ORIGINAL ARTICLE

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A comparative study of the binding properties, dipeptidyl peptidase-4 (DPP-4) inhibitory activity and glucose-lowering efficacy of the DPP-4 inhibitors alogliptin, linagliptin, saxagliptin, sitagliptin and vildagliptin in mice

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Summary

Aims: Since 2006, DPP-4 inhibitors have become established therapy for the treatment of type 2 diabetes. Despite sharing a common mechanism of action, considerable chemical diversity exists amongst members of the DPP-4 inhibitor class, raising the question as to whether structural differences may result in differentiated enzyme inhibition and antihyperglycaemic activity.

Methods: We have compared the binding properties of the most commonly used inhibitors and have investigated the relationship between their inhibitory potency at the level of the enzyme and their acute glucose-lowering efficacy.

Results: Firstly, using a combination of published crystal structures and in-house data, we demonstrated that the binding site utilized by all of the DPP-4 inhibitors assessed was the same as that used by neuropeptide Y, supporting the hypothesis that DPP-4 inhibitors are able to competitively inhibit endogenous substrates for the enzyme. Secondly, we ascertained that the enzymatic cleft of DPP-4 is a relatively large cavity which displays conformational flexibility to accommodate structurally diverse inhibitor molecules. Finally, we found that for all inhibitors, irrespective of their chemical structure, the inhibition of plasma DPP-4 enzyme activity correlates directly with acute plasma glucose lowering in mice.

Conclusion: The common binding site utilized by different DPP-4 inhibitors enables similar competitive inhibition of the cleavage of the endogenous DPP-4 substrates. Furthermore, despite chemical diversity and a range of binding potencies observed amongst the DPP-4 inhibitors, a direct relationship between enzyme inhibition in the plasma and glucose lowering is evident in mice for each member of the classes studied.

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1 | INTRODUCTION

Dipeptidyl peptidase 4 (DPP-4) inhibitors are effective antihyperglycaemic agents by virtue of their ability to inhibit the breakdown of the active form of the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).[1] This results in increased plasma levels of the intact and, thus, biologically active form of both incretin hormones, which results in an improvement of glycaemic control primarily via augmentation of glucose-stimulated insulin secretion and inhibition of glucagon release by the beta and alpha cells of the pancreas, respectively.[2]

In the years since the initial proposal that pharmacological inhibition of DPP-4 might be an effective approach to the treatment of type 2 diabetes,[3] several structurally diverse DPP-4 inhibitors have become established therapeutically, the most widespread of which are sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin. Presently, no less than eleven DPP-4 inhibitors have been approved by regulatory authorities worldwide, although some with more limited geographical availability.[1]

Because the DPP-4 inhibitors are now an established and well-tolerated class of oral antihyperglycaemic agents, there has been increasing interest in understanding their precise mechanism of action including how the binding of the inhibitors to the target enzyme relates to glucose-lowering efficacy, and how this relationship compares between the different inhibitors. To date, however, few studies have been published in which the different DPP-4 inhibitors have been compared directly (ie, head to head), using the same experimental or analytical methods to compare their binding characteristics, enzymatic inhibitory properties and biological efficacy.[4] To address this, we undertook a direct comparison of five commonly used DPP-4 inhibitors (together with a pharmacologically active metabolite of one of them) to assess their molecular interactions with the DPP-4 enzyme in comparison with a known DPP-4 substrate (a fragment of neuropeptide Y, tNPY) and to examine their activity using standardized enzyme inhibition assays in vitro. We also used an acute oral glucose tolerance test (OGTT) in the mouse in vivo to enable a direct head-to-head comparison of the antihyperglycaemic and enzyme inhibitory properties of each inhibitor to be made. We used human recombinant DPP-4 for the X-ray crystallography and murine-based assays for measuring enzyme inhibition and antihyperglycaemic effects. To address some aspects of the potential impact of species differences, we compared the inhibitory potency of the inhibitors in assays using human- and murine-derived enzymes.

In a previous study, Nabeno et al[5] assigned some of the then existing DPP-4 inhibitors into three classes, based upon their binding to the core S1, S2 sites, to the S1' and S2' sites, and to a further "S2 extensive subsite" within the target enzyme. They suggested that differences in duration of action and selectivity for DPP-4 binding versus other related enzymes may result from differences in binding to these specific binding sites. However, it remained unknown as to whether such differences between the inhibitors with respect to their DPP-4 binding characteristics might result in differences in biological efficacy (ie, glucose lowering). As soluble DPP-4 circulates in plasma, measurement of murine plasma DPP-4 activity in treated animals provides an index of in vivo target engagement and acute pharmacodynamics for the various inhibitors and permits assessment of the correlation between the extent of plasma DPP-4 inhibition ex vivo and the corresponding glucose-lowering efficacy in vivo.

Of note, not all DPP-4 inhibitors are metabolically stable in vivo.[6] Both vildagliptin and saxagliptin are known to be metabolized extensively,[7,8] with saxagliptin forming an active metabolite (saxagliptin M2) in vivo.[8] Hence, we also included saxagliptin M2 for in vitro potency measurement and enzyme binding assessment.

2 | RESEARCH DESIGN AND METHODS

2.1 | Materials and methods

2.1.1 | Reagents

Alogliptin, linagliptin, saxagliptin, saxagliptin M2 (active metabolite), sitagliptin and vildagliptin were synthesized in house.

2.1.2 | Animals

All animal procedures described below were performed in accordance with the guidelines of the institutional animal care and use committee of Merck & Co., Inc., Kenilworth, NJ, USA. Male C57BL/6N mice (7-12 weeks of age, 19-26 grams) were purchased from Taconic Farms, Germantown, NY, USA, and were housed 10 per cage and given access to normal diet rodent chow (Teklad 7012) and water ad libitum.

2.1.3 | Structural studies

The soluble domain of human DPP-4 (residues 39-766) was prepared as described.[9] The structures for tNPY, sitagliptin, alogliptin, saxagliptin and linagliptin complexes with DPP-4 have been previously reported.[10-14] To obtain the structures of the corresponding vildagliptin and saxagliptin M2 metabolite complexes with DPP-4, crystals of human DPP-4 were initially obtained in hanging drops formed by mixing 1 μL of protein (10 mg/mL in 20 mmol/L TRIS, pH 8.0, 30 mmol/L NaCl) and 1 μL of precipitant solution

(18%-20% PEG3350, 270-300 mmol/L sodium acetate (NaAc), 100 mmol/L TRIS, pH 8.0). The drops were incubated at room temperature for 2 hours before being microseeded. The inhibitor complexes were formed by soaking the apo crystals overnight in a solution containing 0.5 mmol/L inhibitor in 22% PEG3350, 270-300 mmol/L NaAc, 100 mmol/L TRIS, pH 8.0. Crystals were quickly dipped in cryoprotectant solution (15% glycerol in 22% PEG3350, 270-300 mmol/L NaAc, 100 mmol/L TRIS, pH 8.0) before flash-freezing in liquid nitrogen. Data collections for all structures were carried out using the beamline 17-ID of the Industrial Macromolecular Crystallography Association (IMCA-CAT, Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA). Data were processed using autoPROC (Global Phasing Ltd., Cambridge, UK). The crystal structures were solved by difference Fourier procedures using the 1X70.pdb coordinate file, without insertion of any water molecules or ligand. The ligand was fitted to the available difference electron density using AFITT,[15] and the structures were refined against all available data using REFMA.[16] Coordinates have been deposited with the Protein Data Bank, accession code 6B1O and 6B1E for the saxagliptin M2 and the vildagliptin complex, respectively.

In vitro potency assays for inhibition of DPP-4

Briefly, human DPP-4 activity was assayed in vitro as described previously[16] in a continuous fluorescent (CF) assay in 100 mmol/L HEPES, pH 7.5, 0.1 mg/mL BSA using Gly-Pro-aminomethylcoumarin (AMC) as substrate, which is cleaved by DPP-4 to release the fluorescent AMC leaving group, at 50 μmol/L at 37°C for 30 minutes in the presence of a concentration range of each inhibitor using an excitation wavelength of 360 nm and an emission wavelength of 460 nm (Spectramax, Gemini Molecular Devices, Sunnyvale, CA, USA). Inhibitory potencies for murine DPP-4 were determined by measuring murine plasma DPP-4 activity in a CF assay containing 2% mouse plasma, 100 mmol/L HEPES, pH 7.5, 0.1% BSA and 5 μmol/L Gly-Pro-AMC as substrate at 37°C for 5 minutes (excitation/emission: 360/460 nm). The inhibitory potency (IC_{50}) was calculated for both human and mouse DPP-4 using a method described previously.[17] Summary statistics were calculated as the arithmetic mean and the standard deviation.

2.2 | Oral glucose tolerance test (OGTT) in lean mice

Male C57/Bl6N mice (n = 6-8/group) were randomly assigned to treatment groups and fasted overnight (~18-21 hours). Baseline (t = −60 minutes) blood glucose concentration was determined by glucometer (One Touch Ultra, Lifescan Inc, Wayne, PA, USA) from tail nick blood. Animals were treated orally by gavage with vehicle (0.25% methylcellulose, 5 mL/kg) or test compound at doses ranging from 0.01 to 3.0 mg/kg. Blood glucose concentration was measured 1 hour after treatment (t = 0 minutes) and was immediately followed by administration of dextrose (5 g/kg, 10 mL/kg p.o. by gavage). One group of vehicle-treated mice was challenged with water (instead of dextrose) as a control. Blood glucose levels were determined from tail

bleeds taken at 20, 40, 60 and 120 minutes after dextrose challenge. The blood glucose excursion profile from t = 0 to t = 120 minutes was used to integrate an area under the curve (AUC) and to calculate an average plasma glucose excursion (Cav = AUC/120 minutes) for each treatment.

2.3 | Measurement of murine plasma DPP-4 activity ex vivo

Male C57/Bl6N mice (n = 20-28/group) were randomly assigned to treatment or vehicle groups and fasted overnight (~18-21 hours). Animals were treated orally (gavage) with vehicle (0.25% methylcellulose, 5 mL/kg) or test inhibitors at t = −60 minutes. At t = 0, mice received dextrose (5 g/kg, 10 mL/kg p.o. by gavage). One group of vehicle-treated mice was challenged with water (instead of dextrose) as a control. Ten minutes after dextrose challenge (70 minutes after inhibitor dose), mice were killed and terminal blood samples were collected by cardiocentesis for measurement of plasma DPP-4 inhibition ex vivo. Blood samples were collected into EDTA tubes, and the plasma was prepared by centrifugation. Aliquots of plasma were stored at −70°C until analysis.

Plasma murine DPP-4 activity was measured using a CF assay with the substrate Gly-Pro-AMC. The assay media contained 50% plasma, 50 μmol/L Gly-Pro-AMC and buffer (100 mmol/L HEPES, pH 7.5, 0.1 mg/mL BSA) in a total reaction volume of 50 to 70 μL (depending on the availability of sample). Release of AMC was monitored continuously in a 96-well plate fluorometer (excitation/emission: 360/460 nm; Spectramax, Gemini Molecular Devices). Due to blood volume limitations, baseline (predose) samples were not collected. Therefore, plasma DPP-4 inhibition was calculated as the percentage of DPP-4 activity postdose for each sample relative to the arithmetic mean plasma DPP-4 activity of the vehicle group. Summary statistics were calculated as the arithmetic mean and standard deviation of the percentage DPP-4 activity for each dose group.

2.4 | Relationship between plasma DPP-4 inhibition ex vivo and acute glucose lowering (OGTT) in lean mice

Briefly, a linear mixed-effects mathematical model was developed relating the observed plasma DPP-4 inhibition data collected 70 minutes postdose (10 minutes after dextrose challenge) to the observed baseline average plasma glucose excursion (Cav) OGTT data in the parallel cohort for the pooled data set of all five DPP-4 inhibitors. To determine whether the relationship between acute pharmacodynamics and efficacy might substantially vary by inhibitor, an additional factor for specific inhibitor was also tested in the mixed-effects model for statistical significance (prespecified P value: .05), using Kenward and Roger's method for calculating degrees of freedom. [This analysis was conducted using R v.3.2.3[18] with packages lme4[19] and pbkrtest[20] for model development and testing for significance, and hmisc for plots.[21]]

3 | RESULTS

3.1 | Comparison of DPP-4 inhibitor binding interactions and inhibitory potencies

Figure 1 shows the reported crystal structure of the tNPY substrate fragment bound to DPP-4 (PDB accession code 1R9N; only the first 6 of the 10 amino acid residues are visible in the structure). DPP-4 specifically cleaves dipeptides from the *N*-terminus of peptide substrates that typically have a proline (Pro) or an alanine (Ala) residue in the penultimate position (P1 position, residue Pro2 for this peptide). The substrate P1 residue is bound in the protein S1 site, a hydrophobic pocket near the catalytic Ser630, the small size of which dictates the need for a small amino acid (Ala or Pro) in this position. The *N*-terminus of the peptide substrate (P2 position, residue Tyr1 in this case) is recognized and anchored by interactions with two acidic residues, Glu205 and Glu206, that are part of the protein S2 pocket; this pocket is mostly hydrophobic and includes the side chains of Arg125, Phe357 and Tyr547. Arg125 makes specific hydrogen bond interactions with the carbonyl oxygen atoms of the substrate Tyr1 and Ser3 (P1' residue), and it has been proposed to be responsible for fixing the conformation of the peptide within the catalytic site.[22] The S1' pocket is flat and not very well defined, and in the tNPY structure, the interactions between the P1' residue Ser3 and the S1' residues are mostly nonspecific Van der Waals interactions. The DPP-4 S2' pocket contains a Trp629 residue which forms a hydrophobic wall for the lipophilic P2' residue (Lys4) side chain to interact with.

Figure 2 compares the binding interactions of the assessed commercially available DPP-4 inhibitors with the human enzyme (the associated protein data bank (PDB) accession code is reported under the figure). While there are some differences between the inhibitors in terms of binding potency, both to human DPP-4 and to the highly homologous murine enzyme (Table 1), they all bind essentially in the same catalytic site, thus behaving as substrate competitive inhibitors. All six inhibitors have small hydrophobic moieties that occupy
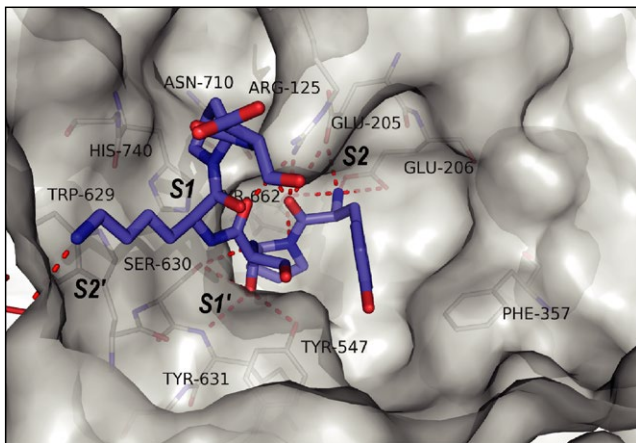
FIGURE 1 Binding of tNPY to human DPP-4 (PDB accession code 1R9N); all the residues relevant to substrate binding and catalysis are labelled, as well as the subsites

the S1 pocket and hydrophilic groups that engage the primary residues involved in substrate recognition and binding, namely the side chains of Glu205, Glu206 and Arg125. Specific interactions with the other regions of the binding pocket vary for the different inhibitors: Sitagliptin[10] (Figure 2A) extends towards Phe357, into a novel pocket formed by a major movement of the side chain of Arg358, the S2 extensive site proposed by Nabeno et al[5] Alogliptin[11] (Figure 2B) interacts mainly with residues in the S1 and S2 pockets. Linagliptin[12] (Figure 2C) extends towards the S2' pocket and engages the side chain of Trp629 through its quinazoline moiety. Saxagliptin[12] (Figure 2D), saxagliptin M2 (Figure 2E) and vildagliptin[22] (Figure 2F) are reversible covalent inhibitors that occupy the S2 pocket.

3.2 | In vitro and ex vivo potencies of DPP-4 inhibitors

The inhibitory activities of the different compounds were determined against both human and murine DPP-4 in vitro and are shown in Table 1. As shown, the inhibitory potency (IC_{50}) was highly consistent between species and spanned a range in human from 0.14 nmol/L for linagliptin to 34 nmol/L for vildagliptin.

3.3 | Comparison of acute antihyperglycaemic activity in lean mice

All of the compounds dose-dependently inhibited plasma DPP-4 activity (Figure 3) with maximal plasma DPP-4 inhibition levels of ~80%-95% observed (uncorrected for dilution of the plasma samples in the DPP-4 activity assay). Associated with the dose-dependent inhibition of DPP-4 activity in treated mice, the average plasma glucose excursion during the OGTT was reduced in a dose-dependent manner, with maximal reduction of >50% compared to the vehicle group.

The results of the linear mixed-effects analysis relating plasma DPP-4 inhibition to acute glucose lowering (Cav) indicate that the amount of glucose lowering could be well described based on the degree of DPP-4 inhibition, and this glucose-lowering effect did not significantly vary by inhibitor (*P*-value: >.3). A calculated slope for the Cav versus DPP-4 inhibition correlation plots of −0.73 (SE: 0.05), indicated that for all DPP-4 inhibitors tested, each incremental 10% of plasma DPP-4 inhibition approximately lowered the average plasma glucose excursion in the OGTT by 7.3 mg/dL irrespective of the structural diversity of the inhibitors (Figure 4).

4 | DISCUSSION

The present study is a direct comparison of the acute pharmacological profile and the binding/inhibitory properties of various DPP-4 inhibitors, assessed using the same assays and analytical methods, thereby allowing for a more accurate comparison than comparing data for the various inhibitors from different reported studies, which typically involved differing methodologies. The fact that NPY, a known biochemical substrate for DPP-4, binds in the same site as the inhibitor
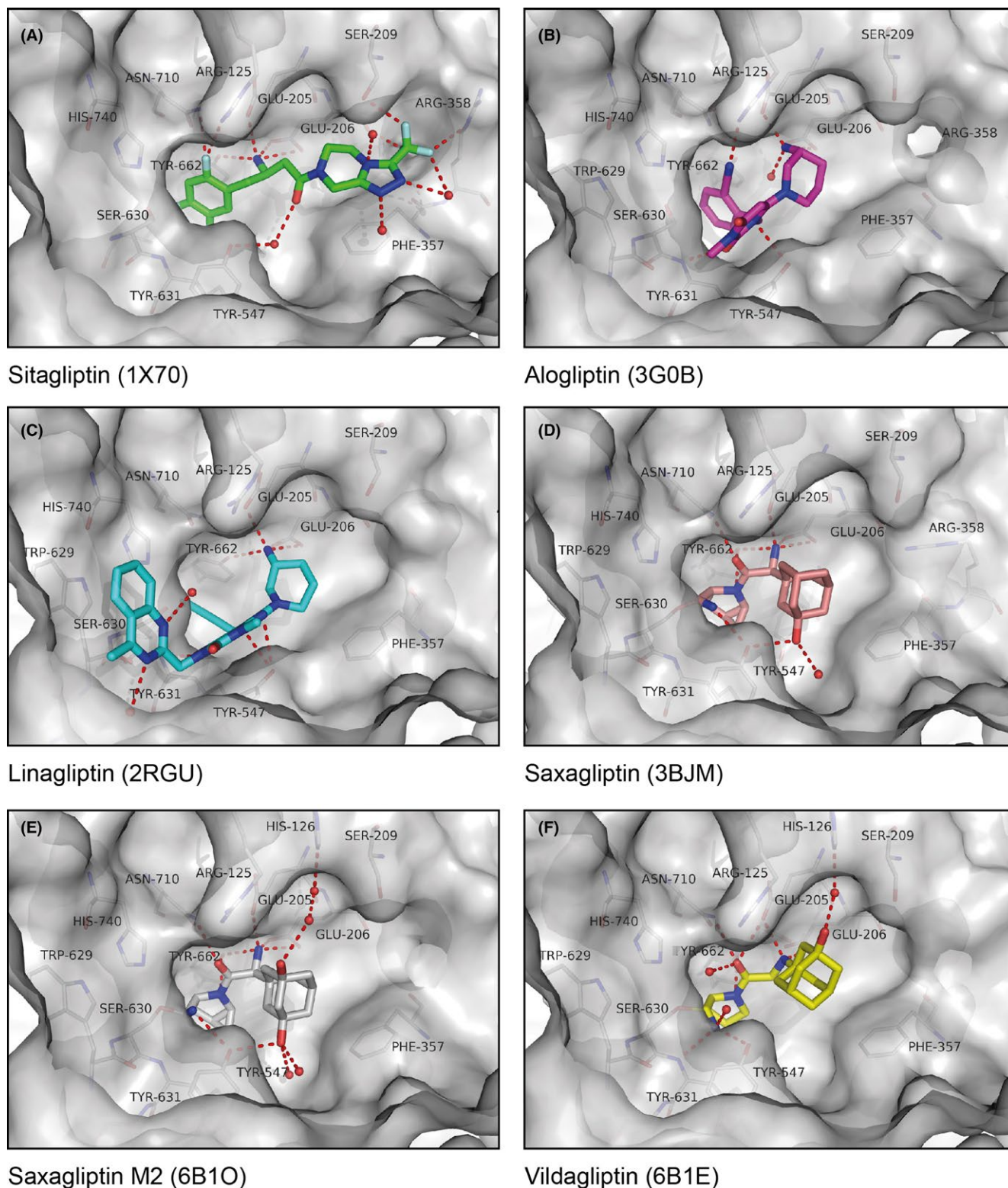
FIGURE 2 Binding sites in human DPP-4 for the six inhibitors described in the text. The corresponding PDB codes are reported in parentheses. (A) Sitagliptin, (B) Alogliptin, (C) Linagliptin, (D) Saxagliptin, (E) Saxagliptin M2, and (F) Vildagliptin

molecules is supportive of the notion that these inhibitors act as competitive inhibitors of physiologically relevant substrates (Figure 1). To date, however, the site of binding of other relevant substrates, such as glucagon-like peptide 1, remains to be characterized, although we conjecture that such physiologically relevant substrates are likely to bind to the same site. The differences between biochemical substrates and physiologically relevant substrates (in terms of glucose lowering) have been reviewed.[23]

From the X-ray crystallography investigations, we observed that although the location of binding was broadly similar for each of the

TABLE 1 Inhibitory potencies of different DPP-4 inhibitors for human and murine DPP-4

	Mean IC$_{50}$, nmol/L (SD)					
	Sitagliptin	**Vildagliptin**	**Saxagliptin**	**Saxagliptin M2**	**Alogliptin**	**Linagliptin**
Human DPP-4	18 (5.8)	34 (7.7)	1.5 (0.1)	3.1 (0.3)	7.5 (0.4)	0.14 (0.13 - 0.14)[a]
Murine DPP-4	20 (19 - 21)[a]	26 (8.4)	1.9 (0.7)	3.9 (3.7 - 4.0)[a]	8.6 (2.2)	0.12 (0.12 - 0.12)[a]

[a]Data are limited to N = 2 replicates; range is shown instead of standard deviation.
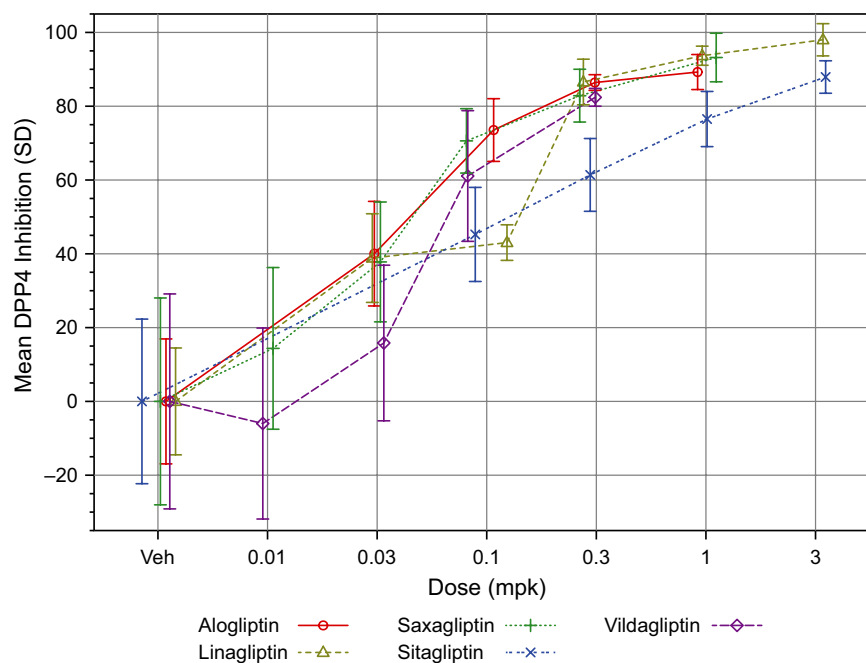
FIGURE 3 Observed mean (SD) DPP-4 inhibition in C57/Bl6N mouse plasma 70 min after oral dose (10 min post-OGTT) for alogliptin, linagliptin, saxagliptin, sitagliptin and vildagliptin

inhibitors tested in that they all occupy the S1 and S2 sites of the enzymatic cleft, some differences were apparent. All inhibitors bound either directly or through a water molecule to the hydroxyl moiety of Tyr547. Conversely, they showed somewhat different interactions with other areas of the binding site (Figure 2), reflecting the fact that the DPP-4 binding pocket is a fairly large cavity characterized by a certain degree of flexibility. The overall similarity of binding to the DPP-4 active site by the different inhibitors might be associated, therefore, with their similar resultant acute pharmacology as antihyperglycaemic agents, albeit with differing inhibitory potencies, both in vitro and ex vivo, as illustrated in the present study. The use of human enzyme for the binding studies and murine-based assays for the in vivo work is justified due to the highly homologous human and murine structures of the DPP-4 enzyme and the similar potencies of the various DPP-4 inhibitors using assays from the two species.[24]

The inhibitory potency data shown in Table 1 illustrate that the relatively high degree of sequence identity (85%) and homology (92%) are reflected by broadly similar potency values when comparing the human and murine enzyme. Regarding the values of potency per se, the results obtained in this study are broadly in agreement with values cited elsewhere.[6] In this context, it is also important to note that despite the observed differences in inhibitory potencies across the

chemically diverse inhibitors in vitro (Table 1), a consistent relationship was found between the degree of inhibition of plasma DPP-4 and the extent of glucose lowering with each inhibitor (Figure 4). It would appear then that, if there are indeed three different classes of DPP-4 inhibitor in terms of binding, as suggested by Nabeno et al,[5] these differences do not seem to be of biological significance in terms of the relationship between plasma DPP-4 inhibition and acute glucose lowering in mice. Nevertheless, it seems plausible that the number of binding sites utilized by the various inhibitors could be a factor in determining selectivity between DPP-4 and other related enzymes, simply because the more the binding sites there are within the target enzyme, the less is the likelihood that similar interactions will also exist in other enzymes. The classifications may, therefore, still be relevant in defining the differences in both the inhibitors' potencies in vitro and their selectivity between different enzymes (IC$_{50}$ differences between inhibition of DPP-4 vs other enzymes (eg, DPP8 and DPP9)).[6]

The correlation between plasma DPP-4 inhibition and acute glucose lowering is a significant observation, which is supportive of the hypothesis that plasma DPP-4 inhibition is a predictive biomarker for acute glucose-lowering efficacy, irrespective of where DPP4-mediated degradation of endogenous GLP-1, GIP and other substrates actually occurs, or of any differences in the nature of
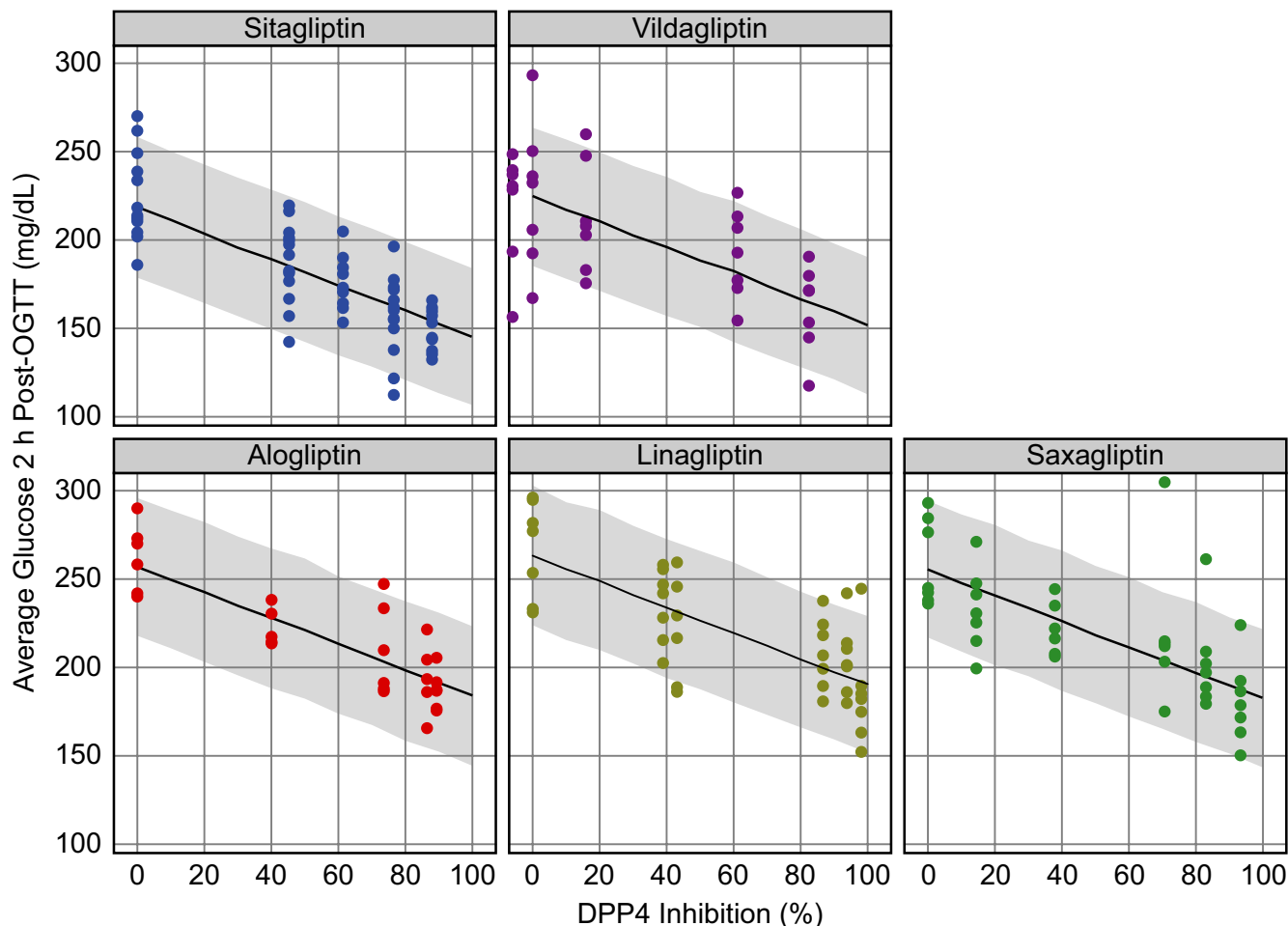
FIGURE 4 Observed 2 hours post-OGTT average plasma glucose vs. plasma DPP-4 inhibition 10 min post-OGTT in C57/Bl6N mice, with model line and 90% prediction interval

inhibitor binding (including differences in binding to the different subsites within the enzymatic cleft or differences in covalent/non-covalent binding properties). While there were no significant differences in the relationship between acute glucose lowering and plasma DPP-4 inhibition for the DPP-4 inhibitors investigated in our study, the methods used were not designed to rule out potentially small differences between these agents. The observation that different DPP4 inhibitors have similar glucose-lowering efficacy in the clinic has been reported in the literature (eg, Park et al[25]) although there is a paucity of directly comparative studies. Moreover, despite the fact that soluble DPP-4 in mixed venous plasma comprises only a small part of the total pool of DPP-4 in the body, the majority being membrane bound, the extent of inhibition of the plasma enzyme does appear to predict glucose-lowering activity well in mice as well as in humans.[26]

In conclusion, our studies show that DPP-4 inhibitors comprise a structurally heterogeneous family of oral antihyperglycaemic agents that share common binding interactions with the enzyme. The finding that structurally diverse DPP-4 inhibitors bind in the same site as the fragment of a biochemically relevant substrate, NPY, supports the notion that the inhibitors act as competitive inhibitors of substrate

binding. Finally, plasma DPP-4 inhibition would appear to be a convenient and accurate biomarker for blood glucose-lowering efficacy in mice, just as it is in humans.[27]

CONFLICT OF INTEREST

All authors are or were employees of MSD A/S or Merck Sharp & Dohme Corp., subsidiaries of Merck & Co., Inc., Kenilworth, NJ, USA

AUTHOR CONTRIBUTIONS

All of the authors are responsible for the work described in this manuscript. JPB, RS, AP, GJE, SSX, AEW and NAT conceived, designed and/or planned the study. JPB, AP, TMK, GS, KADP, JKW, GJE and XZ acquired the data. JPB, RS, TMK, GS, Y-DG, KADP, GJE, DAT and RDC analysed the data. JPB, RS, AP, TMK, Y-DG, GJE, DAT and RDC interpreted the results. JPB, RS, KADP, JKW, GJE, XZ, DAT and RDC drafted the manuscript. JPB, RS, AP, TMK, GS, Y-DG, GJE, SSX, DAT, AEW, NAT and RDC critically reviewed and/or revised the manuscript for important intellectual content. All authors provided final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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