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Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin in vitro and ex vivo when compared to a rapidly-dissociating DPP4 inhibitor

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Abstract

Background: Dipeptidylpeptidase 4 (DPP4) inhibitors have clinical benefit in patients with type 2 diabetes mellitus by increasing levels of glucose-lowering incretin hormones, such as glucagon-like peptide -1 (GLP-1), a peptide with a short half life that is secreted for approximately 1 hour following a meal. Since drugs with prolonged binding to their target have been shown to maximize pharmacodynamic effects while minimizing drug levels, we developed a time-dependent inhibitor that has a half-life for dissociation from DPP4 close to the duration of the first phase of GLP-1 release.

Results: Saxagliptin and its active metabolite (5-hydroxysaxagliptin) are potent inhibitors of human DPP4 with prolonged dissociation from its active site (Ki = 1.3 nM and 2.6 nM, $t_{1/2}$ = 50 and 23 minutes respectively at 37°C). In comparison, both vildagliptin (3.5 minutes) and sitagliptin (< 2 minutes) rapidly dissociated from DPP4 at 37°C. Saxagliptin and 5-hydroxysaxagliptin are selective for inhibition of DPP4 versus other DPP family members and a large panel of other proteases, and have similar potency and efficacy across multiple species.
Inhibition of plasma DPP activity is used as a biomarker in animal models and clinical trials. However, most DPP4 inhibitors are competitive with substrate and rapidly dissociate from DPP4; therefore, the type of substrate, volume of addition and final concentration of substrate in these assays can change measured inhibition. We show that unlike a rapidly dissociating DPP4 inhibitor, inhibition of plasma DPP activity by saxagliptin and 5-hydroxysaxagliptin in an ex vivo assay was not dependent on substrate concentration when substrate was added rapidly because saxagliptin and 5-hydroxysaxagliptin dissociate slowly from DPP4, once bound. We also show that substrate concentration was important for rapidly dissociating DPP4 inhibitors.

Conclusions: Saxagliptin and its active metabolite are potent, selective inhibitors of DPP4, with prolonged dissociation from its active site. They also demonstrate prolonged inhibition of plasma DPP4 ex vivo in animal models, which implies that saxagliptin and 5-hydroxysaxagliptin would continue to inhibit DPP4 during rapid increases in substrates in vivo.

Background

Diabetes is a worldwide epidemic, with the World Health organization estimating that more than 220 million people have diabetes worldwide http://www.who.int/mediacentre/factsheets/fs312/en/index.html, with greater than 90% of those having type 2 diabetes mellitus (T2DM). T2DM is thought to develop as a combination of insulin resistance and pancreatic β-cell failure [1]. Therefore, identification of novel treatments that would increase pancreatic insulin secretion while protecting pancreatic β-cells are of great interest.

Incretin hormones, such as glucagon-like peptide-1 (GLP-1), are secreted from cells in the gastrointestinal (GI) tract into the circulation in response to nutrient

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absorption. They are a major component of the mechanism regulating post-prandial insulin secretion when it is needed following meals [2]. Incretins account for up to 60% of the post-prandial insulin secretion in healthy individuals, but the incretin response is impaired in T2DM [3]. Incretin effects do not lead to insulin release per se, but potentiate the physiological release of insulin from the pancreas in response to increases in plasma glucose. Since GLP-1 has been shown to have the major incretin effect on glucose homeostasis in patients with type 2 diabetes [4], much work has been done to understand the effects of this incretin hormone on normal and pathophysiological glucose homeostasis.

Following its secretion, dipeptidylpeptidase-4 (DPP4) rapidly metabolizes the intact form of GLP-1 (GLP-1_{7-36}) to inactive GLP-1_{9-36} with a half-life of 1 to 2 minutes in vivo [5]. Therefore, two approaches have been taken to increase activity of the incretin axis, parenteral administration of DPP4-resistant GLP-1 analogues or oral administration of DPP4 inhibitors. DPP4 inhibitors have minimal risk of hypoglycemia because they enhance glucose-dependent insulin secretion and glucagon reduction. They are also weight neutral; i.e., they do not promote weight gain that is typically seen with many other antidiabetic agents. DPP4 inhibitors are also effective in combination with several other diabetes drug classes [6-8]. Finally, data from animal models indicate that GLP-1 is a trophic factor for β-cells, and potentiating endogenous incretins with DPP4 inhibitors does increase β-cell function and number, thereby contributing to improvement of β-cell function over the long-term [9].

There are many examples of enzyme inhibitors displaying time-dependence (e.g. [10,11]), with several becoming marketed drugs, including members of the DPP4 inhibitor class [12-14]. In many cases, prolonged pharmacodynamic effects on the target enzyme (when compared to the pharmacokinetics of the drug) confers an advantage over rapidly dissociating compounds, because time-dependent drugs typically require lower plasma levels and reduced drug peak-to-trough ratios, reducing the risk of off-target toxicity [11,15]. In humans, peak GLP-1 secretion occurs during the first phase of secretion, which occurs rapidly following a meal, giving a 2- to 3-fold increase that lasts 30 to 60 minutes [3]. This can be followed by a prolonged phase that gives a small increase in GLP-1 levels above fasting levels for up to 2 hours [reviewed in [16]. Therefore, we hypothesized that if a time-dependent inhibitor has a half-life for dissociation close to the duration of the first phase of GLP-1 secretion, the majority of the enzyme-inhibitor complex would not dissociate during the release of GLP-1 and this would maximize the compound's beneficial effects while minimizing plasma drug levels. DPP4 also has many other substrates in vitro, although only a few have been shown to be cleaved by DPP4 in vivo (reviewed in [17]). Therefore, it would be ideal if binding did not extend past the duration of first phase GLP-1 secretion, such that the inhibitor activity would follow its pharmacokinetics for inhibition of cleavage of other substrates of DPP4, should any such substrates have more prolonged in vivo half-lives relative to GLP-1.

Here we describe the inhibitory properties of saxagliptin and its 5-hydroxy metabolite, which are both slow binding DPP4 inhibitors with extended off-rates from DPP4 at 37°C, similar to the duration of the first phase of release of GLP-1 in vivo. We also use the ex vivo plasma DPP assays that are used in preclinical animal models and the clinic as a biomarker for efficacy, to demonstrate how slow binding compounds such as saxagliptin differ from rapidly dissociating DPP4 inhibitors. We show that saxagliptin does not have a dilution artifact or a large dependence on the pseudo-substrate used in the assay, unlike rapidly dissociating DPP4 inhibitors, and we discuss the significance of these findings.

Results

Saxagliptin is a potent inhibitor of human DPP4 in vitro irrespective of substrate

We measured the IC_{50} for inhibition of substrates across a range of substrate concentrations (10 μM to 1000 μM, dependent on substrate) that straddled the Km for the pseudo-substrate gly-pro-pNA (Km 180 ± 8 μM, kcat 40 ± 9 s^{-1}, room temp, n = 3), and GLP-1 (Km 24 ± 16 μM, kcat 2.9 ± 0.9 s^{-1}; room temp, n = 5), then calculated the Ki for inhibition of cleavage by each substrate (Table 1).

Each of the DPP4 inhibitors tested were equipotent inhibitors of GLP-1 and gly-pro-pNA, as expected for inhibitors that are competitive with substrate and bind in the active site of DPP4. Therefore, we used gly-pro-pNA as a substrate in subsequent experiments. Saxagliptin was approximately 10-fold more potent than vildagliptin or sitagliptin under these conditions at room temperature.

Potency and selectivity of saxagliptin and 5-hydroxysaxagliptin for human enzymes in vitro at 37°C

Routine screening was performed at room temperature. However, as DPP4 inhibition in vivo occurs at 37°C, we measured the potency and selectivity of DPP4 inhibitors in vitro at that temperature (Table 2). The Km and turnover rate of gly-pro-pNA pseudo-substrate for DPP4 increased (Km = 209 ± 18 μM; kcat = 67 ± 4 s^{-1}, n = 3), as did the Ki values for inhibition of DPP4 by DPP4 inhibitors (Table 2). Saxagliptin was 10-fold more potent than either vildagliptin or sitagliptin at 37°C. Saxagliptin generates an active metabolite in vivo [18], 5-hydroxysaxagliptin; it was 2-fold less potent than saxagliptin.

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Table 1 Inhibition of isolated cloned human DPP4 at room temperature

compound	GLP-1 K_i (nM)	gly-pro-pNA K_i (nM)
Saxagliptin	0.41 ± 0.1 (7)	0.45 ± 0.1 (5)
Sitagliptin	2.5 ± 0.7 (4) ***	8 ± 1 (5)***
Vildagliptin	1.5 ± 0.5 (4) ***	7 ± 2 (5)***

mean ± standard deviation (number of independent experiments). ***P < 0.001 versus saxagliptin

The gly-pro-pNA pseudo-substrate is not specific to DPP4 and is also cleaved by other enzymes, including DPP8 (Km = 792 ± 60 μM; kcat = 5.1 ± 0.4 s^{-1}, n = 3) and DPP9 (Km = 221 ± 27 μM; kcat = 3.7 ± 0.7 s^{-1}, n = 3), although the cleavage rate of both enzymes is 20-fold lower than DPP4. Therefore, we also used this substrate to investigate inhibition of DPP8 and DPP9 by DPP4 inhibitors. Saxagliptin is approximately 400-fold selective and 75-fold selective for DPP4 versus DPP8 and DPP9, respectively, with the 5-hydroxymetabolite having approximately twice the selectivity (DPP8 approximately 950-fold and DPP9 approximately 160-fold). In comparison, vildagliptin had 400-fold selectivity for DPP8 and 20-fold selectivity for DPP9, while sitagliptin had 1900-fold selectivity for DPP8 and 3000-fold selectivity for DPP9.

Saxagliptin and 5-hydroxysaxagliptin were tested against multiple other enzymes (at room temperature). Both compounds had > 1000-fold selectivity against FAP, and > 6000-fold selectivity against DPP2 and all other proteases tested: these included neutral endopeptidase, angiotensin converting enzyme, aminopeptidase P, prolidase, prolyl carboxypeptidase, activated protein C, chymotrypsin, factor IXa, Factor VIIa, Factor Xa, Factor XIa, factor XIIa, plasma kallikrein, plasmin, thrombin, tissue kallikrein, tissue plasminogen activator, trypsin and urokinase (data not shown). They also had > 10,000-fold selectivity against a panel of 39 unrelated proteins that included 15 G-protein coupled receptors, 4 nuclear hormone receptors, 6 ion channels, 4 other enzymes and 10 transporters (data not shown).

Potency and selectivity of saxagliptin and 5-hydroxysaxagliptin for inhibition of cynomolgus monkey DPP enzymes in vitro at 37°C

The potency and selectivity for all 4 compounds for inhibition of cynomolgus monkey (rhesus monkey has the same DPP4 DNA sequence) DPP4, DPP8 and DPP9 is shown in Table 3 and is very similar to that found versus the human enzymes.

Similar data were also obtained for mouse and rat enzymes (data not shown). Therefore, we confirmed that the potency and specificity of saxagliptin and its 5-hydroxymetabolite were similar across species in vitro. We did not investigate the effects of DPP4 inhibitors on other peptidases from other species because no effect of saxagliptin and 5-hydroxysaxagliptin were seen on the human proteins tested.

Saxagliptin and 5-hydroxysaxagliptin are long-acting DPP4 inhibitors in vitro

During the course of initial experiments, we noticed that there was time dependence to inhibition of DPP4 by some DPP4 inhibitors. In order to determine time-dependence, we preincubated DPP4 inhibitors with DPP4 and measured the rate of dissociation of DPP4 inhibitors from DPP4 using an 'infinite dilution' method.

The data in table 4 show that saxagliptin and 5-hydroxysaxagliptin have slow binding when tested at 37°C, with $t_{1/2}$ for dissociation of 50 minutes and 23 minutes, respectively. While vildagliptin shows some evidence of slow binding ($t_{1/2}$ = 3.5 minutes), this was much less pronounced. Sitagliptin showed no time dependence (within the limitations of the experimental protocol at < 2 minutes). The time dependence was only found for inhibition of DPP4 and was not seen during experiments investigating the inhibition of DPP8 and DPP9; therefore, these prolonged effects would only relate to inhibition of cleavage of DPP4 substrates by DPP4.

Saxagliptin does not have a dilution artifact in plasma DPP assays in vitro

The ex vivo assay measuring inhibition of plasma DPP4 activity has been used as a key biomarker assay for DPP4 inhibitor assessment by multiple groups in both animal models and in the clinic. Given that the duration of the ex vivo assay is typically between 10 and 20 minutes, there would be no dilution artifact predicted for ex vivo determination of inhibition from saxagliptin dosed animals, because negligible dissociation of saxagliptin from DPP4 would occur over the time frame of the experiment (Table 4 and the rate of dissociation is

Table 2 Inhibition of isolated, cloned human DPP4, DPP8 and DPP9 at 37°C

	DPP4 K_i (nM)	DPP8 K_i (nM)	DPP9 K_i (nM)
Saxagliptin	1.3 ± 0.3 (12)	508 ± 174 (13)	98 ± 44 (11)
5-hydroxysaxagliptin	2.6 ± 1.0 (12)***	2495 ± 727 (14)*	423 ± 64 (12)
Vildagliptin	13 ± 2.8 (12)***	5218 ± 2319 (14)***	258 ± 93 (12)
Sitagliptin	18 ± 1.6 (12)***	33780 ± 5532 (12)***	55142 ± 19414 (11)***

mean ± standard deviation (number of independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 versus saxagliptin

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Table 3 Inhibition of isolated, cloned cynomolgus monkey DPP4, DPP8 and DPP9 at 37°C

	DPP4 K_i (nM)	DPP8 K_i (nM)	DPP9 K_i (nM)
Saxagliptin	1.1 ± 0.2 (14)	390 ± 82 (6)	61 ± 5 (6)
5-hydroxysaxagliptin	2.9 ± 1.1 (13)***	2061 ± 658 (6)***	323 ± 60 (6)*
Vildagliptin	6.8 ± 2.0 (14)***	3692 ± 917 (7)***	125 ± 39 (7)***
Sitagliptin	15.6 ± 3.6 (14)***	21949 ± 17461 (6)***	65757 ± 7966 (6)***

mean ± standard deviation (number of independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 versus saxagliptin

decreased with lower temperature). To confirm this for saxagliptin, we took naive human plasma and compared samples with just the addition of compound to plasma only, to samples where compound was added to both plasma and buffer (such that total concentration of drug was kept constant during the 'dilution step').

Saxagliptin is unaffected by the 3-fold dilution in the human plasma assay (Figure 1a) using ala-pro-AFC as substrate. However, sitagliptin clearly has a dilution artifact (Figure 1b). When sitagliptin was added only to the plasma, the inhibition curve shifted 3-fold to the right compared to when compound was added to both the plasma and the dilution buffer (IC_{50} = 152 ± 41 nM versus 414 ± 116 nM: mean ± s.d., n = 3), except where there is virtually no inhibition or full inhibition, consistent with the 3-fold dilution during substrate addition. This is presumably due to a new equilibrium being rapidly established following dilution, such that the potency of sitagliptin will be underestimated when compound is only present in the plasma. Similar data were also obtained using both cynomolgus and rhesus monkey plasma (data not shown).

Maximum inhibition of plasma DPP4 activity in plasma samples by DPP4 inhibitors differs between species

Untreated human plasma samples gave a plasma DPP enzyme activity rate of 5.0 ± 0.6 nmoles/min per ml plasma (mean ± s.d., n = 3 independent experiments) when ala-pro-AFC was used as substrate. Untreated cynomolgus and rhesus monkey plasma DPP4 rates were similar to those seen in human, with rates of 5.2 ± 0.3 and 7.3 ± 0.2 nmoles/min per ml plasma, respectively. However, the ability of DPP4 inhibitors to inhibit cleaveage of peudo-substrates differs among species. Figure 2 shows that the maximum inhibition of plasma DPP activity seen with saxagliptin was approximately 85% in rhesus and 80% in cynomolgus monkeys, but > 95% in humans (Figure 2). Like human, rodent (mouse and rat) and dog plasma DPP is inhibited > 95% by saxagliptin (data not shown).

Table 4 On and off rates of DPP4 inhibitors at 37°C

Compound	kon, 10^5 M^{-1} s^{-1}	koff, 10^{-5} s^{-1}	$t_{1/2}$ (min.)
	37°C		
Saxagliptin	4.6 ± 0.6	23 ± 1	50
5-hydroxysaxagliptin	0.7 ± 0.1	50 ± 2	23
Vildagliptin	1.2 ± 0.2	330 ± 30	3.5
Sitagliptin	> 100	> 580	< 2

mean ± standard error. Standard errors for kon were calculated from equations (2), (3) and (4), and for koff are from the fits to equation (5)

These effects were shown to be independent of DPP4 inhibitor and similar data were obtained with gly-pro-pNA as the pseudo-substrate (Table 5; Figure 3). Since the pseudo-substrates are not specific for DPP4, presumably these findings reflect a species difference in the relative activity of all the other plasma peptidases that cleave these pseudo-substrates.

The in vitro IC_{50} for inhibition of human, rhesus monkey and cynomolgus monkey plasma DPP activity by saxagliptin, vildagliptin and sitagliptin was similar across species (Table 5). Therefore, although there were different amounts of maximal inhibition among species, DPP4 inhibitors have similar potency in rhesus monkey, cynomolgus monkey and human plasma for inhibition of plasma DPP activity.

Choice of assay affects the IC_{50} measured for inhibition of plasma DPP activity by DPP4 inhibitors at steady-state in vitro

The measured IC_{50} varies with the ratio of substrate concentration to substrate Km for competitive inhibitors (see Methods). Using the assays described here, Km values were calculated as 57 ± 13 μM (n = 3 independent experiments) for the ala-pro-AFC assay, and 180 ± 10 μM (n = 3) for gly-pro-pNA assay in human plasma. Km values were similar in cynomolgus monkey plasma, at 35 ± 6 μM (n = 3) for ala-pro-AFC assay and 134 ± 5 μM (n = 3) for gly-pro-pNA assay. Since the majority of DPP4 inhibitors are competitive with substrate, a difference in substrate concentration will affect the measured IC_{50} of these inhibitors. In the two pseudo-substrate assays we used to measure inhibition of plasma DPP activity, the ratio of Km to substrate concentration is approximately 7-fold in the ala-pro-AFC assay (370 μM substrate concentration), but is only 2-fold in the gly-pro-pNA assay (400 μM substrate concentration). Therefore, using gly-pro-pNA as substrate would give an apparent increase in potency of DPP4 inhibitors compared to ala-pro-AFC. Further, the difference in temperature (30°C versus room temperature) and pH (7.4

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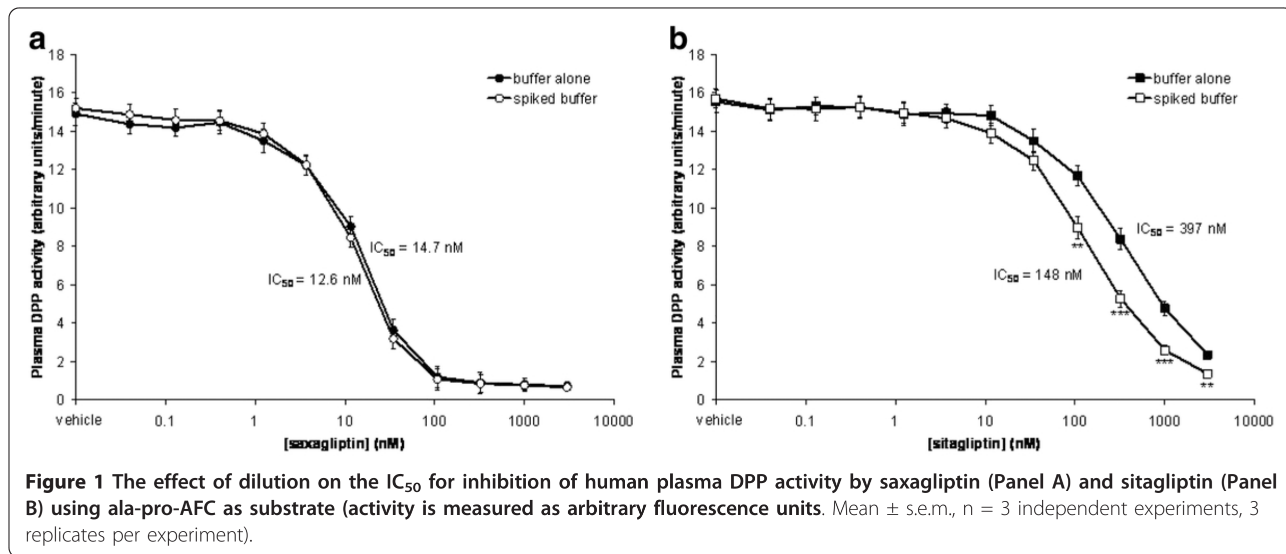

Figure 1 The effect of dilution on the IC_{50} for inhibition of human plasma DPP activity by saxagliptin (Panel A) and sitagliptin (Panel B) using ala-pro-AFC as substrate (activity is measured as arbitrary fluorescence units. Mean ± s.e.m., n = 3 independent experiments, 3 replicates per experiment).

versus 7.9) of the two assays would also affect the measured IC_{50}. Figure 3 shows the inhibition of cynomolgus monkey plasma DPP activity in vitro for saxagliptin and sitagliptin under pseudo-steady-state conditions. There was a small change in IC_{50} for saxagliptin (2.5 ± 0.2 nM to 9.8 ± 0.3 nM, $P < 0.0001$), with a narrow concentration range over which a difference would be seen between the two assays (1 to 10 nM. Figure 3A). This presumably reflects differences in temperature and pH between the two assays. However, at concentrations of sitagliptin between 5 and > 3000 nM, much more inhibition of DPP activity is seen with the gly-pro-pNA assay than with the ala-pro-AFC assay (Figure 3B). Further, the IC_{50} for inhibition in the ala-pro-AFC assay was significantly increased 26-fold compared to the gly-pro-pNA assay, from 17 ± 2 nM to 440 ± 163 nM ($P < 0.0001$). Similar data were obtained using human plasma (data not shown).

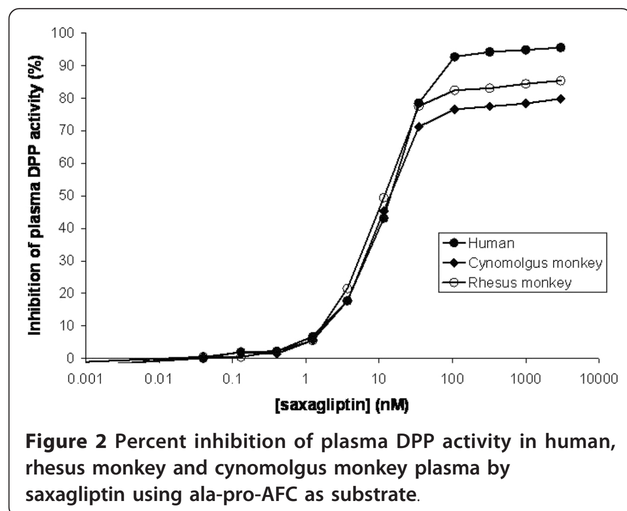

Figure 2 Percent inhibition of plasma DPP activity in human, rhesus monkey and cynomolgus monkey plasma by saxagliptin using ala-pro-AFC as substrate.

Measurement of plasma DPP activity in ex vivo assays

The differences in dissociation rate from DPP4 and the substrate used have substantial implications for measurement of activity following dosing in animals and humans. Figure 4 shows data from an in vivo study where various doses of saxagliptin and sitagliptin were given to cynomolgus monkeys and plasma DPP inhibition was measured after 24 hours, at trough.

When the ala-pro-AFC assay was used to measure plasma DPP inhibition ex vivo, saxagliptin treatment resulted in close to maximal inhibition of the inhibitable plasma DPP activity at its highest doses, with the 1, 3 and 10 mg/kg doses being statistically different from vehicle. However, sitagliptin treatment had no effect on plasma DPP activity at any of the doses. When the gly-pro-pNA assay was run on exactly the same samples, similar results were obtained for saxagliptin and there was no statistical difference between the data obtained with either assay at any dose. In contrast to the ala-pro-AFC assay, sitagliptin treatment gave statistically significant inhibition of plasma DPP activity at 3, 10 and 40 mg/kg doses when compared to vehicle in the gly-pro-pNA assay. Further, plasma DPP inhibition in the gly-pro-pNA assay at the 10 and 40 mg/kg doses were statistically significantly different from those obtained using the ala-pro-AFC assay ($P = 0.07$ for the 3 mg/kg dose). However, the highest dose tested still did not give maximal inhibition of plasma DPP activity. Therefore, choice of assay had significant relevance for the interpretation of inhibition by sitagliptin in this study.

Discussion

Saxagliptin (BMS-477118) is a potent inhibitor of DPP4 that is approximately 10-fold more potent than vildagliptin or sitagliptin. Saxagliptin also has an active

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