Inhibitor selectivity in the clinical application of dipeptidyl peptidase-4 inhibition

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ABSTRACT

DPP-4 (dipeptidyl peptidase-4) degrades the incretin hormones GLP-1 (glucagon-like peptide-1) and GIP (gastric inhibitory polypeptide), decreasing their stimulatory effects on β-cell insulin secretion. In patients with Type 2 diabetes, meal-related GLP-1 secretion is reduced. DPP-4 inhibitors (alogliptin, dutogliptin, linagliptin, saxagliptin, sitagliptin and vildagliptin) correct the GLP-1 deficiency by blocking this degradation, prolonging the incretin effect and enhancing glucose homoeostasis. DPP-4 is a member of a family of ubiquitous atypical serine proteases with many physiological functions beyond incretin degradation, including effects on the endocrine and immune systems. The role of DPP-4 on the immune system relates to its extra-enzymatic activities. The intracytosolic enzymes DPP-8 and DPP-9 are recently discovered DPP-4 family members. Although specific functions of DPP-8 and DPP-9 are unclear, a potential for adverse effects associated with DPP-8 and DPP-9 inhibition by non-selective DPP inhibitors has been posed based on a single adverse preclinical study. However, the preponderance of data suggests that such DPP-8 and DPP-9 enzyme inhibition is probably without clinical consequence. This review examines the structure and function of the DPP-4 family, associated DPP-4 inhibitor selectivity and the implications of DPP-4 inhibition in the treatment of Type 2 diabetes.

INTRODUCTION

DPP-4 (dipeptidyl peptidase-4; E.C. 3.4.14.5) inhibitors, a novel modality for the treatment of Type 2 diabetes, augment glucose homoeostasis by preventing degradation of the incretin hormones GIP (gastric inhibitory polypeptide) and GLP-1 (glucagon-like peptide-1). The latter hormone accounts for the majority of the incretin effect and is essential for regulating both fasting and postprandial plasma glucose by stimulating insulin secretion, supporting pancreatic β-cell proliferation and inhibiting glucagon production by the pancreatic α-cells to decrease glucose production by the liver [1]. In patients with Type 2 diabetes, meal-related GLP-1 secretion is impaired, most probably as a consequence of the disease [2], and enhancement of endogenous incretin levels with DPP-4 inhibitors should help preserve GLP-1 function. Clinical trials of DPP-4 inhibitors have demonstrated significant glycaemic efficacy, including sustained HbA1c (glycated haemoglobin A1c) reductions for up to 2 years in patients treated with monotherapy and combination therapy [1,3,4]. Advantages over existing diabetes treatments include a low risk of hypoglycaemia, a neutral effect on body weight and the potential, based on animal and in vitro studies, for preservation or enhancement of β-cell function [1,3,5,6]. The first

Key words: diabetes, dipeptidyl peptidase-4, enzyme inhibition, immunity, selectivity.

Abbreviations: CI, confidence interval; DPP, dipeptidyl peptidase; DPL, DPP-like; FAP, fibroblast activation protein; FDA, Food and Drug Administration; GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptide; IP-10, interferon-γ-induced protein-10; I-TAC, interferon-inducible T-cell α chemoattractant; MMP-9, matrix metalloproteinase-9; NEP, neutral endopeptidase; NPY, neuropeptide Y; PYY, peptide YY; SDF-1, stromal cell-derived factor-1; 3D, three-dimensional.

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agent in the gliptin class, sitagliptin, received US FDA (Food and Drug Administration) approval in 2006, followed by saxagliptin in 2009. Both sitagliptin and vildagliptin received approval from the EMEA (European Medicines Agency) in 2007. A number of additional DPP-4 inhibitors are in various phases of clinical development. As the understanding of the mechanism of DPP-4 function progresses and competing products become available, an important consideration is the degree of enzyme selectivity exhibited by different agents in this therapeutic class.

DPP-4 is in a family of ubiquitous atypical serine proteases with numerous functions, including roles in nutrition, metabolism, the endocrine and immune systems, cancer growth, bone marrow mobilization and cell adhesion [7]. Given that these many functions are associated with the DPP-4 family, selective interactions might be necessary for optimal efficacy, safety and tolerability. The present review covers the structure and function of the DPP-4 family, mechanisms of DPP-4 inhibition, extra-enzymatic activities independent of catalysis, and the relationship between DPP-4 enzyme inhibition and non-selective DPP inhibition as a means of examining the relevance of selectivity in the clinical application of DPP-4 inhibitors.

DPP-4 FAMILY

The DPP-4 family, a subfamily of the prolyl oligopeptidase superfamily, includes four enzymes, DPP-4, FAP (fibroblast activation protein), DPP-8 and DPP-9, and two non-enzymes, DPP-4-like protein-6 (DPP-6, DPL-1 or DPP-X) and DPP-10 (DPL-2). Members of the DPP-4 family preferentially cleave Xaa-Pro- and Xaa-Ala- dipeptides (where Xaa is any amino acid except proline) from the N-terminus of proteins [8]. The DPP-4 family differentiates itself from the prolyl oligopeptidase superfamily by the presence of two glutamate residues located within the catalytic pocket, which are essential for enzymatic activity [9]. The absence of catalytic activity in DPL-1 and DPL-2 is attributed to a number of amino acid substitutions in the catalytic pocket [10].

DPP-4

DPP-4 is widely distributed, existing both as a membrane-anchored cell-surface peptidase and as a smaller soluble form in blood plasma. In humans, DPP-4 is expressed in epithelial cells, capillary endothelia and lymphocytes. This includes expression in the gastrointestinal tract, biliary tract, exocrine pancreas acinar cells, kidney, thymus, lymph nodes, uterus, placenta, prostate, the adrenal, parotid, sweat and mammary glands, liver, spleen, lungs and brain [11].

A 766-amino-acid protein, DPP-4 is arranged in two domains: an N-terminal β-propeller domain and a C-terminal α/β-hydrolase domain. The two domains form a large cavity that houses the active site (Figure 1) [12]. The tertiary structure of DPP-4 reveals a dimer that may form a dimer of dimers, suggesting a potential for inter-cellular homotypic binding (Figure 2) [13]. The α/β-hydrolase domain is involved in dimerization, and the β-propeller domain is involved in both the dimeric and tetrameric interactions [12].

Both the cell-surface and soluble forms of DPP-4 are catalytically active as dimers. Access to the cavity, and hence to the active site, is via an opening in the centre of the β-propeller or through the larger opening between the propeller and hydrolase domains (Figure 1) [12,13]. Essential to the catalytic activity of DPP-4 are residues Ser630, Asp708 and His740 of the catalytic triad, Tyr547 in the hydrolase domain, and Glu360 and Glu206 in the β-propeller domain [12,14]. The two glutamate residues align the substrate peptide such that only two amino acids with smaller side chains, such as proline and alanine, reach the active serine residue. These features help explain the preferential Xaa-Pro- and Xaa-Ala- cleaving activity and substrate specificity of DPP-4 (Figure 3) [14].

Substrates of DPP-4 include numerous neuropeptides, hormones and chemokines (Table 1). However, although the majority of the substrates identified serve as pharmacological substrates in vitro, relatively few have been determined to be endogenous physiological substrates (defined as peptides whose endogenous circulating levels of intact compared with N-terminally cleaved forms are altered following reduction or elimination of DPP-4 activity in vivo). Both GIP and GLP-1 are endogenous physiological substrates for DPP-4; in rats and mice with targeted genetic inactivation or inhibition of DPP-4, there are increased circulating levels of intact GIP and GLP-1, and levels of intact GIP and GLP-1 are increased relative to their N-terminally truncated forms [15].

Similarly, substance P, a neurotransmitter/neuromodulator associated with many physiological functions, including nociception, pain transmission, smooth muscle contraction, anxiety and stress-related responses, and the chemokines SDF-1α (stromal cell-derived factor-1 α) and SDF-1β, chemoattractants for lymphocytes and monocytes that regulate the development of T- and B-lymphocytes, and the survival of mature lymphocytes, are cleaved by DPP-4 at the N-terminus, and plasma levels of active forms of these substrates are increased in Dpp-4- knockout mice [16]. Whether partial or full reduction of DPP-4 activity due to selective DPP-4 inhibitors results in biologically significant increases in the levels of active SDF-1α/β or substance P at their sites of action in humans, as it does for the incretin hormones, is unknown.

FAP

The enzyme FAP, also known as seprase, is the most similar family member to DPP-4, as it shares a 52% amino acid identity (human enzymes) and
similar substrate specificity. Despite these similarities, DPP-4 and FAP differ markedly in their expression patterns. FAP expression is confined predominantly to activated fibroblasts in diseased tissue, such as fibrotic and epithelial tumours, and invasive cancers, and may be important in wound healing [17]. FAP immunostaining intensity correlates with the histological severity of fibrosis in chronic liver disease, which involves chronic wound healing [18]. FAP has narrow prolyl endopeptidase activity, confined to Z-Gly-Pro-derived substrates, and a dipeptidyl peptidase activity that more effectively hydrolyses H-Ala-Pro- than H-Gly-Pro-derived substrates [19,20].

**DPP-8 and DPP-9**
The other two catalytically active DPP-4 family members, DPP-8 and DPP-9, share 61% amino acid identity with each other, and a 26 and 21% amino acid identity with the protein sequences of DPP-4 and FAP respectively (human enzymes) [21]. In contrast with DPP-4 and FAP, which have an extracellular catalytic domain, both DPP-8 and DPP-9 proteins are localized to the cytoplasm (Figure 4) [21]. DPP-8 expression is up-regulated in activated T-cells. DPP-8 and DPP-9 enzyme activity has been detected in human blood lymphocytes and monocytes [22], and there is some evidence of DPP-8 and DPP-9 expression in mouse pulmonary leucocytes [23]. High levels of DPP-9 are found in cancer cells, normal skeletal muscle, the heart and liver. DPP-8 and DPP-9 hydrolyse H-Ala-Pro- and H-Gly-Pro-derived substrates, although with less efficiency than DPP-4. *In vitro* peptide substrates of DPP-8 or DPP-9 identified to date include GLP-1, GLP-2, NPY (neuropeptide Y), PYY (peptide YY), SDF-1, IP-10 (interferon-γ-induced protein-10) and I-TAC (interferon-inducible T-cell α chemoattractant) (Table 1) [24,25]. However, a physiological substrate or role for the DPP activity of either DPP-8 or DPP-9 remains to be demonstrated *in vivo.*

**Other enzymes involved in DPP-4 substrate degradation**
Although DPP-4 has been established as the primary inactivation enzyme of GLP-1, other enzymes degrade GLP-1, in particular NEP 24.11 (neutral endopeptidase 24.11). NEP 24.11, expressed mainly in the kidney, is a widespread membrane-bound zinc metallopeptidase with broad-spectrum specificity that is involved in the inactivation and renal clearance of peptide hormones [26]. *In vivo* studies have shown that up to 50% of GLP-1 degradation may be due to NEP 24.11 [26]. It has been demonstrated that when DPP-4 and NEP
Figure 2  Schematic representation of the structure of DPP-4


24.11 inhibitors are administered concomitantly, the combined effect is greater than the effect of either inhibitor alone, resulting in significant improvements in the antihyperglycaemic and insulinotropic effects of exogenous GLP-1 [26]. Therefore the relevance of DPP-4 in physiological cleavage of other substrates in vivo may similarly depend on the relative rates of substrate cleavage by DPP-4 compared with other enzymes. This might explain why, even though DPP-4 has nine chemokine substrates, no in vivo role for DPP-4 cleavage of chemokines has been demonstrated. For example, SDF-1 is proteolytically inactivated by NEP, cathepsin G and MMP-9 (matrix metalloproteinase-9) [27]. Further studies utilizing Dpp-4-knockout mice and a DPP-4-selective inhibitor in an appropriate model of cell migration may aid in demonstrating the importance of DPP-4-mediated chemokine degradation.

ENZYMATIC INHIBITION OF THE DPP-4 FAMILY

The leading DPP-4 inhibitors in clinical use or late-phase clinical development are alogliptin, saxagliptin, sitagliptin and vildagliptin. Inhibition of the DPP-4 enzyme based on IC50 (concentration at which there is 50% inhibition of measured activity in vitro, dependent on substrate concentration) and Ki (enzyme–inhibitor dissociation constant, independent of substrate concentration) values for these DPP-4 inhibitors show values in the low

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nanomolar range. In contrast, these inhibitors have a low affinity for DPP-8 and DPP-9 (Table 2), and other proteases are not inhibited by DPP-4 inhibitors. Whether DPP-8 and DPP-9 are inhibited \textit{in vivo} during administration of therapeutic doses of these selective DPP-4 inhibitors remains unknown. No \textit{in vivo} effects have been reported that point to a pharmacological impact of the inhibition of other peptidases [1].

It is important to recognize that comparisons among the different inhibitors are different because a standardized DPP-4 enzyme assay is not used. IC$_{50}$ values depend on the substrate used and the ratio of substrate concentration to its $K_i$ for that enzyme; for rapidly dissociating DPP-4 inhibitors, such as alogliptin and sitagliptin, substrate choice alters the $K_m$ and, therefore, results in different IC$_{50}$ values for a single enzyme [28]. Saxagliptin and vildagliptin have been reported to have slow binding. Therefore a suitable incubation period for the equilibration of enzyme and inhibitor needs to occur prior to substrate addition, otherwise inhibition by these compounds could be severely underestimated. Finally, $K_i$ increases with temperature; thus ambient and physiological assays’ temperatures produce differing data [1].

Another important consideration is that assays measuring plasma DPP-4 activity introduce a dilution step that produces a dilution artifact for rapidly dissociating DPP-4 inhibitors. Methodology in most selectivity papers is incomplete and generally includes non-physiological conditions. Thus there is a major need to adopt a standardized DPP-4 assay and focus upon $K_i$.

As DPP-4 preferentially cleaves substrates with a proline residue at the P1 (1-position of the pyrrolidine ring) and accepts most residues at P2 and in prime side positions, many inhibitors (including a whole class of cyano- and boronic-substituted pyrrolidine DPP inhibitors) incorporate a proline or proline mimetic at P1, with either a reversible or an irreversible electrophilic isostere to form an adduct with the active site Ser$^{630}$ in the hydrolase domain. These are typified by saxagliptin and vildagliptin [1]. Saxagliptin and vildagliptin both contain cyanopyrrolidines that bind to DPP-4 in the S1 pocket. Saxagliptin has been shown by extensive nuclear magnetic resonance, isothermal titration calorimetry and X-ray diffraction-derived structural studies to form a reversible covalent bond between the catalytic hydroxy group of Ser$^{630}$ in DPP-4 with the nitrile carbon atom to hydroxy oxygen bond length of $< 1.3$ Å (where 1 Å = 0.1 nm) [14]. This strong interaction of saxagliptin and vildagliptin with the enzyme may be a major determinant of their inhibitor potency.

Two glutamate residues (Glu$^{205}$ and Glu$^{206}$) in the $\beta$-propeller domain are highly conserved across the DPP family and are essential for its enzymatic activity [9]. The amino groups contained within the DPP-4 inhibitors alogliptin, saxagliptin, sitagliptin and vildagliptin are all believed to interact with these two glutamate residues with different interaction strengths. This interaction has been demonstrated experimentally through single-crystal X-ray diffraction for the bound DPP-4 complexes of sitagliptin [29], saxagliptin [14] and a close analogue of alogliptin [30]. The interactions with Glu$^{205}$ and Glu$^{206}$ are thought to be crucial.
Table 1  DPP-4 substrates

<table>
<thead>
<tr>
<th>Pharmacological substrate</th>
<th>Physiological substrate</th>
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<tbody>
<tr>
<td>Chemokines</td>
<td>Glucagon family</td>
</tr>
<tr>
<td>MIG/CXCL9</td>
<td>GLP-1</td>
</tr>
<tr>
<td>IP-10/CXCL10</td>
<td>GLP-2</td>
</tr>
<tr>
<td>I-TAC/CXCL11</td>
<td>GIP</td>
</tr>
<tr>
<td>LD78/β/CCL3L1</td>
<td>Other</td>
</tr>
<tr>
<td>RANTES/CCLL5</td>
<td>Substance P</td>
</tr>
<tr>
<td>MDC/CCL22</td>
<td>SDF-1β/β/CXCL2</td>
</tr>
<tr>
<td>Eotaxin/CCL11</td>
<td></td>
</tr>
<tr>
<td>GRO-β1/CXCL2</td>
<td></td>
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<tr>
<td>GCP-2/CXCL6</td>
<td></td>
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<tr>
<td>Pancreatic polypeptide family</td>
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<tr>
<td>NPY</td>
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<tr>
<td>PYY</td>
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<tr>
<td>PACAP27</td>
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<tr>
<td>PACAP38</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
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<tr>
<td>Vasostatin-1</td>
<td></td>
</tr>
<tr>
<td>GRP</td>
<td></td>
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<tr>
<td>GRP-(2–27)</td>
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<tr>
<td>GHRH</td>
<td></td>
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<tr>
<td>IGF-1</td>
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<td>PHM</td>
<td></td>
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<tr>
<td>β-Casomorphin-2</td>
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<tr>
<td>Endomorphin-2</td>
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<tr>
<td>Morphiceptin</td>
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<tr>
<td>Enterostatin</td>
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<tr>
<td>Haemorphin-7</td>
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<tr>
<td>β-Type natriuretic peptide</td>
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</table>

Interactions for alogliptin and sitagliptin. Although saxagliptin and vildagliptin both contain a hydroxy-substituted adamantane fragment, with each occupying the S2 pocket of the DPP-4 active site, the carbon-linked adamantane of saxagliptin, as well as its lipophilic cyanocyanopropylpyrrolidine, provide it with a high level of binding efficacy. With regard to vildagliptin, the nitrogen-linked adamantane and smaller S1 pocket-filling cyanopropylpyrrolidine may be factors that limit its potency. Therefore, because saxagliptin has strong interactions with both Ser<sup>630</sup> and Glu<sup>205/206</sup> [14], the combination of these interactions results in its highly potent DPP-4 inhibition compared with alogliptin, sitagliptin and vildagliptin (Table 2).

As the β-propeller domain of DPP-4, DPP-8 and DPP-9 is not as conserved as the α/β-hydrolase domain and the active sites of DPP-4, DPP-8 and DPP-9 differ [24,31,32], selective inhibition of DPP-4 may be achieved. The precise origin of this selectivity must await the crystal structures of DPP-8 and DPP-9. A number of indirect studies, however, have given insight into the differential binding in these peptidases. For instance, a 3D (three-dimensional) quantitative structure-activity relationship study provided a highly predictive CoMFA (comparative molecular field analysis) model [33] and supported findings in other reports [34,35] that the S1 pocket of DPP-4 is smaller than in DPP-8/9, whereas the S1′ site is larger in DPP-4 and more capable of accommodating negatively charged groups. Further subtle analysis suggests that the positions between the S2 and S3 sites are larger in DPP-4, while the S3-binding site itself is smaller in DPP-4 than in DPP-8/9. The S3 site in particular is believed to exhibit the greatest variance between the three enzymes, and the specific interactions around Phe<sup>357</sup> (DPP-4) have been proposed to play an important role in enhancing the selectivity against DPP-8 [33,34]. More recently, Van der Veken et al. [36] reported on a series of compounds with a 4S-azido substituent on the P1 2S-cyanopropylpyrrolidine that were selective for DPP-8/9 over DPP-4. In addition, these authors also describe substituted isoinodoline P1 inhibitors that had significant selectivity for DPP-8/9 over DPP-4 [37]. These results support further the conclusion above that the S1 pocket in DPP-4 is smaller than that in DPP-8 or DPP-9.

Linking proteases with their substrates is crucial for understanding protease function. Two key steps in identifying the role of DPP-4 in glucose homeostasis were the discoveries that GLP-1 is a DPP-4 substrate and that DPP-4 is a major determinant of the half-life of GLP-1 in vivo. Similarly, knowledge of DPP-8 and DPP-9 substrates is crucial for understanding their biological roles. Although the substrate specificity of DPP-8 and DPP-9 is very similar to DPP-4, their cleavage rates are significantly slower than DPP-4 [25].
This difference suggests that these in vitro substrates will not prove to be the biologically relevant substrates of DPP-8 and DPP-9, and that the most readily hydrolysed DPP-8 and DPP-9 substrates are yet to be discovered. Whereas DPP-4 can access chemokines extracellularly, the intracellular location of DPP-8 and DPP-9 makes it unclear whether DPP-8 or DPP-9 makes physical contact with incretin hormones, chemokines or substance P in vivo [25]. Thus the physiological relevance of most substrate cleavage in the DPP family is unclear. Such uncertainty concerning the biological importance of substrate discovery applies to most proteases because substrates have often been identified only by in vitro assay and substrates are generally inactivated by two or more proteases.

Elucidating the effects of DPP-8 or DPP-9 inhibition is difficult because few useful reagents are available. There are no specific substrates, fully selective inhibitors or gene-knockout mice for either enzyme. Therefore comparisons between selective and less selective DPP-4 inhibitors or DPP-8/DPP-9-selective inhibitors have not been made. The availability of compounds selective for DPP-8 and DPP-9 would be useful. Furthermore, in contrast with DPP-4, which is extracellular, DPP-8 and DPP-9 are cytosolic, so for each inhibitor compound the potential effects on DPP-8 and DPP-9 depend upon the intracellular concentration achieved. Moreover, unlike DPP-4 and FAP, DPP-8 and DPP-9 activity diminishes in mildly oxidizing conditions [32] and so may be influenced by intracellular redox state.

The mechanism of toxicity observed in a preclinical study of DPP inhibitors is unclear [38]. Evidence indicating that DPP-8 and DPP-9 are not involved in such toxicity is summarized below. Important to note is that small differences in compound chemistry can produce significant differences in biological effects.

### EXTRA-ENZYMATIC ACTIVITY IN THE DPP-4 FAMILY

Determining the functions of the DPP family is complicated further by the fact that DPP-4, FAP, DPP-8 and DPP-9 possess extra-enzymatic activities, which are not influenced by enzyme inhibitors and are crucial for DPP-4 action in immunity. Mutation of active-site residues has shown that enzyme activity is not required for the observed effects of DPP-4, DPP-8, DPP-9 or FAP overexpression on cell adhesion, migration, proliferation and apoptosis in epithelial and hepatic stellate cell lines [39,40]. Melanoma and lung cancer cells exhibit similar effects when DPP-4 is overexpressed, including increased apoptosis and inhibition of cell migration and anchorage-independent growth, whether transfected with wild-type or enzyme-negative mutant DPP-4 [41,42]. The DPP-4-mediated effect on cell adhesion involves p38 MAPK (mitogen-activated protein kinase) dephosphorylation and β1-integrin [43]. Interestingly, DPP-9 overexpression causes cells to produce less of the extracellular matrix-interacting molecule DDR1 (discoidin domain receptor family 1), a receptor tyrosine kinase activated by collagen binding [40]. DDR1 is an integrin-independent cell adhesion molecule. In vivo
evidence supporting the role of extra-enzymatic rather than enzymatic activity in these processes is primarily derived from experiments comparing nude mice injected with melanoma cells stably expressing wild-type DPP-4 or mutant DPP-4. No differences in tumour progression were observed [44].

Moreover, DPP-4 has been shown to interact with other proteins, including adenosine deaminase, caveolin-1, plasminogen-3ɛ, glypican-3 and fibronectin type III [17]. These interactions do not interfere with the catalytic function of DPP-4 as their sites of interaction are or very likely to be located on the lower outer surface of the DPP-4 protein, distant from the catalytic pocket located on the inside surface of the enzyme [7,45].

Thus DPP-4 extra-enzymatic functions, such as protein–protein interactions, are crucial in these biological roles of the DPP-4 family enzymes. Directly studying extra-enzymatic functions by blocking protein–protein interactions of DPP-4 and its relatives would be interesting, but such reagents are unavailable.

**INTERPRETING ANIMAL TOXICITY DATA**

A preclinical study [38] assessing toxicities in rodents and dogs treated with inhibitors of DPP-8 and DPP-9 (allo-isoleucyl thiazolidine and threo-isoleucyl thiazolidine), a selective DPP-4 inhibitor and non-selective DPP inhibitor, found that selective DPP-4 inhibition has no adverse toxicity effects in rodents and dogs, but the non-selective DPP inhibition resulted in severe toxicities and, in some cases, mortality. Toxicity-related outcomes included death, alopecia, thrombocytopenia, reticulocytopenia and splenomegaly in rodents given 100 mg · kg⁻¹ of body weight · day⁻¹ for up to 2 weeks. Similar toxicities were observed when using a DPP-8 and DPP-9 inhibitor at similar doses in rodents and 10 mg/kg of body weight in dogs. The DPP-8/DPP-9 inhibitors produced similar toxicities in Dpp-4-deficient and wild-type mice, thus the observed toxicities were not due to DPP-4 inhibition [38].

The suggestion that DPP-8 and DPP-9 inhibition produces toxicity in preclinical species is principally disputed by recent results showing that high doses of vildagliptin, producing nearly complete in vivo inhibition of DPP-8 and DPP-9 enzyme activity, yielded no toxicities in rodents [46]. Vildagliptin is a selective DPP-4 inhibitor with modest DPP-8/DPP-9 inhibitory activity, 200-fold selective against DPP-8 and more than 30-fold selective against DPP-9. In that study [46], the DPP-4 inhibitor dose was up to 1500 mg · kg⁻¹ of body weight · day⁻¹ in mice and 900 mg · kg⁻¹ of body weight · day⁻¹ in rats for 13 weeks, resulting in plasma concentrations well above those that would produce 24-h inhibition of both DPP-8 and DPP-9. Although the initial observations were interpreted as indicating that inhibition of DPP-8 and DPP-9 is toxic in rodents and dogs [38], compounds associated with the toxicities were close structural peptidic analogues of each other, with shared stereochemistry of the unnatural (L-allo) form of isoleucine, and the observed toxicities could have been related to the common structural feature, completely independent of DPP-8/DPP-9 inhibition [46].

Concordantly, an absence of gastrointestinal toxicity with DPP-8 and DPP-9 inhibition in dogs has been observed using a non-selective DPP inhibitor compound ‘G’ [47]. Compound ‘G’ was shown to be cell-permeant, thereby having access to DPP-8 and DPP-9 in the cytosol. The dose of 600 mg/kg of body weight in dogs caused compound ‘G’ to reach plasma levels 1000-fold above the IC₅₀ for both DPP-8 and DPP-9, and caused inhibition of DPP-8 and DPP-9 throughout the gastrointestinal tract and brain. In that study, the allo-isoleucyl isoindoline derivative that inhibits DPP-8 and DPP-9 again produced toxicities similar to those reported previously [38]. However, compound ‘G’, despite inhibiting DPP-8/DPP-9 in vivo far more than that allo-isoleucyl isoindoline derivative, was nontoxic [47]. These findings strongly suggest that the report of toxicity associated with certain DPP inhibitor compounds relates to properties of those compounds rather than inhibition of DPP-4, DPP-8 or DPP-9.

Additional preclinical assessment of potential clinical candidates is important to understand further the significance of DPP-4 inhibitor selectivity, and the effects of DPP-8/DPP-9 inhibition at pharmacologically relevant plasma concentrations. The serum concentrations of clinically available DPP-4 inhibitors are unlikely to approach that of the DPP-8/DPP-9-selective inhibitors used in in vitro studies.

**DPP-4-RELATED IMMUNE RESPONSES**

DPP-4 is also known as CD26, a cell-surface marker for T-cell activation that has a co-stimulatory role in T-cell activation. Several lines of evidence indicate that its DPP-4 enzyme activity is not involved in CD26-mediated T-cell activation and proliferation [11]. The most recent study has shown that T-cell-dependent antibody responses and cytotoxic T-cell responses are not affected when DPP-4 is selectively inhibited in mice. Moreover, these immune responses are the same in Dpp-4-knockout and wild-type mice [51]. Previous in vitro analyses concorded with that study. In transfected Jurkat cells, inactivating DPP-4 by substitution of the catalytic serine with alanine residues has no effect on CD26-dependent T-cell activation [48]. All of the DPP enzyme inhibitors that inhibit T-cell proliferation in vitro inhibit DPP-8 and DPP-9 in addition to, and often more than, DPP-4 [38]. The DPP-4-negative Fischer344 rat strain has no defects in in vitro responses to mitogens or antigens [49]. Moreover, suppression of in vitro responses
to mitogens or *Mycobacterium tuberculosis* antigen by the non-selective DPP inhibitor Lys(Z(NO$_2$))-thiazolidide, is equally effective in wild-type lymphocytes and cells from the DPP-4-negative Fischer344 rat strain [50], demonstrating that such effects do not relate to DPP-4. These results indicate that DPP-4 proteolytic activity is not a prerequisite for the T-cell-activating or co-stimulating properties of DPP-4/CD26.

The suppression of *in vitro* T-cell proliferation by the non-selective DPP inhibitory pyrrolidides (e.g. Lys(Z(NO$_2$))-pyrrolidide) appears to be mediated by DPP-8 and/or DPP-9, because these pyrrolidides are two to three logs more potent against DPP-8 and DPP-9 than DPP-4 [38]. In addition, the DPP-8/DPP-9-selective inhibitor compound ($S$-3$R$)-2-(2-amino-3-methyl-1-oxopentan-1-yl)-1,3-dihydro-2H-isindole hydrochloride, but not the DPP-4-selective inhibitor compound ($S$-2-[4-[[[($S$)-3-amino-4-(2,5-difluorophenyl)-1-oxobutyl]-2-pyrrolidinyl]carbonyl]amino)methyl]phenoxo]-3-methylbutanoic acid trifluoroacetate attenuates proliferation and IL-2 (interleukin-2) release in *in vitro* human T-cell activation [38]. These are the central findings illustrating that DPP-4 catalytic activity is not required for T-cell activation. Immunological effects observed previously with several DPP inhibitors in preclinical models may indirectly indicate a role for DPP-8 and DPP-9 enzyme activity in immune responses, but this question requires further investigation, including separating the roles of DPP-8 from DPP-9.

**DPP-4 INHIBITORS IN CLINICAL DEVELOPMENT**

Among the three DPP-4 inhibitors currently in clinical use, saxagliptin, sitagliptin and vildagliptin, or those in late-stage clinical development (Table 3), no indication of DPP-8/DPP-9-related adverse events has been observed in respective extensive clinical development programmes. Although adverse preclinical findings observed in one study of DPP inhibition have been reported [38], the aforementioned lack of clinical adverse events is the most relevant information when considering the use of selective DPP-4 inhibitors in the treatment of Type 2 diabetes.

DPP-4 inhibitors are generally well tolerated. In monotherapy trials, the overall incidence of adverse effects with vildagliptin and sitagliptin has been similar between DPP-4 inhibitor and comparator groups. The most common side effects reported in DPP-4 inhibitor clinical trials are nasopharyngitis and headache. A recent meta-analysis of sitagliptin and vildagliptin reported a small increased risk of nasopharyngitis (6.4% for DPP-4 inhibitor compared with 6.1% for comparator; risk ratio, 1.2 [95% CI (confidence interval), 1.0–1.4]) and headache (5.1% for DPP-4 inhibitor compared with 3.9% for comparator; risk ratio, 1.4 [95% CI, 1.1–1.7]) [3]. Although not considered a class effect, rare cases of mild hepatic dysfunction have been reported with vildagliptin leading to a request to monitor liver enzyme in patients treated with vildagliptin (see European Public Assessment Report at http://www.ema.europa.eu/humandocs/Humans/EPAR/galvus/galvus.htm) and very rare serious hypersensitivity reactions, including Stevens–Johnson syndrome, have occurred with sitagliptin (see http://www.januvia.com/sitagliptin/januvia/hcp/januvia/safety_profile/safety_profile.jsp?WT.svl=2). In a pooled five-study analysis up to week 24, hypersensitivity-related events were recorded in 1.5% of patients who received 2.5- and 5-mg doses of saxagliptin (see http://packageinserts.bms.com/pi/pi_onglyza.pdf). These are not exceptional issues in a new drug class.

**CONCLUSIONS**

The understanding that DPP-4 is the major inactivator of GLP-1 prompted the clinical development and application of DPP-4 inhibitors for the treatment of Type 2 diabetes to improve glycaemic control by increasing GLP-1 longevity. Clinical trials indicate that the selective DPP-4 inhibitors alogliptin, saxagliptin, sitagliptin and vildagliptin are generally well tolerated, with the benefits of increases in active incretin hormones, less circulating glucagon and the possibility of preserved or enhanced β-cell function. As the DPP-4 family are ubiquitous serine proteases with numerous functions, the roles of DPP-4 as well as its inhibitory effects continue to be studied extensively. Although DPP-4 and the related enzymes DPP-8 and DPP-9 form a family of proline-targeted serine proteases and have a close structural similarity, they also have a number of dissimilarities. One notable dissimilarity is that DPP-4 is extracellular, whereas DPP-8 and DPP-9 are present exclusively in the cytoplasm. In addition, *in vitro* and *in vivo* models have demonstrated that the roles of DPP-4 in the immune system are independent of the protease activity of DPP-4 (GLP-1 and GIP inactivation/metabolism). A single adverse preclinical study led to controversy concerning non-selective inhibition of DPP-4 and the potential for adverse effects associated with inhibition of DPP-8 and DPP-9. However, recent studies reviewed here indicate that enzyme inhibition of DPP-8 and DPP-9 probably lacks an adverse clinical consequence and that these observed toxicities were probably due to the non-enzymatic actions of these compounds.

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