughan, W. P. Gai, M. D. Gorrell and C. A. Abbott, *Biochim. Biophys. Acta*, 2006, **1764**, 33–43.
50. K. Takimoto, Y. Hayashi, X. Ren and N. Yoshimura, *Biochem. Biophys. Res. Commun.*, 2006, **348**, 1094–1100.
51. H. Zhu, Z. M. Zhou, L. Lu, M. Xu, H. Wang, J. M. Li and J. H. Sha, *Asian J. Androl.*, 2005, **7**, 245–255.
52. M. D. Gorrell and D. M. T. Yu, in *Trends in Protein Research*, ed. J. W. Robinson, Nova Science Publishers, Inc., New York, 2005. in pp. 1 – 78.
53. P. O'Brien and B. F. O'Connor, *Biochim. Biophys. Acta*, 2008, **1784**, 1130–1145.
54. X. M. Wang, T. W. Yao, N. A. Nadvi, B. Osborne, G. W. McCaughan and M. D. Gorrell, *Front. Biosci.*, 2008, **13**, 3168–3180.
55. E. Boonacker and C. J. Van Noorden, *Eur. J. Cell Biol.*, 2003, **82**, 53–73.
56. M. L. Sulda, C. A. Abbott and M. Hildebrandt, *Adv. Exp. Med. Biol.*, 2006, **575**, 197–206.
57. C. F. Deacon, R. D. Carr and J. J. Holst, *Front. Biosci.*, 2008, **13**, 1780–1794.
58. C. H. McIntosh, *Front. Biosci.*, 2008, **13**, 1753–1773.
59. J. A. Lovshin and D. J. Drucker, *Nat. Rev. Endocrinol.*, 2009, **5**, 262–269.
60. W. N. Dinjens, J. ten-Kate, J. T. Wijnen, E. P. van-der-Linden, C. J. Beek, M. H. Lenders, P. M. Khan and F. T. Bosman, *J. Biol. Chem.*, 1989, **264**, 19215–19220.
61. M. D. Gorrell, *Clin. Sci.*, 2005, **108**, 277–292.
62. A. J. Kenny, A. G. Booth, S. G. George, J. Ingram, D. Kershaw, E. J. Wood and A. R. Young, *Biochem. J.*, 1976, **157**, 169–182.
63. P. Aemaimanan, N. Sattayasai, N. Wara-aswapati, W. Pitiphat, W. Suwannarong, S. Prajaneh and S. Taweechaisupapong, *J. Periodontol.*, 2009, **80**, 1809–1814.

64. Y. Ogawa, M. Kanai-Azuma, Y. Akimoto, H. Kawakami and R. Yanoshita, *Biol. Pharm. Bull.*, 2008, **31**, 1059–1062.
65. J. E. Scherberich, J. Wiemer and W. Schoeppe, *Eur. J. Clin. Chem. Clin. Biochem.*, 1992, **30**, 663–668.
66. F. Perner, T. Gyuris, G. Rakoczy, E. Sarvary, D. Gorog, F. Szalay, I. Kunos, L. Szonyi, M. Peterfy and L. Takacs, *J. Lab. Clin. Med.*, 1999, **134**, 56–67.
67. M. Maes, I. De Meester, S. Scharpe, R. Desnyder, R. Ranjan and H. Y. Meltzer, *Acta Psychiatr. Scand.*, 1996, **93**, 1–8.
68. O. J. Cordero, D. Ayude, M. Nogueira, F. J. Rodriguez-Berrocal and M. P. de la Cadena, *Br. J. Cancer*, 2000, **83**, 1139–1146.
69. O. J. Cordero, F. J. Salgado and M. Nogueira, *Cancer Immunol. Immunother.*, 2009, **58**, 1723–1747.
70. N. Busso, N. Wagtmann, C. Herling, V. Chobaz-Peclat, A. Bischof-Delaloye, A. So and E. Grouzmann, *Am. J. Pathol.*, 2005, **166**, 433–442.
71. Z. Tamaki, M. Kubo, N. Yazawa, Y. Mimura, R. Ashida, M. Tomita, Y. Tada, T. Kawashima and K. Tamaki, *J. Dermatol. Sci.*, 2008, **52**, 67–69.
72. M. A. Fletcher, X. R. Zeng, K. Maher, S. Levis, B. Hurwitz, M. Antoni, G. Broderick and N. G. Klimas, *PLoS One*, 2010, **5**, e10817.
73. C. Morimoto and S. F. Schlossman, *Immunol. Rev.*, 1998, **161**, 55–70.
74. F. Bühling, D. Kunz, D. Reinhold, A. J. Ulmer, M. Ernst, H. D. Flad and S. Ansorge, *Nat. Immun.*, 1994, **13**, 270–279.
75. F. Bühling, U. Junker, D. Reinhold, K. Neubert, L. Jager and S. Ansorge, *Immunol. Lett.*, 1995, **45**, 47–51.
76. D. Reinhold, U. Bank, F. Bühling, K. Neubert, T. Mattern, A. J. Ulmer, H. D. Flad and S. Ansorge, *Immunobiology*, 1993, **188**, 403–414.
77. M. Cuchacovich, H. Gatica, P. Vial, J. Yovanovich, S. V. Pizzo and M. Gonzalez-Gronow, *Clin. Diagn. Lab. Immunol.*, 2002, **9**, 1253–1259.
78. D. Marguet, L. Baggio, T. Kobayashi, A. M. Bernard, M. Pierres, P. F. Nielsen, U. Ribet, T. Watanabe, D. J. Drucker and N. Wagtmann, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 6874–6879.
79. S. L. Conarello, Z. Li, J. Ronan, R. S. Roy, L. Zhu, G. Jiang, F. Liu, J. Woods, E. Zycband, D. E. Moller, N. A. Thornberry and B. B. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 6825–6830.
80. N. Frerker, K. Raber, F. Bode, T. Skripuletz, H. Nave, C. Klemann, R. Pabst, M. Stephan, J. Schade, G. Brabant, D. Wedekind, R. Jacobs, A. Jorns, U. Forssmann, R. H. Straub, S. Johannes, T. Hoffmann, L. Wagner, H. U. Demuth and S. von Horsten, *Clin. Chem. Lab. Med.*, 2009, **47**, 275–287.
81. C. Klemann, J. Schade, R. Pabst, S. Leitner, J. Stiller, S. von Horsten and M. Stephan, *Clin. Exp. Immunol.*, 2009, **155**, 357–365.
82. W. N. Dinjens, J. Ten Kate, J. A. Kirch, H. J. Tanke, E. P. Van der Linden, H. F. Van den Ingh, G. J. Van Steenbrugge, P. Meera Khan and F. T. Bosman, *J. Pathol.*, 1990, **160**, 195–201.

83. T. Bogenrieder, C. L. Finstad, R. H. Freeman, C. N. Papandreou and H. I. Scher, A. P. Albino, V. E. Reuter and D. M. Nanus, *Prostate*, 1997, **33**, 225–232.
84. J. ten Kate, H. F. van den Ingh, P. M. Khan and F. T. Bosman, *Int. J. Cancer*, 1986, **37**, 479–485.
85. A. N. Houghton, A. P. Albino, C. Cordon-Cardo, L. J. Davis and M. Eisinger, *J. Exp. Med.*, 1988, **167**, 197–212.
86. U. V. Wesley, A. P. Albino, S. Tiwari and A. N. Houghton, *J. Exp. Med.*, 1999, **190**, 311–322.
87. W. J. Rettig, P. Garin-Chesa, H. R. Beresford, H. F. Oettgen, M. R. Melamed and L. J. Old, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 3110–3114.
88. P. Garin-Chesa, L. J. Old and W. J. Rettig, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 7235–7239.
89. W. L. Monsky, C. Y. Lin, A. Aoyama, T. Kelly, S. K. Akiyama, S. C. Mueller and W. T. Chen, *Cancer Res.*, 1994, **54**, 5702–5710.
90. J. Niedermeyer, M. Kriz, F. Hilberg, P. Garin-Chesa, U. Bamberger, M. C. Lenter, J. Park, B. Viertel, H. Puschner, M. Mauz, W. J. Rettig and A. Schnapp, *Mol. Cell. Biol.*, 2000, **20**, 1089–1094.
91. M. T. Levy, G. W. McCaughan, C. A. Abbott, J. E. Park, A. M. Cunningham, E. Muller, W. J. Rettig and M. D. Gorrell, *Hepatology*, 1999, **29**, 1768–1778.
92. M. T. Levy, G. W. McCaughan, G. Marinos and M. D. Gorrell, *Liver*, 2002, **22**, 93–101.
93. X. M. Wang, D. M. Yu, G. W. McCaughan and M. D. Gorrell, *Hepatology*, 2005, **42**, 935–945.
94. W. J. Rettig, S. L. Su, S. R. Fortunato, M. J. Scanlan, B. K. Raj, P. Garin-Chesa, J. H. Healey and L. J. Old, *Int. J. Cancer*, 1994, **58**, 385–392.
95. B. R. Klass, A. O. Grobbelaar and K. J. Rolfe, *Postgrad. Med. J.*, 2009, **85**, 9–14.
96. D. Pohlers, J. Brenmoehl, I. Löffler, C. K. Muller, C. Leipner, S. Schultze-Mosgau, A. Stallmach, R. W. Kinne and G. Wolf, *Biochim. Biophys. Acta*, 2009, **1792**, 746–756.
97. C. Margadant and A. Sonnenberg, *EMBO Rep.*, 2010, **11**, 97–105.
98. S. B. Jakowlew, *Cancer Metastasis Rev.*, 2006, **25**, 435–457.
99. P. S. Acharya, A. Zukas, V. Chandan, A. L. Katzenstein and E. Pure, *Hum. Pathol.*, 2006, **37**, 352–360.
100. A. Goodwin and G. Jenkins, *Biochem. Soc. Trans.*, 2009, **37**, 849–854.
101. K. N. Lee, K. W. Jackson, V. J. Christiansen, C. S. Lee, J. G. Chun and P. A. McKee, *Blood*, 2006, **107**, 1397–1404.
102. M. J. Scanlan, B. K. Raj, B. Calvo, P. Garin-Chesa, M. P. Sanz-Moncasi, J. H. Healey, L. J. Old and W. J. Rettig, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 5657–5661.
103. G. Ghersi, H. Dong, L. A. Goldstein, Y. Yeh, L. Hakkinen, H. S. Larjava and W. T. Chen, *J. Biol. Chem.*, 2002, **277**, 29231–29241.

104. G. Ghersi, Q. Zhao, M. Salamone, Y. Yeh, S. Zucker and W. T. Chen, *Cancer Res.*, 2006, **66**, 4652–4661.
105. H. C. Cheng, M. Abdel-Ghany and B. U. Pauli, *J. Biol. Chem.*, 2003, **278**, 24600–24607.
106. J. Kameoka, T. Tanaka, Y. Nojima, S. F. Schlossman and C. Morimoto, *Science*, 1993, **261**, 466–469.
107. I. De Meester, G. Vanham, L. Kestens, G. Vanhoof, E. Bosmans, P. Gigase and S. Scharpé, *Eur. J. Immunol.*, 1994, **24**, 566–570.
108. A. C. Girardi, B. C. Degray, T. Nagy, D. Biemesderfer and P. S. Aronson, *J. Biol. Chem.*, 2001, **276**, 46671–46677.
109. Y. Torimoto, N. H. Dang, E. Vivier, T. Tanaka, S. F. Schlossman and C. Morimoto, *J. Immunol.*, 1991, **147**, 2514–2517.
110. M. Gonzalez-Gronow, S. Kaczowka, G. Gawdi and S. V. Pizzo, *Front. Biosci.*, 2008, **13**, 1610–1618.
111. R. P. Dong, K. Tachibana, M. Hegen, Y. Munakata, D. Cho, S. F. Schlossman and C. Morimoto, *J. Immunol.*, 1997, **159**, 6070–6076.
112. M. Gonzalez-Gronow, M. S. Hershfield, F. X. Arredondo-Vega and S. V. Pizzo, *J. Biol. Chem.*, 2004, **279**, 20993–20998.
113. A. C. Girardi, F. Knauf, H. U. Demuth and P. S. Aronson, *Am. J. Cell Physiol.*, 2004, **287**, C1238–1245.
114. A. C. Girardi, L. E. Fukuda, L. V. Rossoni, G. Malnic and N. A. Reboucas, *Am. J. Physiol. Renal Physiol.*, 2008, **294**, F414–422.
115. M. Gonzalez-Gronow, U. K. Misra, G. Gawdi and S. V. Pizzo, *J. Biol. Chem.*, 2005, **280**, 27173–27178.
116. M. Gonzalez-Gronow, H. E. Grenett, M. R. Weber, G. Gawdi and S. V. Pizzo, *Biochem. J.*, 2001, **355**, 397–407.
117. V. V. Artym, A. L. Kindzelskii, W. T. Chen and H. R. Petty, *Carcinogenesis*, 2002, **23**, 1593–1601.
118. H. W. Smith and C. J. Marshall, *Nat. Rev. Mol. Cell Biol.*, 2010, **11**, 23–36.
119. C. H. Tang and Y. Wei, *Cell. Mol. Life Sci.*, 2008, **65**, 1916–1932.
120. S. Killeen, A. Hennessey, Y. El Hassan and B. Waldron, *Drug News Perspect.*, 2008, **21**, 107–116.
121. S. Ullisse, E. Baldini, S. Sorrenti and M. D’Armiento, *Curr. Cancer Drug Targets*, 2009, **9**, 32–71.
122. J. R. Bjelke, J. Christensen, P. F. Nielsen, S. Branner, A. B. Kanstrup, N. Wagtman and H. B. Rasmussen, *Biochem. J.*, 2006, **396**, 391–399.
123. H. J. Lee, Y. S. Chen, C. Y. Chou, C. H. Chien, C. H. Lin, G. G. Chang and X. Chen, *J. Biol. Chem.*, 2006, **281**, 38653–38662.
124. H. K. Tang, H. Y. Tang, S. C. Hsu, Y. R. Chu, C. H. Chien, C. H. Shu and X. Chen, *Arch. Biochem. Biophys.*, 2009, **485**, 120–127.
125. M. R. Pitman, R. I. Menz and C. A. Abbott, *Biol. Chem.*, 2010, **391**, 959–972.
126. V. Dubois, A. M. Lambeir, P. Van der Veken, K. Augustyns, J. Creemers, X. Chen, S. Scharpe and I. De Meester, *Front. Biosci.*, 2008, **13**, 3558–3568.

127. J. Park, H. M. Knott, N. A. Nadvi, C. A. Collyer, X. M. Wang, W. B. Church and M. D. Gorrell, *Open Enzym. Inhib. J.*, 2008, **1**, 52–60.
128. T. Tsukahara, S. Ishiura and H. Sugita, *J. Biol. Chem.*, 1990, **265**, 21448–21453.
129. V. Fülöp, Z. Szeltner, V. Renner and L. Polgar, *J. Biol. Chem.*, 2001, **276**, 1262–1266.
130. V. Dubois, C. Van Ginneken, H. De Cock, A. M. Lambeir, P. Van der Veken, K. Augustyns, X. Chen, S. Scharpé and I. De Meester, *J. Histochem. Cytochem.*, 2009, **57**, 531–541.
131. D. M. Yu, K. Ajami, M. G. Gall, J. Park, C. S. Lee, K. A. Evans, E. A. McLaughlin, M. R. Pitman, C. A. Abbott, G. W. McCaughan and M. D. Gorrell, *J. Histochem. Cytochem.*, 2009, **57**, 1025–1040.
132. J. Stremenova, E. Krepela, V. Mares, J. Trim, V. Dbaly, J. Marek, Z. Vanickova, V. Lisa, C. Yea and A. Sedo, *Int. J. Oncol.*, 2007, **31**, 785–792.
133. N. Frerker, L. Wagner, R. Wolf, U. Heiser, T. Hoffmann, J. U. Rahfeld, J. Schade, T. Karl, H. Y. Naim, M. Alfalah, H. U. Demuth and S. von Horsten, *Peptides*, 2007, **28**, 257–268.
134. S. Ansorge, U. Bank, A. Heimburg, M. Helmuth, G. Koch, J. Tadge, U. Lendeckel, C. Wolke, K. Neubert, J. Faust, P. Fuchs, D. Reinhold, A. Thielitz and M. Tager, *Clin. Chem. Lab. Med.*, 2009, **47**, 253–261.
135. M. B. Maes, V. Dubois, I. Brandt, A. M. Lambeir, P. Van der Veken, K. Augustyns, J. D. Cheng, X. Chen, S. Scharpe and I. De Meester, *J. Leukocyte Biol.*, 2007, **81**, 1252–1257.
136. D. M. Yu, X. M. Wang, G. W. McCaughan and M. D. Gorrell, *FEBS J.*, 2006, **273**, 2447–2460.
137. D. M. Yu, X. M. Wang, K. Ajami, G. W. McCaughan and M. D. Gorrell, *Adv. Exp. Med. Biol.*, 2006, **575**, 63–72.
138. C. Rummey and G. Metz, *Proteins*, 2007, **66**, 160–171.
139. K. Aertgeerts, S. Ye, M. G. Tennant, M. L. Kraus, J. Rogers, B. C. Sang, R. J. Skene, D. R. Webb and G. S. Prasad, *Protein Sci.*, 2004, **13**, 412–421.
140. M. Engel, T. Hoffmann, S. Manhart, U. Heiser, S. Chambre, R. Huber, H. U. Demuth and W. Bode, *J. Mol. Biol.*, 2006, **355**, 768–783.
141. G. Fischer, J. Heins and A. Barth, *Biochim. Biophys. Acta*, 1983, **742**, 452–462.
142. R. Thoma, B. Löffler, M. Stihle, W. Huber, A. Ruf and M. Hennig, *Structure*, 2003, **11**, 947–959.
143. R. Mentlein, *Regul. Pept.*, 1999, **85**, 9–24.
144. N. S. Kang, J. H. Ahn, S. S. Kim, C. H. Chae and S. E. Yoo, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 3716–3721.
145. Y. S. Chen, C. H. Chien, C. M. Goparaju, J. T. Hsu, P. H. Liang and X. Chen, *Protein Expression Purif.*, 2004, **35**, 142–146.
146. O. Kleifeld, A. Doucet, U. auf dem Keller, A. Prudova, O. Schilling, R. K. Kainthan, A. E. Starr, L. J. Foster, J. N. Kizhakkedathu and C. M. Overall, *Nat. Biotechnol.*, **28**, 281–288.

147. C. F. Deacon, A. H. Johnsen and J. J. Holst, *J. Clin. Endocrinol. Metab.*, 1995, **80**, 952–957.
148. R. Mentlein, B. Gallwitz and W. E. Schmidt, *Eur. J. Biochem.*, 1993, **214**, 829–835.
149. T. J. Kieffer, C. H. McIntosh and R. A. Pederson, *Endocrinology*, 1995, **136**, 3585–3596.
150. D. J. Drucker, *Nat. Clin. Pract. Endocrinol. Metab.*, 2005, **1**, 22–31.
151. J. J. Holst, T. Vilsboll and C. F. Deacon, *Mol. Cell. Endocrinol.*, 2009, **297**, 127–136.
152. A. M. Lambeir, P. Proost, S. Scharpe and I. De Meester, *Biochem. Pharmacol.*, 2002, **64**, 1753–1756.
153. J. A. Pospisilik, S. A. Hinke, R. A. Pederson, T. Hoffmann, F. Rosche, D. Schlenzig, K. Glund, U. Heiser, C. H. McIntosh and H. Demuth, *Regul. Pept.*, 2001, **96**, 133–141.
154. S. A. Hinke, J. A. Pospisilik, H. U. Demuth, S. Mannhart, K. Kuhn-Wache, T. Hoffmann, E. Nishimura, R. A. Pederson and C. H. S. McIntosh, *J. Biol. Chem.*, 2000, **275**, 3827–3834.
155. A. M. Lambeir, C. Durinx, P. Proost, J. Van Damme, S. Scharpe and I. De Meester, *FEBS Lett.*, 2001, **507**, 327–330.
156. D. J. Drucker, Q. Shi, A. Crivici, M. Sumner-Smith, W. Tavares, M. Hill, L. DeForest, S. Cooper and P. L. Brubaker, *Nat. Biotechnol.*, 1997, **15**, 673–677.
157. B. Hartmann, M. B. Harr, P. B. Jeppesen, M. Wojdemann, C. F. Deacon, P. B. Mortensen and J. J. Holst, *J. Clin. Endocrinol. Metab.*, 2000, **85**, 2884–2888.
158. I. Nausch, R. Mentlein and E. Heymann, *Biol. Chem. Hoppe-Seyler*, 1990, **371**, 1113–1118.
159. M. Bouras, J. F. Huneau, C. Luengo, C. Erlanson-Albertsson and D. Tome, *Peptides*, 1995, **16**, 399–405.
160. J. B. Pesquero, G. N. Jubilut, C. J. Lindsey and A. C. Paiva, *J. Hypertens.*, 1992, **10**, 1471–1478.
161. I. Brandt, A. M. Lambeir, J. M. Ketelslegers, M. Vanderheyden, S. Scharpe and I. De Meester, *Clin. Chem.*, 2006, **52**, 82–87.
162. L. A. Frohman, T. R. Downs, E. P. Heimer and A. M. Felix, *J. Clin. Invest.*, 1989, **83**, 1533–1540.
163. L. Boulanger, P. Roughly and P. Gaudreau, *Peptides*, 1992, **13**, 681–689.
164. J. Bongers, T. Lambros, M. Ahmad and E. P. Heimer, *Biochim. Biophys. Acta*, 1992, **1122**, 147–153.
165. X. Y. Zhang, I. De Meester, A. M. Lambeir, L. Dillen, W. Van Dongen, E. L. Esmans, A. Haemers, S. Scharpe and M. Claeys, *J. Mass Spectrom.*, 1999, **34**, 255–263.
166. E. Heymann and R. Mentlein, *FEBS Lett.*, 1978, **91**, 360–364.
167. S. Ahmad, L. Wang and P. E. Ward, *J. Pharmacol. Exp. Ther.*, 1992, **260**, 1257–1261.
168. I. Nausch and E. Heymann, *J. Neurochem.*, 1985, **44**, 1354–1357.

169. R. Mentlein, P. Dahms, D. Grandt and R. Kruger, *Regul. Pept.*, 1993, **49**, 133–144.
170. D. Grandt, P. Dahms, M. Schimiczek, V. E. Eysselein, J. Reeve, Jr. and R. Mentlein, *Med. Klin.*, 1993, **88**, 143–145.
171. Z. Zukowska-Grojec, E. Karwatowska-Prokopczuk, W. Rose, J. Rone, S. Movafagh, H. Ji, Y. Yeh, W. T. Chen, H. K. Kleinman, E. Grouzmann and D. S. Grant, *Circ. Res.*, 1998, **83**, 187–195.
172. M. Kikuchi, K. Fukuyama and W. L. Epstein, *Arch. Biochem. Biophys.*, 1988, **266**, 369–376.
173. E. Boonacker, S. Elferink, A. Bardai, J. Wormmeester and C. J. Van Noorden, *BioTechniques*, 2003, **35**, 766–768, 770, 772 passim.
174. C. Tiruppathi, Y. Miyamoto, V. Ganapathy and F. H. Leibach, *Am. J. Physiol.*, 1993, **265**, g81–g89.
175. M. Iwan, B. Jarmolowska, K. Bielkowicz, E. Kostyra, H. Kostyra and M. Kaczmarek, *Peptides*, 2008, **29**, 1042–1047.
176. B. Hartrodt, K. Neubert, G. Fischer, U. Demuth, T. Yoshimoto and A. Barth, *Pharmazie*, 1982, **37**, 72–73.
177. R. Shane, S. Wilk and R. J. Bodnar, *Brain Res.*, 1999, **815**, 278–286.
178. C. Sakurada, S. Sakurada, T. Hayashi, S. Katsuyama, K. Tan-No and T. Sakurada, *Biochem. Pharmacol.*, 2003, **66**, 653–661.
179. A. P. Bird, J. R. Faltinek and A. H. Shojaei, *J Control Release*, 2001, **73**, 31–36.
180. M. Barsun, N. Jajcanin, B. Vukelic, J. Spoljaric and M. Abramic, *Biol. Chem.*, 2007, **388**, 343–348.
181. M. Cohen, I. Fruitier-Arnaudin and J. M. Piot, *Biochimie*, 2004, **86**, 31–37.
182. T. Oravec, M. Pall, G. Roderiquez, M. D. Gorrell, M. Ditto, N. Y. Nguyen, R. Boykins, E. Unsworth and M. A. Norcross, *J. Exp. Med.*, 1997, **186**, 1865–1872.
183. P. Proost, I. De Meester, D. Schols, S. Struyf, A. M. Lambeir, A. Wuyts, G. Opdenakker, E. De Clercq, S. Scharpe and J. Van Damme, *J. Biol. Chem.*, 1998, **273**, 7222–7227.
184. A. M. Lambeir, P. Proost, C. Durinx, G. Bal, K. Senten, K. Augustyns, S. Scharpe, J. Van Damme and I. De Meester, *J. Biol. Chem.*, 2001, **276**, 29839–29845.
185. A. Ludwig, F. Schiemann, R. Mentlein, B. Lindner and E. Brandt, *J. Leukocyte Biol.*, 2002, **72**, 183–191.
186. S. Struyf, P. Proost, D. Schols, E. De Clercq, G. Opdenakker, J. P. Lenaerts, M. Detheux, M. Parmentier, I. De Meester, S. Scharpe and J. Van Damme, *J. Immunol.*, 1999, **162**, 4903–4909.
187. T. Ohtsuki, O. Hosono, H. Kobayashi, Y. Munakata, A. Souta, T. Shioda and C. Morimoto, *FEBS Lett.*, 1998, **431**, 236–240.
188. P. Proost, S. Struyf, D. Schols, C. Durinx, A. Wuyts, J. P. Lenaerts, E. De Clercq, I. De Meester and J. Van Damme, *FEBS Lett.*, 1998, **432**, 73–76.

189. T. Shioda, H. Kato, Y. Ohnishi, K. Tashiro, M. Ikegawa, E. E. Nakayama, H. Hu, A. Kato, Y. Sakai, H. Liu, T. Honjo, A. Nomoto, A. Iwamoto, C. Morimoto and Y. Nagai, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 6331–6336.
190. P. Proost, S. Struyf, D. Schols, G. Opdenakker, S. Sozzani, P. Allavena, A. Mantovani, K. Augustyns, G. Bal, A. Haemers, A. M. Lambeir, S. Scharpé, J. Van Damme and I. De Meester, *J. Biol. Chem.*, 1999, **274**, 3988–3993.
191. P. Proost, P. Menten, S. Struyf, E. Schutyser, I. De Meester and J. Van Damme, *Blood*, 2000, **96**, 1674–1680.
192. K. Ajami, M. R. Pitman, C. H. Wilson, J. Park, R. I. Menz, A. E. Starr, J. H. Cox, C. A. Abbott, C. M. Overall and M. D. Gorrell, *FEBS Lett.*, 2008, **582**, 819–825.
193. R. Yazbeck, G. S. Howarth and C. A. Abbott, *Cytokine Growth Factor Rev.*, 2009, **20**, 175–184.
194. B. Chandrasekharan, V. Bala, V. L. Kolachala, M. Vijay-Kumar, D. Jones, A. T. Gewirtz, S. V. Sitaraman and S. Srinivasan, *PLoS One*, 2008, **3**, e3304.
195. G. H. Ballantyne, *Obes. Surg.*, 2006, **16**, 651–658.
196. K. Abe, J. U. Tilan and Z. Zukowska, *Curr. Top. Med. Chem.*, 2007, **7**, 1704–1709.
197. J. Duhault, M. Boulanger, S. Chamorro, J. A. Boutin, O. Della Zuana, E. Douillet, J. L. Fauchere, M. Feletou, M. Germain, B. Husson, A. M. Vega, P. Renard and F. Tisserand, *Can. J. Physiol. Pharmacol.*, 2000, **78**, 173–185.
198. A. Kanatani, J. Ito, A. Ishihara, H. Iwaasa, T. Fukuroda, T. Fukami, D. J. MacNeil, L. H. Van der Ploeg and M. Ihara, *Regul. Pept.*, 1998, **75-76**, 409–415.
199. D. A. Keire, P. Mannon, M. Kobayashi, J. H. Walsh, T. E. Solomon and J. R. Reeve, Jr., *Am J Physiol Gastrointest. Liver Physiol.*, 2000, **279**, G126–131.
200. G. Ghersi, W. Chen, E. W. Lee and Z. Zukowska, *Peptides*, 2001, **22**, 453–458.
201. R. L. Batterham, M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone and S. R. Bloom, *Nature*, 2002, **418**, 650–654.
202. R. Geiss-Friedlander, N. Parmentier, U. Moller, H. Urlaub, B. J. Van den Eynde and F. Melchior, *J. Biol. Chem.*, 2009, **284**, 27211–27219.
203. H. Yokosawa, S. Endo, Y. Ogura and S. Ishii, *Biochem. Biophys. Res. Commun.*, 1983, **116**, 735–742.
204. L. H. Wang, S. Ahmad, I. F. Benter, A. Chow, S. Mizutani and P. E. Ward, *Peptides*, 1991, **12**, 1357–1364.
205. J. B. Byrd, K. Touzin, S. Sile, J. V. Gainer, C. Yu, J. Nadeau, A. Adam and N. J. Brown, *Hypertension*, 2008, **51**, 141–147.
206. J. B. Byrd, A. Shreevatsa, P. Putlur, D. Foretia, L. McAlexander, T. Sinha, M. D. Does and N. J. Brown, *J. of Allergy Clin. Immunol.*, 2007, **120**, 403–408.

207. N. J. Brown, S. Byiers, D. Carr, M. Maldonado and B. A. Warner, *Hypertension*, 2009, **54**, 516–523.
208. K. B. Helle, *Cardiovasc. Res.*, **85**, 9–16.
209. H. Shimizu, K. Masuta, K. Aono, H. Asada, K. Sasakura, M. Tamaki, K. Sugita and K. Yamada, *Clin. Chim. Acta*, 2002, **316**, 129–135.
210. M. Vanderheyden, J. Bartunek and M. Goethals, *Eur. J. Heart Fail.*, 2004, **6**, 261–268.
211. G. S. Katsanos, A. Anogeianaki, C. Orso, S. Tete, V. Salini, P. L. Antinolfi and G. Sabatino, *J. Biol. Regul. Homeost. Agents*, 2008, **22**, 93–98.
212. K. Ebner and N. Singewald, *Amino Acids*, 2006, **31**, 251–272.
213. E. Katsouni, P. Sakkas, A. Zarros, N. Skandali and C. Liapi, *Peptides*, 2009, **30**, 1586–1591.
214. J. L. Hylden and G. L. Wilcox, *J. Pharmacol. Exp. Ther.*, 1983, **226**, 398–404.
215. M. Maes, I. De Meester, G. Vanhoof, S. Scharpe, E. Bosmans, C. Vandervorst, R. Verkerk, B. Minner, E. Suy and J. Raus, *Biol. Psych.*, 1991, **30**, 577–586.
216. M. Maes, F. Goossens, S. Scharpe, J. Calabrese, R. Desnyder and H. Y. Meltzer, *Psychiatry Res.*, 1995, **58**, 217–225.
217. M. Maes, A. H. Lin, S. Bonaccorso, F. Goossens, A. Van Gastel, R. Pioli, L. Delmeire and S. Scharpe, *J. Affect. Disord.*, 1999, **53**, 27–34.
218. P. Busek, J. Stremenova, E. Krepela and A. Sedo, *Physiol. Res.*, 2008, **57**, 443–449.
219. D. S. Jessop, A. Fassold, C. Wolff, R. Hofbauer, A. Chover-Gonzalez, L. J. Richards and R. H. Straub, *Ann. N. Y. Acad. Sci.*, **1193**, 117–122.
220. A. Olivo Rdo, F. Teixeira Cde and P. F. Silveira, *Biochem. Pharmacol.*, 2005, **69**, 1441–1450.
221. A. Janecka, R. Staniszevska, K. Gach and J. Fichna, *Peptides*, 2008, **29**, 2066–2073.
222. R. Guieu, E. Fenouillet, C. Devaux, Z. Fajloun, L. Carrega, J. M. Sabatier, N. Sauze and D. Marguet, *Behav. Brain Res.*, 2006, **166**, 230–235.
223. J. Pil, P. Van der Veken, G. Bal, K. Augustyns, A. Haemers and J. Tytgat, *Biochem. Pharmacol.*, 2004, **67**, 1887–1895.
224. D. Schols, P. Proost, S. Struyf, A. Wuyts, I. De Meester, S. Scharpe, J. Van Damme and E. De Clercq, *Antiviral Res.*, 1999, **40**, 189–190.
225. P. Proost, E. Schutyser, P. Menten, S. Struyf, A. Wuyts, G. Opdenakker, M. Detheux, M. Parmentier, C. Durinx, A. M. Lambeir, J. Neyts, S. Liekens, P. C. Maudgal, A. Billiau and J. Van Damme, *Blood*, 2001, **98**, 3554–3561.
226. P. Proost, A. Mortier, T. Loos, J. Vandercappellen, M. Gouwy, I. Ronsse, E. Schutyser, W. Put, M. Parmentier, S. Struyf and J. Van Damme, *Blood*, 2007, **110**, 37–44.

227. Y. X. Sun, E. A. Pedersen, Y. Shiozawa, A. M. Havens, Y. Jung, J. Wang, K. J. Pienta and R. S. Taichman, *Clin. Exp. Metastasis*, 2008, **25**, 765–776.
228. M. G. Narducci, E. Scala, A. Bresin, E. Caprini, M. C. Picchio, D. Remotti, G. Ragone, F. Nasorri, M. Frontani, D. Arcelli, S. Volinia, G. A. Lombardo, G. Baliva, M. Napolitano and G. Russo, *Blood*, 2006, **107**, 1108–1115.
229. M. Wolf, S. Albrecht and C. Marki, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 1185–1198.
230. T. Hoffmann, J. Faust, K. Neubert and S. Ansorge, *FEBS Lett.*, 1993, **336**, 61–64.
231. S. Morel, F. Levy, O. Burlet-Schiltz, F. Brasseur, M. Probst-Kepper, A. L. Peitrequin, B. Monsarrat, R. Van Velthoven, J. C. Cerottini, T. Boon, J. E. Gairin and B. J. Van den Eynde, *Immunity*, 2000, **12**, 107–117.
232. F. Levy, L. Burri, S. Morel, A. L. Peitrequin, N. Levy, A. Bachi, U. Hellman, B. J. Van den Eynde and C. Servis, *J. Immunol.*, 2002, **169**, 4161–4171.
233. N. A. Yates, E. G. Deyanova, W. Geissler, M. C. Wiener, J. R. Sachs, K. K. Wong, N. A. Thornberry, R. S. Roy, R. E. Settlege and R. C. Hendrickson, *Int. J. Mass Spectrom.*, 2007, **259**, 174–183.
234. J. E. Park, M. C. Lenter, R. N. Zimmermann, P. Garin-Chesa, L. J. Old and W. J. Rettig, *J. Biol. Chem.*, 1999, **274**, 36505–36512.
235. V. J. Christiansen, K. W. Jackson, K. N. Lee and P. A. McKee, *Arch. Biochem. Biophys.*, 2007, **457**, 177–186.
236. M. M. Jost, J. Lamerz, H. Tammen, C. Menzel, I. De Meester, A. M. Lambeir, K. Augustyns, S. Scharpe, H. D. Zucht, H. Rose, M. Jurgens, P. Schulz-Knappe and P. Budde, *Biochem. Pharmacol.*, 2009, **77**, 228–237.
237. R. Yazbeck, G. S. Howarth and C. A. Abbott, *Trends Pharmacol. Sci.*, 2009, **30**, 600–607.
238. A. Thielitz, D. Reinhold, R. Vetter, U. Bank, M. Helmuth, R. Hartig, S. Wrenger, I. Wiswedel, U. Lendeckel, T. Kahne, K. Neubert, J. Faust, C. C. Zouboulis, S. Ansorge and H. Gollnick, *J. Invest. Dermatol.*, 2007, **127**, 1042–1051.
239. D. Reinhold, A. Goihl, S. Wrenger, A. Reinhold, U. C. Kuhlmann, J. Faust, K. Neubert, A. Thielitz, S. Brocke, M. Tager, S. Ansorge and U. Bank, *Clin. Chem. Lab. Med.*, 2009, **47**, 268–274.
240. J. Schade, M. Stephan, A. Schmiedl, L. Wagner, A. J. Niestroj, H. U. Demuth, N. Frerker, C. Klemann, K. A. Raber, R. Pabst and S. von Horsten, *J. Histochem. Cytochem.*, 2008, **56**, 147–155.
241. R. Yazbeck, M. L. Sulda, G. S. Howarth, A. Bleich, K. Raber, S. von Horsten, J. J. Holst and C. A. Abbott, *Inflamm. Bowel Dis.*, 2010, **16**, 1340–1351.
242. D. Williams-Herman, E. Round, A. S. Swern, B. Musser, M. J. Davies, P. P. Stein, K. D. Kaufman and J. M. Amatruda, *BMC Endocr. Disord.*, 2008, **8**, 14.

243. G. A. Herman, C. Stevens, K. Van Dyck, A. Bergman, B. Yi, M. De Smet, K. Snyder, D. Hilliard, M. Tanen, W. Tanaka, A. Q. Wang, W. Zeng, D. Musson, G. Winchell, M. J. Davies, S. Ramael, K. M. Gottesdiener and J. A. Wagner, *Clin. Pharmacol. Ther.*, 2005, **78**, 675–688.
244. N. A. Thornberry and A. E. Weber, *Curr. Top. Med. Chem.*, 2007, **7**, 557–568.
245. D. J. Augeri, J. A. Robl, D. A. Betebenner, D. R. Magnin, A. Khanna, J. G. Robertson, A. Wang, L. M. Simpkins, P. Taunk, Q. Huang, S. P. Han, B. Abboa-Offei, M. Cap, L. Xin, L. Tao, E. Tozzo, G. E. Welzel, D. M. Egan, J. Marcinkeviciene, S. Y. Chang, S. A. Biller, M. S. Kirby, R. A. Parker and L. G. Hamann, *J. Med. Chem.*, 2005, **48**, 5025–5037.
246. K. Traynor, *Am. J. Health Syst. Pharm.*, 2009, **66**, 1513.
247. S. Thareja, S. Aggarwal, P. Malla, D. Haksar, T. R. Bhardwaj and M. Kumar, *Mini Rev. Med. Chem.*, 2010, **10**, 759–765.
248. E. B. Villhauer, J. A. Brinkman, G. B. Naderi, B. F. Burkey, B. E. Dunning, K. Prasad, B. L. Mangold, M. E. Russell and T. E. Hughes, *J. Med. Chem.*, 2003, **46**, 2774–2789.
249. H. He, P. Tran, H. Yin, H. Smith, Y. Batard, L. Wang, H. Einolf, H. Gu, J. B. Mangold, V. Fischer and D. Howard, *Drug Metab. Dispos.*, 2009, **37**, 536–544.
250. C. Mathieu and E. Degrande, *Vasc. Health Risk Manag.*, 2008, **4**, 1349–1360.
251. J. Feng, Z. Zhang, M. B. Wallace, J. A. Stafford, S. W. Kaldor, D. B. Kassel, M. Navre, L. Shi, R. J. Skene, T. Asakawa, K. Takeuchi, R. Xu, D. R. Webb and S. L. Gwaltney, 2nd, *J. Med. Chem.*, 2007, **50**, 2297–2300.
252. L. Thomas, M. Eckhardt, E. Langkopf, M. Tadayyon, F. Himmelsbach and M. Mark, *J. Pharmacol. Exp. Ther.*, 2008, **325**, 175–182.
253. Y. Horie, N. Hayashi, K. Dugi and M. Takeuchi, *Trials*, 2009, **10**, 82.
254. K. M. Johnson, *Curr. Opin. Investig. Drugs*, **11**, 455–463.
255. H. M. Pattzi, S. Pitale, M. Alpizar, C. Bennett, A. M. O’Farrell, J. Li, J. M. Cherrington and H. P. Guler, *Diabetes Obes. Metab.*, 2010, **12**, 348–355.
256. B. F. Burkey, P. K. Hoffmann, U. Hassiepen, J. Trappe, M. Juedes and J. E. Foley, *Diabetes Obes. Metab.*, 2008, **10**, 1057–1061.
257. J. J. Wu, H. K. Tang, T. K. Yeh, C. M. Chen, H. S. Shy, Y. R. Chu, C. H. Chien, T. Y. Tsai, Y. C. Huang, Y. L. Huang, C. H. Huang, H. Y. Tseng, W. T. Jiaang, Y. S. Chao and X. Chen, *Biochem. Pharmacol.*, 2009, **78**, 203–210.
258. W. T. Jiaang, Y. S. Chen, T. Hsu, S. H. Wu, C. H. Chien, C. N. Chang, S. P. Chang, S. J. Lee and X. Chen, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 687–691.
259. P. Van der Veken, A. Soroka, I. Brandt, Y. S. Chen, M. B. Maes, A. M. Lambeir, X. Chen, A. Haemers, S. Scharpe, K. Augustyns and I. De Meester, *J. Med. Chem.*, 2007, **50**, 5568–5570.

260. S. Van Goethem, P. Van der Veken, V. Dubois, A. Soroka, A. M. Lambeir, X. Chen, A. Haemers, S. Scharpe, I. De Meester and K. Augustyns, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 4159–4162.
261. K. Narra, S. R. Mullins, H. O. Lee, B. Strzemkowski-Brun, K. Magalong, V. J. Christiansen, P. A. McKee, B. Egleston, S. J. Cohen, L. M. Weiner, N. J. Meropol and J. D. Cheng, *Cancer Biol. Ther.*, 2007, **6**, 1691–1699.
262. S. Adams, G. T. Miller, M. I. Jesson, T. Watanabe, B. Jones and B. P. Wallner, *Cancer Res.*, 2004, **64**, 5471–5480.
263. C. Y. Edosada, C. Quan, C. Wiesmann, T. Tran, D. Sutherland, M. Reynolds, J. M. Elliott, H. Raab, W. Fairbrother and B. B. Wolf, *J. Biol. Chem.*, 2006, **281**, 7437–7444.
264. S. Welt, C. R. Divgi, A. M. Scott, P. Garin-Chesa, R. D. Finn, M. Graham, E. A. Carswell, A. Cohen, S. M. Larson and L. J. Old and *et al.*, *J. Clin. Oncol.*, 1994, **12**, 1193–1203.
265. S. Welt, C. R. Divgi, N. Kemeny, R. D. Finn, A. M. Scott, M. Graham, J. S. Germain, E. C. Richards, S. M. Larson and H. F. Oettgen, *J. Clin. Oncol.*, 1994, **12**, 1561–1571.
266. A. M. Scott, G. Wiseman, S. Welt, A. Adjei, F. T. Lee, W. Hopkins, C. R. Divgi, L. H. Hanson, P. Mitchell, D. N. Gansen, S. M. Larson, J. N. Ingle, E. W. Hoffman, P. Tanswell, G. Ritter, L. S. Cohen, P. Bette, L. Arvay, A. Amelsberg, D. Vlock, W. J. Rettig and L. J. Old, *Clin. Cancer Res.*, 2003, **9**, 1639–1647.
267. R. D. Hofheinz, S. E. al-Batran, F. Hartmann, G. Hartung, D. Jager, C. Renner, P. Tanswell, U. Kunz, A. Amelsberg, H. Kuthan and G. Stehle, *Onkologie*, 2003, **26**, 44–48.
268. J. W. Marquis, J. Maresca, K. Hillier, S. Zimmerman, C. Eckelman, W. Joyal and J. J. Babich, *J. Nucl. Med.*, 2009, **50**(Supplement 2), 1563.
269. E. Ostermann, P. Garin-Chesa, K. H. Heider, M. Kalat, H. Lamche, C. Puri, D. Kerjaschki, W. J. Rettig and G. R. Adolf, *Clin. Cancer Res.*, 2008, **14**, 4584–4592.
270. M. Loeffler, J. A. Kruger, A. G. Niethammer and R. A. Reisfeld, *J. Clin. Invest.*, 2006, **116**, 1955–1962.
271. C. Garcia-Aparicio, M. C. Bonache, I. De Meester, A. San-Felix, J. Balzarini, M. J. Camarasa and S. Velazquez, *J. Med. Chem.*, 2006, **49**, 5339–5351.
272. C. Garcia-Aparicio, A. Diez-Torrubia, J. Balzarini, A. M. Lambeir, S. Velazquez and M. J. Camarasa, *Antiviral Res.*, 2007, **76**, 130–139.
273. S. Aggarwal, W. N. Brennen, T. P. Kole, E. Schneider, O. Topaloglu, M. Yates, R. J. Cotter and S. R. Denmeade, *Biochemistry*, 2008, **47**, 1076–1086.
274. A. M. Lebeau, W. N. Brennen, S. Aggarwal and S. R. Denmeade, *Mol. Cancer Ther.*, 2009, **8**, 1378–1386.

- 275. T. Inamoto, T. Yamochi, K. Ohnuma, S. Iwata, S. Kina, S. Inamoto, M. Tachibana, Y. Katsuoka, N. H. Dang and C. Morimoto, *Clin. Cancer Res.*, 2006, **12**, 3470–3477.
- 276. T. Inamoto, T. Yamada, K. Ohnuma, S. Kina, N. Takahashi, T. Yamochi, S. Inamoto, Y. Katsuoka, O. Hosono, H. Tanaka, N. H. Dang and C. Morimoto, *Clin. Cancer Res.*, 2007, **13**, 4191–4200.
- 277. K. Ohnuma, T. Ishii, S. Iwata, O. Hosono, H. Kawasaki, M. Uchiyama, H. Tanaka, T. Yamochi, N. H. Dang and C. Morimoto, *Immunology*, 2002, **107**, 325–333.
- 278. L. Ho, U. Aytac, L. C. Stephens, K. Ohnuma, G. B. Mills, K. S. McKee, C. Neumann, R. LaPushin, F. Cabanillas, J. L. Abbruzzese, C. Morimoto and N. H. Dang, *Clin. Cancer Res.*, 2001, **7**, 2031–2040.
- 279. M. Gonzalez-Gronow, H. E. Grenett, G. Gawdi and S. V. Pizzo, *Exp. Cell Res.*, 2005, **303**, 22–31.

Proteinases as Drug Targets

RSC Drug Discovery Series

Editor-in-Chief:

Professor David Thurston, *London School of Pharmacy, UK*

Series Editors:

Dr David Fox, *Pfizer Global Research and Development, Sandwich, UK*

Professor Salvatore Guccione, *University of Catania, Italy*

Professor Ana Martinez, *Instituto de Quimica Medica-CSIC, Spain*

Dr David Rotella, *Montclair State University, USA*

Advisor to the Board:

Professor Robin Ganellin, *University College London, UK*

Titles in the Series:

- 1: Metabolism, Pharmacokinetics and Toxicity of Functional Groups: Impact of Chemical Building Blocks on ADMET
- 2: Emerging Drugs and Targets for Alzheimer's Disease; Volume 1: Beta-Amyloid, Tau Protein and Glucose Metabolism
- 3: Emerging Drugs and Targets for Alzheimer's Disease; Volume 2: Neuronal Plasticity, Neuronal Protection and Other Miscellaneous Strategies
- 4: Accounts in Drug Discovery: Case Studies in Medicinal Chemistry
- 5: New Frontiers in Chemical Biology: Enabling Drug Discovery
- 6: Animal Models for Neurodegenerative Disease
- 7: Neurodegeneration: Metallostasis and Proteostasis
- 8: G Protein-Coupled Receptors: From Structure to Function
- 9: Pharmaceutical Process Development: Current Chemical and Engineering Challenges
- 10: Extracellular and Intracellular Signaling
- 11: New Synthetic Technologies in Medicinal Chemistry
- 12: New Horizons in Predictive Toxicology: Current Status and Application
- 13: Drug Design Strategies: Quantitative Approaches
- 14: Neglected Diseases and Drug Discovery
- 15: Biomedical Imaging: The Chemistry of Labels, Probes and Contrast Agents
- 16: Pharmaceutical Salts and Cocrystals
- 17: Polyamine Drug Discovery
- 18: Proteinases as Drug Targets

How to obtain future titles on publication:

A standing order plan is available for this series. A standing order will bring delivery of each new volume immediately on publication.

For further information please contact:

Book Sales Department, Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge, CB4 0WF, UK

Telephone: +44 (0)1223 420066, Fax: +44 (0)1223 420247, Email: books@rsc.org

Visit our website at <http://www.rsc.org/Shop/Books/>

Proteinases as Drug Targets

Edited by

Ben Dunn

*Department of Biochemistry and Molecular Biology, University of Florida,
Gainesville, FL, USA*

RSC Publishing

RSC Drug Discovery Series No. 18

ISBN: 978-1-84973-049-5

ISSN: 2041-3203

A catalogue record for this book is available from the British Library

© Royal Society of Chemistry 2012

All rights reserved

Apart from fair dealing for the purposes of research for non-commercial purposes or for private study, criticism or review, as permitted under the Copyright, Designs and Patents Act 1988 and the Copyright and Related Rights Regulations 2003, this publication may not be reproduced, stored or transmitted, in any form or by any means, without the prior permission in writing of The Royal Society of Chemistry or the copyright owner, or in the case of reproduction in accordance with the terms of licences issued by the Copyright Licensing Agency in the UK, or in accordance with the terms of the licences issued by the appropriate Reproduction Rights Organization outside the UK. Enquiries concerning reproduction outside the terms stated here should be sent to The Royal Society of Chemistry at the address printed on this page.

The RSC is not responsible for individual opinions expressed in this work.

Published by The Royal Society of Chemistry,
Thomas Graham House, Science Park, Milton Road,
Cambridge CB4 0WF, UK

Registered Charity Number 207890

For further information see our web site at www.rsc.org

Contents

Chapter 1	Dipeptidyl Peptidases: Substrates and Therapeutic Targeting in Human Health and Disease	1
	<i>Claire H. Wilson and Catherine A. Abbott</i>	
1.1	Introduction	1
1.2	Post-Proline-Cleaving Enzymes	2
1.3	DP4-Like Gene Family and Related Enzymes	3
1.3.1	Homology and Gene Structure	6
1.4	Protease Members of the DP4-Like Gene Family	6
1.4.1	DP4 and FAP: Extracellular Proteins	6
1.4.2	DP8 and DP9: Intracellular Proteins	10
1.5	Protein Structure, Active Site Entry, and Substrate Specificity	13
1.6	DP Substrates: Biological Relevance to Human Health and Disease	16
1.6.1	Glucose Homeostasis and Nutrient Metabolism	16
1.6.2	Cardiovascular Effects	19
1.6.3	Nociception and Neuropsychiatric Disorders	20
1.6.4	Immune System and Disorders	21
1.6.5	Tissue Repair and Cancer	22
1.7	Therapeutic Targeting of the DPs	23
1.7.1	Targeting Enzymatic Function	24
1.7.2	Targeting Non-Enzymatic Function	27
1.8	Conclusion	28
	References	29

RSC Drug Discovery Series No. 18

Proteinases as Drug Targets

Edited by Ben Dunn

© Royal Society of Chemistry 2012

Published by the Royal Society of Chemistry, www.rsc.org

Chapter 2	The Metalloproteases Meprin α and β: Pathophysiological Roles in Inflammation, Cardiovascular Disease, Cancer, and Fibrosis	44
	<i>Christoph Becker-Pauly</i>	
2.1	Introduction	44
2.1.1	Structural Features of Meprins	46
2.1.2	Activation of Meprins	46
2.1.3	Substrate and Cleavage Specificity of Meprin α and β	46
2.2	Pathophysiological Relevance of Meprins	49
2.2.1	Inflammatory Disease	49
2.2.2	Angiogenesis and Cancer	51
2.2.3	Fibrosis	53
2.3	Inhibitors of Meprin α and β	55
	References	58
Chapter 3	Glutamate Carboxypeptidase II as a Therapeutic Target	62
	<i> Klára Hlouchová, Cyril Bařinka and Jan Konvalinka</i>	
3.1	Introduction	62
3.2	Gene and mRNA	63
3.2.1	GCPII mRNA	64
3.3	GCPII Localization	64
3.3.1	Protein Expression in Human and Animal Tissues	65
3.3.2	Cellular Localization	69
3.4	GCPII Biology	69
3.5	GCPII Homologs	70
3.5.1	Human Paralogs	70
3.6	Structure and Enzymology	71
3.6.1	Enzymology	71
3.6.2	Structure	72
3.7	GCPII Inhibitors and Ligands	76
3.7.1	Low-Molecular-Weight Ligands	76
3.7.2	Macromolecules	81
3.8	Applications	81
3.8.1	Experimental Anti-Neurodegeneration Therapy	81
3.8.2	Cancer	83
3.9	Conclusions and Future Perspectives	85
	References	86

Chapter 4	The Role of Proteolytically Inactive Serine Proteases from <i>Sarcoptes scabiei</i> in Complement Evasion	96
	<i>Simone Reynolds and Katja Fischer</i>	
4.1	Scabies and Associated Bacterial Skin Infections	96
4.2	Host Immune Response to Scabies Infection	98
4.3	<i>Sarcoptes scabiei</i> Gene Discovery	99
4.4	Scabies Mite Inactive Protease Paralogues (SMIPP-Ss)	101
4.4.1	Scabies Mite Inactive Protease Paralogues Inhibit Complement	102
4.4.2	Complement-Subversion Strategies in Other Parasitic Organisms	104
4.5	Inactive Proteases in Other Organisms	107
4.5.1	Functions of Inactive Proteases	108
4.5.2	Mode of Action of Inactive Enzymes	110
4.6	Conclusion	111
	Acknowledgment	112
	References	112
Chapter 5	Targeting Dipeptidyl Peptidase-4 (DPP-4) and Fibroblast Activation Protein (FAP) for Diabetes and Cancer Therapy	118
	<i>F. M. Keane, S. Chowdhury, T.-W. Yao, N. A. Nadvi, M. G. Gall, Y. Chen, B. Osborne, A. J. V. Ribeiro, W. B. Church, G. W. McCaughan, M. D. Gorrell and D. M. T. Yu</i>	
5.1	Introduction	118
5.2	DPP-4	119
5.2.1	Functions of DPP-4	119
5.2.2	Distribution of DPP-4	120
5.2.3	Structure of DPP-4	121
5.2.4	Substrates of DPP-4	122
5.2.5	DPP-4 Inhibitors in Disease Therapy	124
5.3	FAP	129
5.3.1	Functions of FAP	129
5.3.2	Distribution of FAP	130
5.3.3	Structure of FAP	130
5.3.4	Substrates of FAP	132
5.3.5	Targeting FAP for Cancer Therapy	132
5.4	Recent Developments in the DPP-4 Protease Family	135
5.5	Conclusion	136
	Acknowledgments	136
	References	136

Chapter 6	Discovery of the Cysteine Protease Cathepsin B as a Drug Target for Alzheimer's Disease	145
	<i>Vivian Hook, Mark Kindy and Gregory Hook</i>	
6.1	Introduction: Alzheimer's Disease and β -Amyloid	145
6.2	Strategies to Identify Protease(s) Cleaving at the Wild-Type β -Secretase Site of APP	147
6.2.1	Specificity of Peptide Substrate Cleavage by Proteases	149
6.2.2	Design of Wild-Type β -Secretase Peptide Substrate, Based on the P2-P1 Residues at the Wild-Type β -Secretase Site of APP	149
6.3	Cathepsin B is Identified as a Candidate β -Secretase in Secretory Vesicles for Production of A β Peptides	150
6.3.1	Regulated Secretory Vesicles Produce the Majority of Secreted A β	150
6.3.2	Wild-Type β -Secretase Substrate Leads to Identification of Cathepsin B in A β -Producing Secretory Vesicles	151
6.3.3	Distinct Cleavage Specificity of Cathepsin B for the Wild-Type β -Secretase Site of APP, But Not the Swedish Mutant β -Secretase Site	151
6.4	Inhibition or Knockout of Cathepsin B Reduces A β Production	154
6.4.1	Inhibition of Cathepsin B Reduces A β in Regulated Secretory Vesicles of Neurons	154
6.4.2	Cathepsin B Inhibitor Treatment of Guinea Pigs, Expressing Human Wild-Type APP, Reduces Brain A β	154
6.4.3	Inhibition of Cathepsin B Reduces Brain A β and Improves Memory Deficits in a Mouse Model Expressing Human APP with the Wild-Type β -Secretase Site, But Not in Mice Expressing APP with the Swedish Mutant Site	154
6.4.4	Knockout of the Cathepsin B Gene Results in Reduction in Brain A β in Mice Expressing APP with the Wild-Type β -Secretase Site, But Not in Mice Expressing the Swedish Mutant Site of APP	157
6.4.5	Cathepsin B Degradation of A β is Not Significant <i>In Vivo</i>	161

6.5 Hypothesis that Cathepsin B and BACE1 Participate as Dual β -Secretases for A β Production	162
6.5.1 BACE1 Prefers to Cleave the Swedish Mutant β -Secretase Site, Rather Than the Wild-Type β -Secretase Site of APP	162
6.5.2 BACE1 Knockout in Swedish APP-Expressing Mice	163
6.5.3 β -Secretase Activity in BACE1 Knockout Mice	163
6.5.4 A β Production and Secretion in Neurons of BACE1 Knockout Mice	164
6.5.5 BACE1 and A β Production in Mice Expressing Normal Compared to Very High Expression Levels of APP: Relationship to AD Patients Expressing Physiological Levels of APP	164
6.6 Cathepsin B Inhibitors as Potential Therapeutic Agents for the Majority of AD Patients Expressing Wild-Type APP	165
Acknowledgments	165
References	165

Chapter 7 <i>Plasmodium falciparum</i> Neutral Aminopeptidases: Development of Novel Anti-Malarials by Understanding Enzyme Structure	169
<i>Donald Gardiner, John Dalton and Sheena McGowan</i>	
7.1 Introduction	169
7.1.1 Falciparum Malaria: The Health Burden	169
7.1.2 A Continuing Need for New Targets for Novel Anti-Malarials	170
7.1.3 <i>Pf</i> Neutral Aminopeptidases as New Drug Targets	172
7.2 <i>Pf</i> Neutral Aminopeptidases	173
7.2.1 <i>Pf</i> A-M1 Alanyl Aminopeptidase	173
7.2.2 <i>Pf</i> A-M17 Leucyl Aminopeptidase	176
7.3 Structure–Activity–Relationship Data and Drug Design	180
7.3.1 Current Inhibitors of the Neutral Aminopeptidases	180
7.3.2 Structure–Activity Relationships and Future Rational Drug Design	182
7.4 Conclusions	183
Acknowledgments	183
References	183

Chapter 8	Blood-Feeding Human Hookworm Proteases	186
	<i>A. Loukas, N. Ranjit, D. A. Pickering and M. S. Pearson</i>	
8.1	Introduction	186
8.2	Proteases of Adult-Stage Hookworms	187
8.2.1	Aspartic Proteases	189
8.2.2	Cysteine Proteases	191
8.2.3	Metalloproteases	192
8.2.4	Aminopeptidases and Exopeptidases	193
8.3	Proteases of Larval-Stage Hookworms	194
8.4	Concluding Remarks	195
	Acknowledgments	195
	References	195
Chapter 9	Pharmacological Targeting of Human Tissue Kallikrein-Related Peptidases	199
	<i>Georgios Pampalakis and Georgia Sotiropoulou</i>	
9.1	Introduction	199
9.1.1	Human Tissue Kallikrein-Related Peptidases	199
9.1.2	Why Should We Target KLKs? Association of KLK with Disease	200
9.1.3	Structure of KLKs	203
9.1.4	Identification of Substrates and Endogenous Inhibitors	204
9.2	Peptide Modulators	205
9.2.1	Synthetic LEKTI Domains	205
9.2.2	Cyclic Peptide Inhibitors	205
9.2.3	Peptide Activators of KLKs	205
9.2.4	Depsipeptides	207
9.3	Inhibitors Designed Based on Bioscaffolds	208
9.3.1	Engineered Sunflower Trypsin Inhibitor (SFTI)	208
9.3.2	Mutant Serpins	208
9.4	Synthetic Inhibitors Based on Modified Peptide-Substrates or Small Molecules	209
9.4.1	KLK1 Inhibitors	212
9.4.2	KLK3/PSA Inhibitors	213
9.4.3	Imaging Based on KLK3/PSA	216
9.4.4	KLK7 Inhibitors	217
9.4.5	General Modulators of KLKs	218
9.5	Antibody-Derived Inhibitors	219
9.5.1	Potential of Antibody Phage Display Strategy	219
9.5.2	Antibodies Directed Against KLKs	219
9.6	Future Directions	221
	References	222

Chapter 10 Structural Mechanisms of Inactivation in Proteolytically Inactive Serine Proteases from <i>Sarcoptes scabiei</i>	229
<i>Katja Fischer, James A. Irving, Robert Pike and Ashley M. Buckle</i>	
10.1 Scabies	229
10.1.1 Scabies Mite Inactivated Protease Paralogues (SMIPPs)	230
10.2 Crystal Structural Analysis of SMIPPs	230
10.2.1 SMIPPs Adopt the Chymotrypsin-Like Serine Protease Fold	230
10.2.2 Structural Diversity Within the Active Site and Throughout the Molecules	232
10.3 Peptide Phage Display Screening Does Not Identify Binding Partners	233
10.4 Restoration of the Catalytic Triad by Mutagenesis Does Not Rescue Protease Activity	234
10.5 Phylogenetic Analysis of the SMIPP Family	234
10.6 SMIPPs Do Not Function by Competing for Substrates with Host Proteases That Play a Role in Host Defense Mechanisms	235
10.6.1 Putative Alternative Binding Sites	237
10.6.2 Sequence Conservation in the SMIPP Family	237
10.7 Conclusions	240
Acknowledgments	240
References	240
 Chapter 11 The Aspartic Proteinases from the Malaria Parasite: Structure and Function of the Plasmepsins	 242
<i>Ben M. Dunn</i>	
11.1 Introduction	242
11.1.1 Tropical Diseases and the Need for Drug Discovery	242
11.2 Malaria	244
11.2.1 Plasmepsins as Targets for Drug Discovery Against Malaria	245
11.2.2 Plasmepsin Gene Family in the Malaria Parasite	245
11.3 Digestive Vacuole Enzymes	246
11.3.1 Plasmepsin I	246
11.3.2 Plasmepsin II	248
11.3.3 HAP, the Histo-Aspartic Plasmepsin	252
11.3.4 Plasmepsin IV	253

11.4	Non-Digestive Vacuole Plasmepsins	255
11.4.1	Plasmepsin V	256
11.4.2	Plasmepsin IX and Plasmepsin X	257
11.5	Inhibitor Development	258
11.5.1	HIV PR Inhibitors and Malaria	262
11.6	Summary	263
	Acknowledgments	263
	References	263

Subject Index	270
----------------------	------------