# Dipeptide Boronic Acid Inhibitors of Dipeptidyl Peptidase IV: Determinants of Potency and in Vivo Efficacy and Safety

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Dipeptidyl peptidase IV (DPP-IV; E.C. 3.4.14.5), a serine protease that degrades the incretin hormones GLP-1 and GIP, is now a validated target for the treatment of type 2 diabetes. Dipeptide boronic acids, among the first, and still among the most potent DPP-IV inhibitors known, suffer from a concern over their safety. Here we evaluate the potency, in vivo efficacy, and safety of a selected set of these inhibitors. The adverse effects induced by boronic acid-based DPP-IV inhibitors are essentially limited to what has been observed previously for non-boronic acid inhibitors and attributed to cross-reactivity with DPP8/9. While consistent with the DPP8/9 hypothesis, they are also consistent with cross-reactivity with some other intracellular target. The results further show that the potency of simple dipeptide boronic acid-based inhibitors can be combined with selectivity against DPP8/9 in vivo to produce agents with a relatively wide therapeutic index (>500) in rodents.

## Introduction

Dipeptidyl peptidase IV (DPP-IV[a]; E.C. 3.4.14.5) is a serine protease widely distributed in mammalian cells and tissues.[1] It is found, for example, on endothelial cells lining the vasculature and lung, on epithelial cells of the intestine, kidney and liver, on T cells, and in soluble form in the serum. DPP-IV cleaves dipeptides from the amino terminus of peptides containing proline or alanine at the penultimate position.[2] The list of potential substrates is large and includes many biologically important hormones, neuropeptides, chemokines, and cytokines.[3] Of particular interest is DPP-IV's established role in degrading the incretin hormone, glucagon-like peptide-1 (GLP-1), which is secreted by the L-cells of the gut in response to nutrient ingestion.[4,5] GLP-1 stimulates glucose-induced insulin biosynthesis and secretion, while inhibiting glucagon secretion, gastric emptying, and food intake—all of which help to lower blood glucose levels.[6,7] Recent findings suggest that GLP-1 may also have regenerative effects on $\beta$-cell mass and function, a highly desirable activity promising sustainable benefits in islet function.[8,9] The use of GLP-1 itself as a therapeutic agent, however, is not practical, owing to the rapidity with which it is inactivated by DPP-IV in vivo.[10] Inhibitors of DPP-IV are therefore of considerable interest as a way to enhance the activity of endogenous GLP-1. Indeed, they have demonstrated antidiabetic activities in both animals and humans, with one inhibitor (sitagliptin) now approved by the United States Food and Drug Administration (FDA) for treatment of type 2 diabetes.[11]

Owing to the large number of bioactive peptides that are potential DPP-IV substrates, the safety of DPP-IV inhibitors has been of particular concern, especially as a number of DPP-IV inhibitors have induced adverse effects (AEs) in animals. The AEs include hair loss, anemia, enlarged spleen, pruritus, skin lesions, bloody diarrhea, emesis, and even mortality.[12] Inhibition of DPP-IV itself, however, is unlikely to be responsible for most, or perhaps any, of the AEs because DPP-IV knockout mice and rats appear normal.[13] Also, there seems to be no correlation between either selective DPP-IV inhibitor potency and severity of AEs or suppression of DPP-IV activity in vivo and onset of AEs.[12] Off-target activity is therefore the more likely cause. Thornberry and co-workers from Merck have claimed that DPP8 and DPP9 are the off-target sites. Their hypothesis is based on nonselective DPP-IV inhibitors and a DPP8/9-selective inhibitor producing similar AEs in rats and dogs, in contrast to a DPP-IV-selective inhibitor producing no AEs.[12]

Although the DPP8/9 hypothesis appears to have gained wide acceptance, it cannot yet be regarded as established. Rosenblum and co-workers, using activity-based profiling in dogs, recently reported that single doses of one inhibitor induced AEs without significantly inhibiting DPP8/9, while another inhibited DPP8/9 significantly without producing AEs.[14] Although these results are plainly inconsistent with the DPP8/9 hypothesis, they do not rule it out entirely because they apply only to the rapid-onset, acute GI-type toxicity, as the animals received only single doses of the inhibitors. Thus, they leave open the possibility that the DPP8/9 hypothesis applies to AEs generated by longer term, chronic dosing. However, Burkey and co-workers did examine longer term AEs arising from chronic dosing.[15] In this study, high doses of vildagliptin were given once a day for 13 weeks to mice and rats. The doses were high enough to maintain plasma levels of vildagliptin several-fold higher than necessary to completely inhibit both DPP8 and DPP9 over the course of the experiment. No adverse effects were observed. Again, these results are plainly inconsistent with the DPP8/9 hypothesis, but not unequivocal, because, unlike the Rosenblum work, inhibition of DPP8/9 in the animals was not directly demonstrated. Thus, it leaves open the possibility that, in spite of the high plasma levels, vildagliptin fails to find its way to DPP8/9 in vivo, perhaps owing to the lack of cell-penetrating ability. Nevertheless, these studies provide sufficient evidence to question the

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[a] Abbreviations: DASH, DPP-IV activity and/or structural homologue; GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase IV; DPP2, dipeptidyl peptidase 2; FAP, fibroblast activation protein; DPP8, dipeptidyl peptidase 8; DPP9, dipeptidyl peptidase 9; $K_i$, inhibition constant; OGTT, oral glucose tolerance test; $IC_{50}$, median inhibition concentration; IC-$IC_{50}$, intracellular median inhibition concentration; MTD, maximum tolerated dose; TI, therapeutic index.

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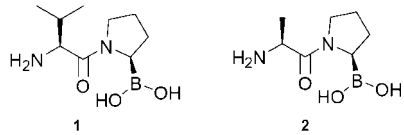
**Table 1.** Inhibition of Dipeptidases by Xaa-boroPro and Xaa-boroAla Inhibitors and Selectivity for DPP-IV

| cmpd | name | $K_i^{DPP\text{-}IV}$, (nM) | $K_i^{DPP8}$, (nM) | $K_i^{DPP9}$, (nM) | selectivity[a] DPP8 | selectivity[a] DPP9 |
|---|---|---|---|---|---|---|
| **1** | Val-boroPro | 00.18 ± 0.03 | 1.5 ± 0.2 | 0.76 ± 0.10 | 8.3 | 4.2 |
| **2** | Ala-boroPro | 0.027 ± 0.020 | 2.0 ± 0.1 | 0.53 ± 0.08 | 74 | 19 |
| **3** | Glu-boroPro | 1.8 ± 0.3 | 11 ± 1.4 | 2.6 ± 0.2 | 6.1 | 1.4 |
| **4** | Gly-boroPro | 0.40 ± 0.04 | 3.1 ± 0.8 | 0.41 ± 0.05 | 7.8 | 1.0 |
| **5** | Glu-boroAla | 8.3 ± 0.9 | 880 ± 110 | 2100 ± 90 | 110 | 250 |
| **6** | Val-boroAla | 3.9 ± 0.4 | 96 ± 13 | 50 ± 3.6 | 27 | 13 |
| **7** | Gln-boroAla | 17 ± 0.3 | 140 ± 18 | 260 ± 10 | 8.2 | 15 |
| **8** | Ac-Val-boroPro | 3300 ± 290 | 8400 ± 530 | 4300 ± 450 | 2.5 | 1.3 |

[a] Fold selectivity for DPP-IV is equal to $K_i$(DPP8/9)/$K_i$(DPP-IV).

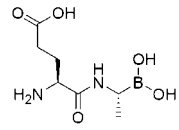
**Chart 1.** Structures of Selected Dipeptide Boronic Acid Inhibitors of DPP-IV



**1** Val-boroPro



**2** Ala-boroPro



**5** Glu-boroAla

DPP8/9 hypothesis. Thornberry and co-workers themselves have acknowledged that their findings are not unequivocal in implicating DPP8/9.[12] Perhaps DPP8/9 serve as an imperfect surrogate marker for reactivity against yet some other target that is responsible for the AEs.

Dipeptide boronic acids of the type Xaa-boroPro or Xaa-boroAla were among the first, and remain among the most potent, DPP-IV inhibitors known (Table 1), having $K_i$ values in the picomolar range.[16] Val-boroPro (**1**) and Ala-boroPro (**2**), for example, have $K_i$ values for DPP-IV of 180 pM and 27 pM, respectively, making them 100- to 1000-fold more potent than DPP-IV inhibitors in clinical use or currently known to be in development.[17] The advantage such boronic acid-based inhibitors of DPP-IV offer in potency has been offset by concerns over potential toxicity. The fear is that compounds of this class are too reactive to be selective and will have a greater tendency to cross-react not only with DPP8/9 and related enzymes, but with other targets as well. Very little experimental evidence, however, is available with which to evaluate this concern.

For compounds of this class, the greatest amount of information is available for **1**. This DPP-IV inhibitor has been in phase 3 clinical trials, but, interestingly, for cancer—not type 2 diabetes.[18] It is clearly a nonselective DPP-IV inhibitor, inhibiting DPP8/9, FAP, DPP2 and some other DASH family enzymes essentially as potently as it inhibits DPP-IV.[12] And **1** is quite toxic, especially to rats. Indeed, it was used in the Merck study as an example of a nonselective, and therefore toxic, DPP-IV inhibitor.[12] Early on, however, we discovered that **2** is far less toxic than **1**. Rats tolerated doses of **2** >200-fold above the $LD_{50}$ of **1** quite well. But no effort was made at the time to identify what dose of **2** would induce AEs, or how these would compare to those induced by **1**. Based on its structure (Chart 1), one would expect **2** to be a potent inhibitor of DPP8/9 as well as of DPP-IV. Here we present data confirming that this is the case. Compound **2** is as potent against DPP8/9 as compound **1**. What then is the basis for the apparent large difference in toxicities between the two compounds?

The overall goal of this work is to better understand the factors affecting the toxicity of boronic acid-based DPP-IV inhibitors. In particular, we ask what the basis is for the difference in toxicity between **1** and **2** and what the significance is of cross-reactivity with DPP8/9. Is the latter a major determinant of toxicity for boronic acid-based DPP-IV inhibitors, as appears to be the case for other classes of DPP-IV inhibitors, or do boronic acids have other, perhaps multiple, off-target activities?[12]

For this study it was our hope to find a simple dipeptide boronic acid DPP-IV inhibitor that is selective against DPP8/9. To this end the substrate specificities of DPP8 and DPP9 were determined using peptide libraries and mass spectrometry. The information was then employed to select a group of dipeptide boronic acids for synthesis and characterization. In general, the substrate and inhibitor specificities corresponded very well, and ultimately led to the identification of reasonably potent and selective dipeptide boronic acid inhibitor of DPP-IV. The in vivo potency, efficacy and toxicity of **1** and **2** were examined and compared with that of a DPP-IV-selective boronic acid inhibitor, Glu-boroAla (**5**), and the results are reported here.

## Results and Discussion

**Substrate Specificity.** Among DASH family enzymes the substrate specificities of DPP-IV and fibroblast-activating protein (FAP) are the best defined. Both exhibit a strong preference for cleaving after Pro, and to a much lesser extent, Ala. At the $P_2$ position DPP-IV is relatively promiscuous, accepting all residues, but exhibiting a slight preference for hydrophobic and basic residues, whereas FAP prefers Ile, Pro, and Arg.[19] A major difference between these two enzymes is that DPP-IV is strictly an aminopeptidase, cleaving after Pro only if the $P_2$ residue has a free amino group, whereas FAP has endopeptidase activity, although limited to Gly-Pro sequences.[20] DPP8 and DPP9, like DPP-IV, FAP, and DASH family enzymes, exhibit a preference for hydrolyzing substrates after Pro, and less efficiently after Ala.[21−23] Beyond this, and despite the growing interest in DPP8 and DPP9, their substrate specificities are not well defined. Recent experiments, using chromogenic positional-scanning combinatorial dipeptide libraries, suggest that DPP8 may be more discriminating than DPP-IV in its preference for hydrophobic and basic residues at $P_2$.[24] To date, there is relatively little information on the $P_2$ substrate specificity of DPP9, and contradictory data on its endopeptidase activity.[25−27] In order to guide the design of a DPP-IV-selective boronic acid inhibitor, the substrate specificities of DPP8 and DPP9 were determined using peptide libraries and mass spectrometry.

To determine $P_1$ specificity, DPP8 and DPP9 were incubated with the peptide library $NH_2$-A-X-FSWS-$NH_2$, where X represents every natural amino acid except Cys, and the cleavage products measured using mass spectrometry (Figure 1A). The results confirm that both DPP8 and DPP9, like DPP-IV, will remove a dipeptide from the amino-terminus only if the

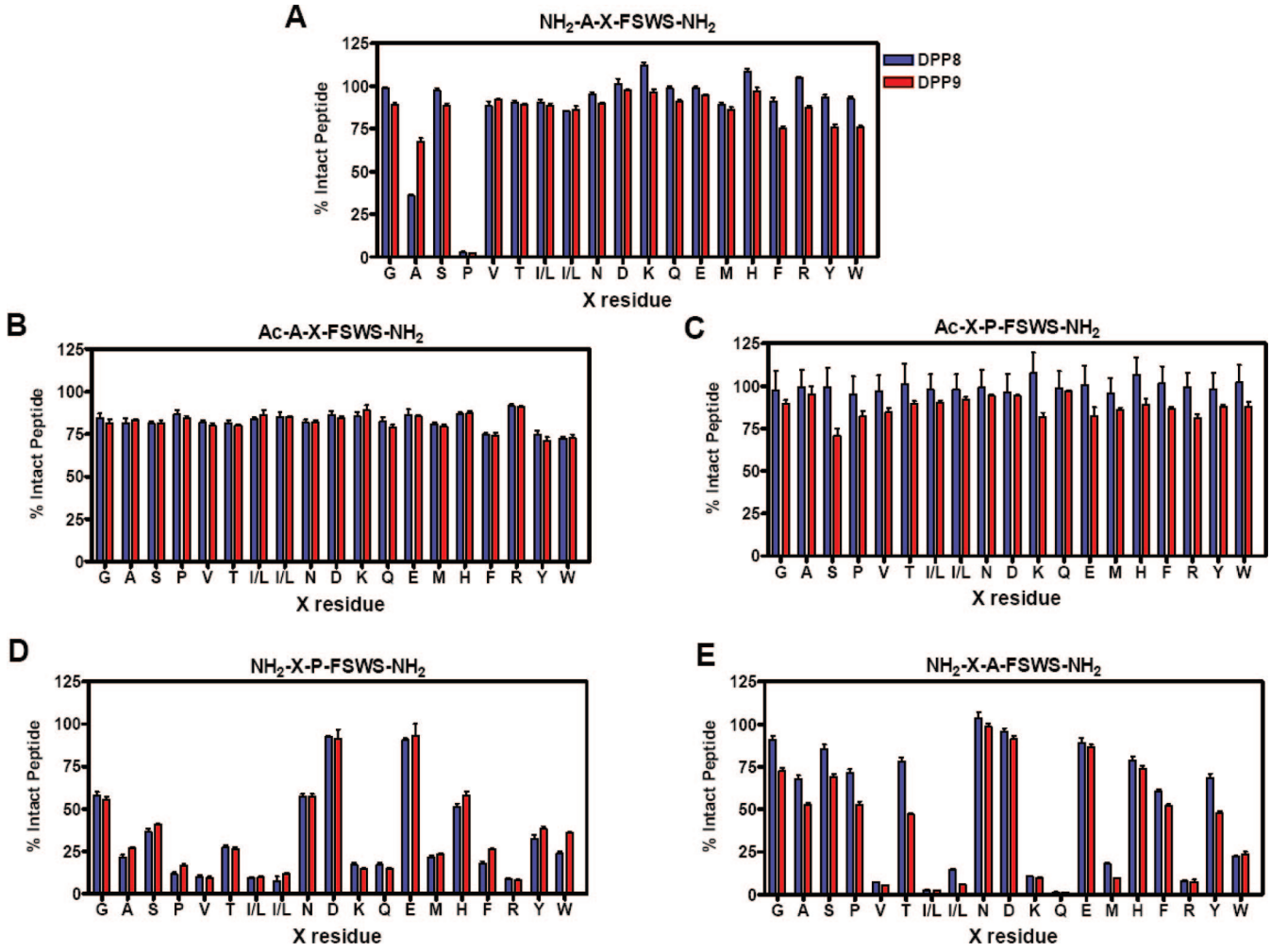



**Figure 1.** Substrate specificities of human DPP8 (3.0 $\mu$M) and DPP9 (8.0 $\mu$M) determined with peptide libraries (1.1 mM). (A) $P_1$ specificity using $NH_2$-A-X-FSWS-$NH_2$, endopeptidase activity using (B) Ac-A-X-FSWS-$NH_2$ and (C) Ac-X-P-FSWS-$NH_2$, and $P_2$ specificity using (D) $NH_2$-X-P-FSWS-$NH_2$ and (E) $NH_2$-X-A-FSWS-$NH_2$ peptide libraries. Each graph is labeled with the hexapeptide library used for the digestion. X denotes all amino acids except Cys. For all bar graphs, blue denotes DPP8 and red denotes DPP9.

penultimate residue is Pro or, though with less efficacy, Ala—essentially agreeing with previous reports.[21−23]

To determine if either DPP8 or DPP9 have endopeptidase activity, each recombinant protein was incubated with N-blocked peptide libraries degenerate in the penultimate position (Ac-A-X-FSWS-$NH_2$) or the N-terminal position (Ac-X-P-FSWS-$NH_2$), and the cleavage products were measured by mass spectroscopy (Figure 1B,C). The results show that neither DPP8 nor DPP9 exhibit any significant endopeptidase activity, and therefore are more like DPP-IV than FAP. This finding is consistent with homology modeling of DPP8 and DPP9 based on a crystal structure of DPP-IV, which indicates that DPP8 and DPP9 resemble DPP-IV in having an Asp (Asp663 in DPP-IV) near the double Glu motif in the "EE-α-helix", in place of the Ala found in FAP (Ala657).[28] It has been proposed that this Asp to Ala difference accounts for FAP's endopeptidase activity by providing sufficient conformational freedom to accommodate R-Gly-Pro sequences.[29]

To determine $P_2$ specificity, DPP8 and DPP9 were incubated with peptide libraries containing a degenerate free amino-terminal residue with either Pro or Ala at the penultimate or $P_1$ position (i.e., $NH_2$-X-P-FSWS-$NH_2$ or $NH_2$-X-A-FSWS-$NH_2$) (Figure 1D,E). The results show that with Pro at $P_1$, both DPP8 and DPP9, like DPP-IV, accept most residues at $P_2$, but have preferences in the following order: hydrophobic residues (M,V,I,L), basic residues (R,K), Gln (Q), and Pro (P) > Ala (A), aromatic residues (F,W,Y), Ser (S), and Thr (T) > Gly (G), Asn (N), and His (H). Both DPP8 and DPP9, like DPP-IV, tolerate negatively charged side chains (E,D) least well at $P_2$, with DPP8 and DPP9 being even more discriminatory against these residues than DPP-IV.[24] These results essentially agree with recent data of Lee et al., obtained using activated chromogenic substrates, which indicate that DPP8 prefers hydrophobic and basic residues at $P_2$.[24] With Ala at $P_1$, both DPP8 and DPP9 appear to have an even stronger preference at $P_2$ for hydrophobic and basic residues and Gln over Gly, Ser, Asn, Asp, and Glu than with Pro at $P_1$ (Figure 1E).

**Dipeptide Boronic Acid Inhibitor Selectivity.** The trends observable in the $K_i$ values of the dipeptide boronic acid inhibitors listed in Table 1 closely parallel those of the substrate specificity profiles. For example, dipeptides with boroPro (**1**−**4**) or boroAla (**5**−**7**) in the $P_1$ position are quite good inhibitors of all three enzymes, with the Xaa-boroPro inhibitors (**1**−**4**) being more potent than the corresponding Xaa-boroAla inhibitors (**5**−**7**). Glu appears to be among the residues accepted least well in the inhibitor $P_2$ position by DPP8 and DPP9, whether $P_1$ is boroPro or boroAla (**3** and **5**). Blocking the N-terminus (e.g., Ac-Val-boroPro (**8**)) essentially eliminates inhibitor potency against all three enzymes—again paralleling the substrate specificities. Although all three enzymes prefer Pro to

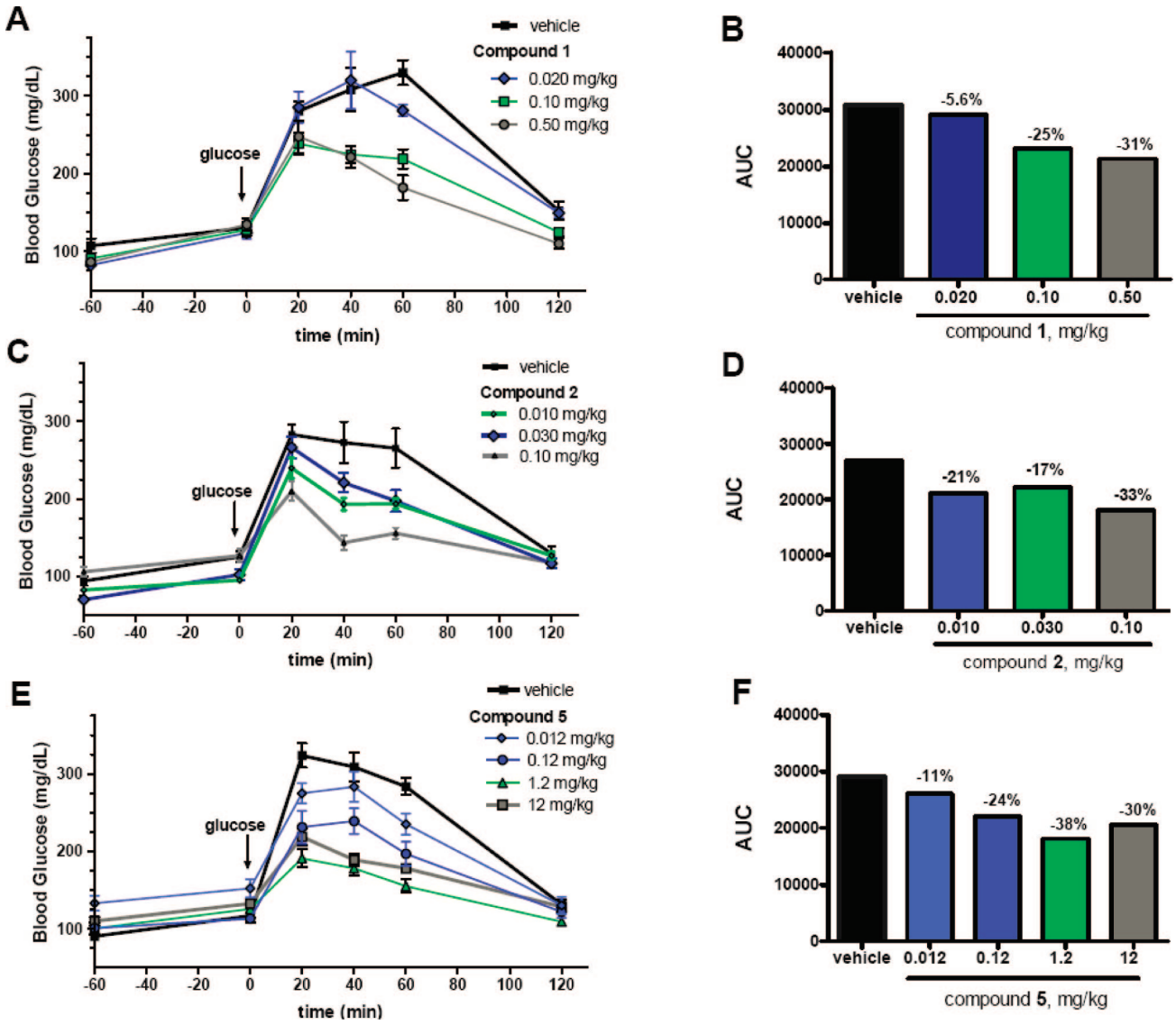



**Figure 2.** Oral glucose tolerance test (OGTT) of male C57BL/6 mice dosed orally with **1** (A and B), **2** (C and D), or **5** (E and F). (A) **1** or vehicle, (C) **2** or vehicle, or (E) **5** or vehicle was administered 60 min prior to an oral glucose challenge (5 g/kg). (B, D, F) The glucose area under the curve (AUC) was determined from 0 to 120 min. Blood glucose change from baseline and reduction of AUC were determined by comparing to vehicle AUC.

Ala in $P_1$, substrate specificity profiles indicate that the preference is greater for DPP8 and DPP9 than for DPP-IV. A similar trend is seen for Glu at $P_2$. These observations suggest that Glu at $P_2$ combined with boroAla at $P_1$ should yield a dipeptide boronic acid inhibitor with selectivity for DPP-IV over DPP8 and DPP9. This is indeed the case, as Glu-boroAla (**5**) exhibits selectivity which is 110-fold for DPP-IV over DPP8 and 250-fold for DPP-IV over DPP9 (Table 1). Although **5** is 46-fold less potent against DPP-IV than **1** and 310-fold less potent than **2**, with a $K_i$ of 8.3 nM, **5** is still a very effective inhibitor of DPP-IV.

**In Vivo Potency and Efficacy of 1, 2, and 5.** The ability of compounds **1**, **2**, and **5** to lower blood glucose in response to an oral glucose tolerance test or "challenge" (OGTT) was assessed in normal mice. Following a 20-h fast, 7−8 male C57BL/6 (normal) mice were dosed with either vehicle or one of the three inhibitors by oral gavage at various dosages. 60 min later the mice were administered 5 g/kg of glucose orally, and the blood glucose levels were measured at 5 intervals over a 120 min time period. Figure 2 shows that all three inhibitors lowered the glucose excursion in a dose-dependent manner. The relative effectiveness in lowering the AUC (area under curve) was in the same order as the in vitro $K_i$ values: **2** > **1** > **5**. The differences, however, are not great. Compound **2** appears to be ∼5-fold more effective than **1**, which in turn appears to be ∼2-fold more effective than **5**. Notice that **5**, the least potent and least effective at lowering the AUC, is nevertheless quite potent, as a dose as low as 0.012 mg/kg gives a statistically significant 11% lowering of the AUC.

Plasma DPP-IV inhibition as a function of oral dose was also measured in a separate experiment using Sprague−Dawley rats. Each inhibitor was given to 4−6 rats via oral gavage and plasma DPP-IV activity measured 6 times over a 24 h period (Figure 3). Each compound inhibited plasma DPP-IV activity in a dose-dependent manner. Compounds **1** and **2** were considerably more effective than **5**, both in the magnitude of the suppression relative to dose and in its longevity.

Nevertheless, **5** was quite effective, sustaining 80% inhibition for 4 h at doses as low as 6.0 mg/kg. Although the results are not directly comparable to the OGTT data because the latter was carried out in mice, whereas the enzyme inhibition studies

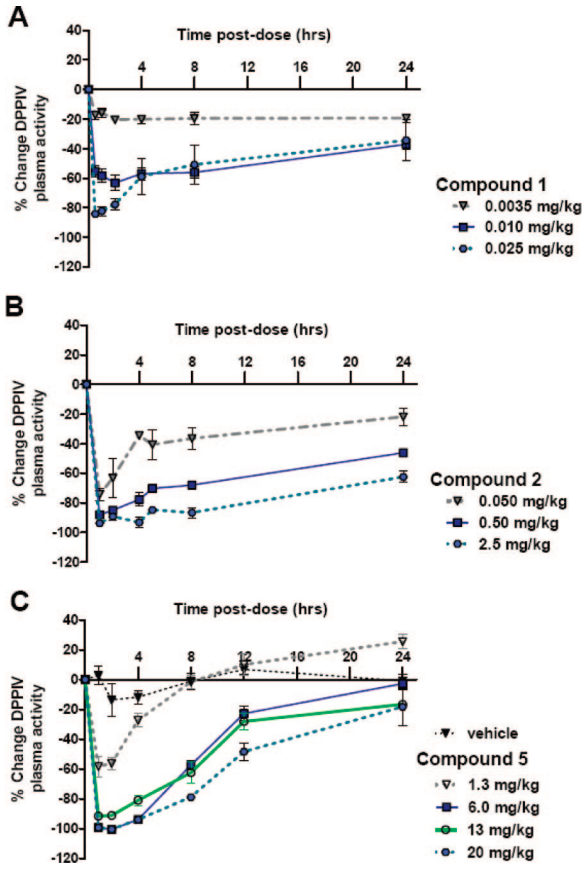



**Figure 3.** Effects of **1**, **2**, and **5** on plasma DPP-IV activity. (A) **1** or (B) **2** alone or (C) **5** or vehicle were administered to Sprague−Dawley rats and blood was collected up to 24-h postadministration of inhibitor. Percent change of DPP-IV plasma activity was determined by comparing plasma activity of treated sample to predose activity.

were carried out rats, we have found that enzyme inhibition data from mice and rats correlate reasonably well.

**Toxicity.** As a measure of toxicity, the maximum tolerated doses (MTD) of **1**, **2**, and **5** were determined in escalating single-dose studies, using Sprague−Dawley rats (Table 2). As expected, **1** was tolerated least well, with an MTD of 0.025 mg/kg. The initial signs of toxicity were observed 3−4 h postdose, and consisted mainly of severely decreased activity. Necropsy performed on lethargic animals showed vasocongestion of all abdominal and thoracic organs, and presence of clear fluid in stomach and small intestines, indicative of gastrointestinal (GI) toxicity. Compound **2** exhibited an MTD of ≥5 mg/kg − at least 200-fold greater than that of **1**. Several rats that received more than 5 mg/kg exhibited lethargy and were sacrificed and necropsied. Fluid and gas were found in the intestine and cecum, again indicative of GI toxicity. Rats receiving compound **5** exhibited no adverse effects until doses exceeded 500 mg/kg, yielding an MTD > $2.0 \times 10^4$-fold that of **1** and between 13- to 100-fold greater than that of **2**. At 900 mg/kg of **5**, 3 of 6 animals died−1 male 3 days postadministration, and 1 male and 1 female 4 days postadministration. There were no obvious signs of premortem suffering or other adverse side effects. Necropsies revealed that the deceased rats had fluid-filled stomachs and small intestines, indicative of gastrointestinal toxicity. All other organs appeared normal.

**Inhibition of DPP9 Within Intact 293T Cells (Intracellular-$IC_{50}$).** DPP-IV is expressed on cell membranes or secreted into the plasma, whereas DPP8 and DPP9 are intracellular enzymes. The cell-penetrating ability of DPP-IV inhibitors may therefore be as important, if not more so, than their inhibition constants in determining selectivity against DPP8/9. A cell assay was therefore designed to assess and compare the abilities of **1**, **2**, and **5** to inhibit DPP9 in intact cells. For this assay, HEK293T cells that overexpress DPP9 were employed to enhance detection of intracellular DPP9 activity. Although only inhibition of DPP9 was measured, in seems highly likely that this measurement also serves as a measure of intracellular DPP8 inhibition, because none of the three inhibitors discriminates significantly between DPP8 and DPP9 (Table 1).

All three compounds demonstrated dose-dependent abilities to inhibit DPP9 within the cells (Figure 4). We will here refer to the concentration of inhibitor external to the cell needed to achieve 50% inhibition of the DPP9 within the cell as "IC-$IC_{50}$" to avoid confusion with normal $IC_{50}$. **1** was the most effective with an IC-$IC_{50}$ of 6.8 $\mu$M, followed by **2**, with an IC-$IC_{50}$ about 50-fold higher at 360 $\mu$M (Table 3). **5** was the least effective, with concentrations as high as 1 mM achieving only ∼14% inhibition of intracellular DPP9, giving an estimated IC-$IC_{50}$, of ∼7000 $\mu$M, at least 20-fold higher than **2,** and 1,000-fold higher than **1** (Table 2). It is important to note that IC-$IC_{50}$ is not a measure of cell permeability. Like $IC_{50}$, it is a composite of factors, including cell permeability, potency against DPP9, rate and equilibrium of the intramolecular cyclization reaction (see Experimental Section), and inhibitor stability, both intrinsic and intracellular.[30]

**Mechanism of Toxicity.** Despite vast differences in toxicity measured by MTDs, greater than 20000-fold between **1** and **5**, the adverse effects encountered above the MTDs are remarkably similar for all three boronic acid inhibitors, suggesting a common mechanism of toxicity. Furthermore, these adverse effects are comparable to those reported for L-*allo*-isoleucine thiazolidine (**9**) and L-*threo* isoleucine isoindoline (**10**), and therefore probably arise from the same cause (Table 2). Compound **9** is a nonselective DPP-IV inhibitor, while compound **10** is a more selective DPP-IV inhibitor examined in the Merck study.[12] Since neither is a boronic acid, the toxicities observed with **1, 2**, and **5** may not be attributable to an off-target activity unique to boronic acids.

A comparison of the in vitro potencies of **1**, **2**, and **5** as inhibitors of DPP8/9 and their MTD values does not support the conclusion that DPP8/9 inhibition is the common underlying cause of toxicity for these inhibitors. Potency against DPP9 decreases in the order **2** > **1** > **5**, with relative $K_i$ values of 1:3:4000. In contrast, toxicities decrease in the order **1** > **2** > **5**, with relative MTDs of 1:200:20000 (Table 2). However, a better correlation exists between the IC-$IC_{50}$ and MTD values (Table 3). Intracellular potency against DPP9, as measured by IC-$IC_{50}$ values, decrease in the order **1** > **2** > **5**, the same as for the MTDs, showing that intracellular potency of these inhibitors tracks their toxicities qualitatively.

A quantitative correlation of the relative IC-$IC_{50}$ (1:50:∼1000) versus relative MTD (1:200:20000), however, is less than perfect. It seems that the IC-$IC_{50}$ measurements tend to increasingly underestimate safety, as measured by the MTD with increasing IC-$IC_{50}$ values. This could well reflect a key difference between in vivo and in vitro measurements. In the animal, the concentration of the inhibitor would be more likely to decline with time through excretion and through metabolic pathways such as the CYP450 enzymes, for example, than in the cell culture system.[31] It therefore seems reasonable that an increase in a measured IC-$IC_{50}$ in cell culture should lead to an even greater increase in the MTD in the animal.

**Table 2.** Comparison of DPP-IV Inhibitors

| cmpd | rel $K_i^{DPP-IV}$ | rel $K_i^{DPP9}$ | rel IC-$IC_{50}$ | MTD (mg/kg) | rel safety | eff dose (mg/kg)[a] | TI[b] |
|---|---|---|---|---|---|---|---|
| Val-boroPro(**1**) | 7 | 3 | 1 | 0.025 | 1 | 0.50 | 0.050 |
| Ala-boroPro(**2**) | 1 | 1 | 50 | 5 ≤ MTD <38 | 200−1500 | 0.10 | 50−380 |
| Glu-boroAla(**5**) | 300 | 4000 | ∼1000 | 500 ≤ MTD <900 | 20000−36000 | 1.2 | 420−750 |
| *threo*-Ile thia (**9**)[c] | 7000 | 3000[d] | nd | >700 | >28000 | ∼20 | >35 |
| *allo*-Ile thia (**10**)[c] | 7000 | 600[d] | nd | <300 | <12000 | ∼20 | <15 |

[a] Effective dose (eff dose) defined as dose required for maximal reduction of AUC in OGTT for compounds **1**, **2**, and **5**. For compounds **9** and **10**, effective dose is maximal reduction of AUC in OGTT from Lankas et al. (estimated). Historical data from **9** and **10** shown for comparison.[12] [b] Therapeutic index (TI) defined as MTD/eff dose. [c] *K*i, $IC_{50}$, and MTD values for compounds **9** and **10** obtained from Lankas et al. 12 [d] $IC_{50}$ values.

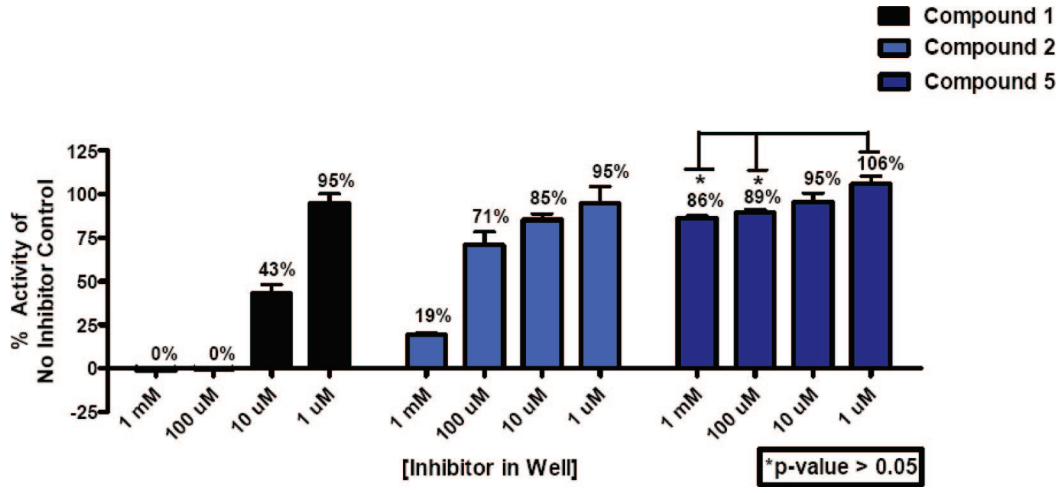



**Figure 4.** Inhibition of DPP9 expressed in intact HEK293T cells by compounds **1**, **2**, and **5**. Compounds **1**, **2**, and **5** or vehicle were added to media of cells overexpressing DPP9 for 2 h, washed, harvested, and lysed. Dipeptidyl peptidase activity was determined by comparing activity of treated sample to that of no-inhibitor control.

**Table 3.** Molar Concentration Comparison of IC-$IC_{50}$ for DPP9 and Maximum Tolerate Doses (MTD) of Compounds in Sprague-Dawley Rats

| cmpd | IC-$IC_{50}$ ($\mu$M) | MTD ($\mu$M) |
|---|---|---|
| Val-boroPro (**1**) | 6.8 | 0.12 |
| Ala-boroPro (**2**) | 360 | 27 ≤ MTD < 210 |
| Glu-boroAla (**5**) | ∼7000 | 23000 ≤ MTD < 41000 |

The above correlation is based on *relative* MTD and IC-$IC_{50}$ values, not *absolute* molar values of the MTDs and IC-$IC_{50}$. The problem, of course, is that it is difficult to translate the MTDs into in vivo molar concentrations. Even if the in vivo concentration were measured, they would change with time and could differ from one tissue to another. For our purposes, a rough approximation can be obtained by assuming each inhibitor is about equally bioavailable when given orally and that it becomes essentially evenly distributed within the rat. The in vivo DPP-IV inhibition and OGTT data indicate that the first assumption is probably valid. The molar MTDs calculated with these assumptions are listed in Table 3, which shows that the molar MTDs and IC-$IC_{50}$ correlate reasonably well with respect to **2** (27 to 210 $\mu$M, versus 360 $\mu$M) and **5** (23000 to 41000 $\mu$M, versus ∼7000 $\mu$M), but less well with respect to **1** (0.12 $\mu$M, versus 6.8 $\mu$M). Considering the complexity of the system, the MTDs and IC-$IC_{50}$ correlate surprisingly well, in both relative and absolute terms. Overall, these results are consistent with off-target inhibition of DPP8/DPP9 as the principal cause of the adverse effects of dipeptide boronic acid-based DPP-IV inhibitors. However, they are also consistent with off-target interaction with yet some other intracellular enzyme, especially if inhibition of DPP8/9 serves as a surrogate marker for the ability to inhibit this other intracellular target.

**Therapeutic Index.** "Therapeutic Index" (TI) is defined here as the ratio of the MTD to the minimal dose that gives maximum lowering of the AUC following an OGTT (Table 2). On the basis of this definition, **1** has a TI < 1, whereas the equally potent and nonselective inhibitor, **2**, has a respectable TI = 50−380. The greater TI for **2** compared to **1** can be ascribed in large measure to its being less effective at gaining access to and inhibiting DPP9 within the cell. The more selective **5** has an even wider TI of 420−750, which can be attributed to both its in vitro selectivity as well as to a deficiency in cell-permeating ability, at least relative to **1**. Although the nonboronic acid inhibitors **9** and **10** have reasonably high MTDs of <300 mg/kg and >700 mg/kg, respectively, the TIs for these compounds of <15 and >35 are nevertheless quite low − lower than the TIs of the boronic acid inhibitors, **2** and **5**. This, of course, is due to their lower potency against DPP-IV. Interestingly, the TIs of **9** and **10** are higher than expected on the basis of their $K_i$ and $IC_{50}$ values for DPP-IV and DPP8/9, which may also reflect a deficiency in cell-penetrating ability, although IC-$IC_{50}$ for these compounds were not determined.

## Conclusions

Boronic acid-based inhibitors of DPP-IV do not exhibit any unique or untoward toxicities. All adverse effects observed were also observed for nonboronic acid inhibitors. While consistent with cross-inhibition of DPP8/9 as the underlying cause, the adverse effects observed here are also consistent with cross-reactivity with some other intracellular target. Here we also show that the intrinsic potency of dipeptide boronic acids as DPP-IV inhibitors can be combined with selectivity against DPP8/9 in vivo to achieve a wide therapeutic index.

## Experimental Section

**Materials and Analysis.** *H*-Ala-Pro-paranitroanilide was purchased from Bachem. The peptide libraries $NH_2$-XPFSWS-$NH_2$,

Ac-XPFSWS-$NH_2$, $NH_2$-AXFSWS-$NH_2$, Ac-AXFSWS-$NH_2$, and $NH_2$-XAFSWS-$NH_2$, where X represents all natural amino acids except Cys, were synthesized by the Tufts University Core Facility. Dipeptide boronic acid derivatives compounds **1**−**4** were synthesized following literature procedures.[32,33] The boroAla-containing dipeptide derivatives (compounds **5**−**7**) were prepared by coupling the appropriate *N*-Boc protected amino acids to L-boroAla-pn·HCl in the presence of HATU and DIPEA followed by simultaneous removal of the protective groups Boc and pinanediol via $BCl_3$ treatment.[34] Compound **8** was synthesized by coupling Boc-Val-OH to L-boroPro-pn·HCl using the same conditions, except that the *N*-Boc protection was selectively removed by HCl in dioxane to yield Val-boroPro-pn. Further acetylation of this intermediate with acetyl chloride in the presence of DIPEA, followed by subsequent removal of the pinane group via $BCl_3$ afforded the target compound **8**.

NMR spectra of the compounds in $D_2O$ solution were recorded on a Bruker Avance 300 MHz spectrometer. Chemical shifts were reported relative to DSS for $^1H$ and $^{13}C$ and vs boric acid for $^{11}B$ NMR. Mass spectra and HPLC retention times were recorded on a Hewlett-Packard HP LC/MSD system with UV detector (monitoring at 215 and 254 nm), using a Discovery C18 569232-U RP-HPLC column (12.5 cm, 4.6 mm, 5 $\mu$m) with solvent gradient A) water (0.1% TFA) and B) acetonitrile (0.08% TFA) flowing at 0.5 mL/min. Unless otherwise noted, all HPLC retention times are given for an eluent gradient 2% B for the first 5 min, then from 2% to 98% B over 10 min, then 98%B maintained for another 10 min. HRMS were performed by the Technical Services of the University of Michigan. All characterization data for compounds **1** to **8** were listed as below.

**H - (*S*) - Val - (*R*) - boroPro (1).** $^1H$ NMR ($D_2O$) $\delta$ 0.98 (d, $J$ = 6.9 Hz, 3H, $CH_3CHCH_3$), 1.08 (d, $J$ = 6.9 Hz, 3H, $CH_3CHCH_3$), 1.60 − 2.34 (m, 5H, $CH_3CHCH_3$ and $BCHCH_2CH_2$), 3.03 − 3.09 (m, 1H, $CH_2CHB$), 3.43 − 3.75 (m, 2H, $CH_2CH_2N$), 4.12 (d, $J$ = 6.3 Hz, 1H, $H_2NCHCO$).$^{13}C$ NMR ($D_2O$) $\delta$ 18.36, 20.70, 29.29, 29.42, 31.37, 50.24, 51.03, 59.73, 169.67. $^{11}B$ NMR ($D_2O$) $\delta$ 10.96. LC-MS ($ESI^+$) $m/z$ (rel intensity): 393.3, ($[2 \times (M - H_2O) + H]^+$, 60); 197.1, ($[M - H_2O + H]^+$, 100). tr = 11.1 min. HRMS: calcd for $C_9H_{18}BN_2O_2$, $[M - H_2O + H]^+$, 197.1461; found, 197.1462.

**H - (*S*) - Ala - (*R*) - boroPro (2).** $^1H$ NMR ($D_2O$) $\delta$ 1.48 (d, $J$ = 6.9 Hz, 3H, $CH_3CHNH_2$), 1.67 − 2.15 (m, 4H, $BCHCH_2CH_2$), 3.03 − 3.09 (m, 1H, $CH_2CHB$), 3.41 − 3.72 (m, 2H, $CH_2CH_2N$), 4.31 (q, $J$ = 6.9 Hz, 1H, $H_2NCHCO$).$^{13}C$ NMR ($D_2O$) $\delta$ 17.53, 29.38, 29.45, 49.76, 50.56, 51.08, 170.61. $^{11}B$ NMR ($D_2O$) $\delta$ 11.31. LC-MS ($ESI^+$) $m/z$ (rel intensity): 337.2, ($[2 \times (M - H_2O) + H]^+$, 100); 169.0, ($[M - H_2O + H]^+$, 23). tr = 5.9 min. HRMS: calcd for $C_7H_{14}BN_2O_2$, $[M - H_2O + H]^+$, 169.1148; found, 169.1142.

**H - (*S*) - Glu - (*R*) - boroPro (3).** $^1H$ NMR ($D_2O$) $\delta$ 1.48 (d, $J$ = 6.9 Hz, 3H, $CH_3CHNH_2$), 1.69 − 2.23 (m, 6H, $BCHCH_2CH_2$ and $CH_2CO_2H$), 2.56 − 2.60 (m, 2H, $CH_2CH_2CO_2H$), 3.05 − 3.11 (m, 1H, $CH_2CHB$), 3.43 − 3.75 (m, 2H, $CH_2CH_2N$), 4.39 (t, $J$ = 5.9 Hz, 1H, $H_2NCHCO$).$^{13}C$ NMR ($D_2O$) $\delta$ 27.28, 29.36, 29.40, 31.23, 50.13, 51.28, 53.50, 169.22, 179.00. $^{11}B$ NMR ($D_2O$) $\delta$ 11.29. LC-MS ($ESI^+$) $m/z$ (rel intensity): 227.0, ($[M - H_2O + H]^+$, 100); 209.0, ($[M - 2H_2O + H]^+$, 61). tr = 7.1 min.

**H - Gly - (*R*) - boroPro (4).** $^1H$ NMR ($D_2O$) $\delta$ 1.69 − 2.07 (m, 4H, $BCHCH_2CH_2$), 3.03 − 3.05 (m, 1H, $CH_2CHB$), 3.36 − 3.50 (m, 2H, $CH_2CH_2N$), 3.88 (s, 2H, $H_2NCH_2CO$).$^{13}C$ NMR ($D_2O$) $\delta$ 29.13, 29.53, 42.89, 49.11, 50.12, 166.89. $^{11}B$ NMR ($D_2O$) $\delta$ 11.03. LC-MS ($ESI^+$) $m/z$ (rel intensity): 309.2, ($[2 \times (M - H_2O) + H]^+$, 100); 155.2, ($[M - H_2O + H]^+$, 53). tr = 5.2 min. HRMS: calcd for $C_6H_{12}BN_2O_2$, $[M - H_2O + H]^+$, 155.0992; found, 155.0985.

**H - (*S*) - Glu - (*R*) - boroAla (5).** $^1H$ NMR ($D_2O$) $\delta$ 1.09 (d, $J$ = 7.5 Hz, 3H, $CH_3CHB$), 2.17 (q, $J$ = 7.1 Hz, 2H, $CH_2CO_2H$), 2.49 − 2.64 (m, 2H, $CH_2CH_2CO_2H$), 2.96 (q, $J$ = 7.5 Hz, 1H, $CH_3CHB$), 4.07 (t, $J$ = 6.7 Hz, 1H, $H_2NCHCO$).$^{13}C$ NMR ($D_2O$) $\delta$ 16.93, 27.88, 31.43, 40.92, 53.27, 172.24, 178.13. $^{11}B$ NMR ($D_2O$) $\delta$ 6.80. LC-MS ($ESI^+$) $m/z$ (rel intensity): 383.2, ($[2 \times (M - H_2O) + H]^+$, 33); 201.1 ($[M - H_2O + H]^+$, 100). tr = 4.1 min.

**H - (*S*) - Val - (*R*) - boroAla (6).** $^1H$ NMR ($D_2O$) $\delta$ 0.96 (d, $J$ = 6.9 Hz, 3H, $CH_3CHCH_3$), 0.97 (d, $J$ = 6.9 Hz, 3H, $CH_3CHCH_3$), 1.09 (d, $J$ = 7.5 Hz, 3H, $CH_3CHB$), 2.09 − 2.21 (m, 1H, $CH_3CHCH_3$), 2.82 (q, $J$ = 7.5 Hz, 1H, $CH_3CHB$), 3.73 (d, $J$ = 6.3 Hz, 1H, $H_2NCHCO$). $^{11}B$ NMR ($D_2O$) $\delta$ 5.50. LC-MS ($ESI^+$) $m/z$ (rel intensity): 341.2, ($[2 \times (M - H_2O) + H]^+$, 100); 171.3 ($[M - H_2O + H]^+$, 11). tr = 6.3 min (the eluent gradient was 2% B for the first 10 min, then from 2% to 98% B over 10 min, which was maintained for the next 10 min).

**H - (*S*) - Gln - (*R*) - boroAla (7).** $^1H$ NMR ($D_2O$) $\delta$ 1.18 (d, $J$ = 7.5 Hz, 3H, $CH_3CHB$), 2.12 − 2.19 (m, 2H, $CH_2CONH_2$), 2.40 − 2.47 (m, 2H, $CH_2CH_2CONH_2$), 2.97 (q, $J$ = 7.5 Hz, 1H, $CH_3CHB$), 4.03 (t, $J$ = 6.6 Hz, 1H, $H_2NCHCO$). $^{11}B$NMR ($D_2O$) $\delta$ 8.00. LC-MS ($ESI^+$) $m/z$ (rel intensity): 399.2 ($[2 \times (M - H_2O) + H]^+$, 100), 200.2 ($[M - H_2O + H]^+$, 60); tr = 3.9 min.

**N- Ac - (*S*) - Val - (*R*) - boroPro (8).** $^1H$ NMR ($D_2O$) $\delta$ 0.88 − 0.95 (m, 6H, $CH_3CHCH_3$), 1.63 − 2.10 (m, 7H, $CH_3CHCH_3$, $BCHCH_2CH_2$ and $CH_3CONH$), 2.92 − 2.98 (m, 1H, $CH_2CHB$), 3.46 − 3.88 (m, 2H, $CH_2CH_2N$), 4.36 (d, $J$ = 6.3 Hz, 1H, $H_2NCHCO$). LC-MS ($ESI^+$) $m/z$ (rel intensity): 279.2, ($[M + Na]^+$, 6); 239.2, ($[M - H_2O + H]^+$, 100). tr = 12.6 min.

**Protease Cloning, Expression, and Purification.** pcDNA3.1/DPP8/V5-His and pcDNA3.1/DPP9/V5-His were generated by Invitrogen Life Technologies by amplifying the targets from one of their human FL placental libraries. Clones corresponding to accession numbers AF221634 (DPP8) and AF542510 (DPP9) were confirmed by full-length DNA sequencing. HEK293T cells grown in 10% Fetal Bovine Serum (Hyclone) were transiently transfected with 10 $\mu$g plasmids encoding recombinant proteases using Lipofectamine 2000 reagent (Invitrogen). Cells ($1 \times 10^7$) expressing each protein were lysed in 1.5 mL of 50 mM sodium phosphate at pH 7.8, and 30−40 $\mu$L of supernatant was used for $K_i$ determination. No significant activity against Ala-Pro-pNA was detected in untransfected cell lysates. For substrate specificity assays, we purified the recombinant proteases by lysing cells ($2 \times 10^7$) expressing each protein in 50 mM sodium phosphate, 300 mM NaCl, and 5 mM imidazole at pH 8.0, and incubating supernatant with Ni-NTA beads (Qiagen). Beads were washed with the same buffer containing 20 mM imidazole, and protein was eluted using the same buffer with 250 mM imidazole. Enzyme activity was used to monitor eluted fractions. SDS-PAGE showing the purities of enzyme preparations is obtainable as Supporting Information. Protein concentrations were determined by the Bradford assay (Biorad).

**Peptide Library Digest.** Peptide libraries (1.1 mM) were incubated for 24 h with either purified DPP8/V5-His (3.0 $\mu$M) or DPP9/V5-His (8.0 $\mu$M) in 50 mM sodium phosphate at pH 7.8 and 37 °C. The reaction was quenched by addition of 10% HCl. The samples were analyzed by reverse-phase HPLC-MS on a Thermo Finnigan LCQ Duo, quantifying the peaks in the resulting base peak chromatograms. Relative cleavage amounts were determined by comparing the postquench abundance of intact peptides to those in the initial library.

**Inhibition Kinetics.** $K_i$ values were determined as previously reported.[35] Such measurements are not straightforward for this class of compounds because, in addition to being "tight" and "slow" binding inhibitors, they undergo a pH dependent conformational change from an open chain and inhibitory form at low pH (e.g., 2.0) to a cyclic and inactive structure at high (e.g., physiological pH (7.2)).[30] The half-life of the cyclization reaction can vary from ∼30 min to several hours, and the equilibrium constant from for example ∼3-fold in favor of the cyclic structure for Glu-boroAla to ∼2000-fold for Ala-boroPro. The previously reported method for measuring $K_i$ values takes into account these processes and yields a value of $K_i$ for the open chain active species even thought the kinetics are done at pH 7.8 where this form is not favored.[36] DPP-IV was purified from human placenta.[37] DPP8 and DPP9 lysates were obtained as described above and used for $K_i$ measurement. No endogenous activity against Ala-Pro-pNA was detected at concentrations used in untransfected HEK293T cell lysates and all data was corrected for.

**Intact Cell Assay (Determining IC-$IC_{50}$).** HEK293T cells were transiently transfected with pcDNA3.1/DPP9/V5-His as described

above. Approximately 48 h post-transfection, $\sim 10^7$ cells were treated with varying doses of compounds **1**, **2**, and **5** (1 mM, 100 $\mu$M, 10 $\mu$M, 1 $\mu$M) in media. The inhibitors were incubated at pH 2.0 overnight to ensure they were fully in the active open-chain configuration. The inhibitors were then adjusted to pH 7.8 and immediately added to the intact cells and allowed to incubate for 2 h. Cells were washed 5 times with PBS, then harvested, resuspended in 50 mM sodium phosphate at pH 7.8, sonicated, and centrifuged to collect supernatant. Total protein concentrations were quantified via Bio-Rad Protein Assay to ensure equal loading of DPP9 to 96-well plates. DPP9 activity was measured using Ala-Pro-pNA and reading the absorbance of cleaved pNA at 405 nm. Percent inhibition was calculated by comparing activity to a no-inhibitor control. IC-$IC_{50}$ values were determined by a nonlinear regression fit of the data to a sigmoidal dose−response curve using GraphPad Prism.

**Oral Glucose Tolerance Test in Lean Mice.** Male C57BL/6 mice (7−8 weeks of age) from Charles River Laboratories, Wilmington, MA were housed 4 per cage and given access to normal rodent chow (Teklad) and water. Mice ($n$ = 7−8/group) were fasted overnight (~18−21 h). Baseline ($t$ = −60 min) blood glucose concentrations were determined by glucometer from blood obtained from tail nick. Animals were then orally treated with vehicle (0.25% methylcellulose, pH 2, 10 mL/kg), compound **1** (0.50, 0.10, and 0.020 mg/kg, 10 mL/kg), compound **2** (0.1, 0.030, and 0.010 mg/kg, 10 mL/kg), or compound **5** (12, 1.2, 0.12, and 0.012 mg/kg, 10 mL/kg). Note that the vehicle and test article solutions were at pH 2.0 to ensure that the test articles are fully in the open chain, active configuration on dosing, although we have demonstrated that the results do not differ if the test articles are given in the cyclic form−most likely because the low pH of the stomach rapidly converts the cyclic structures back into the open chain configurations. Blood glucose concentration was measured 1 h after treatment, and mice were then treated orally with glucose (5 g/kg, 10 mL/kg). Serial blood glucose concentrations were also measured at $t$ = 20, 40, 60, and 120 min after challenge with glucose. The blood glucose excursion profile from $t$ = 0 to $t$ = 120 min was used to integrate the area under the curve (AUC) for each treatment.

**Plasma DPP-IV Activity Inhibition in Sprague−Dawley Rats.** Administration of compounds **1** and **2** to Sprague−Dawley rats was performed by MDS Pharma Services, Saint-Laurent, Quebec, Canada also at pH 2.0 as in the OGTT experiments described above. Male Sprague−Dawley rats (4 per dose) from Charles River Canada, St-Constant, QC, Canada were fasted overnight. A predose sample was collected via jugular venipuncture under isoflurane anesthesia. Compounds **1** or **2** were administered orally by gavage at a dose volume of 2 mL/kg. Following dose administration, blood samples were collected as above at $t$ = 0.5, 1, 2, 4, 8, and 24 h postdose. Blood samples were placed on ice pending centrifugation (3200 g for 10 min at 4 °C). Following centrifugation, plasma was harvested and stored at −20 °C pending shipment. Plasma DPP-IV activity was measured using a Molecular Devices SPECTRAmax $340PC^{384}$ plate reader following 1 h incubation at 25 °C. A typical reaction well has 10 $\mu$L serum sample and 150 $\mu$L of 3 mM H-Ala-Pro-pNA (Bachem) in 0.1 M Hepes, 0.14 M NaCl, pH 8.0. Relative DPP-IV activity was based on the $A_{410}$ of the predose serum sample. For compound **5**, male Sprague−Dawley rats (7−8 weeks) from Charles River Laboratories, Wilmington, MA were housed 3 per cage and given access to normal rat chow (Teklad) and water. A predose blood sample was collected via tail bleed immediately prior to oral treatment with vehicle (pH 2 water) or compound **5** (20, 13, 6.0, 1.0 mg/kg, 10 mL/kg; 6 per dose). Blood samples were collected into heparin tubes at $t$ = 1, 2, 4, 8, 12, and 24 h after administration of compound **5**. Plasma was harvested by centrifugation and stored at −80 °C until analysis. Plasma DPP-IV activity was measured using a continuous fluorometric assay with the substrate Gly-Pro-AMC. A typical reaction well contains 5 $\mu$L plasma sample, 5 $\mu$L incubation buffer (80 mM $MgCl_2$, 25 mM HEPES, 140 mM NaCl, 1% BSA, pH 7.8), and 10 $\mu$L 100 $\mu$M Gly-Pro-AMC. The reaction is incubated for 20 min at room temperature in the dark, and the liberation of AMC is measured using an excitation wavelength of 360 nm with emission at 460 nm by a $VICTOR^3V$ 1420 Multilabel Counter plate reader (Perkin-Elmer). All data are reported as % change DPP-IV activity = - $100(1 - A_{ts}/A_c)$. $A_{ts}$ = activity treated sample; $A_c$ = activity control.

**Rat Toxicity Studies.** Sprague−Dawley rats (7−8 weeks) from Charles River Laboratories, Wilmington, MA were housed 3 per cage. At 8−9 weeks of age, rats were sham dosed with vehicle (pH 2 water, 5 mL/kg) for two days and then administered compounds **2** and **5** by oral gavage (5 mL/kg, 3 male and 3 female per dose) at pH 2.0 as described above for the OGTT experiments. Animals were observed for physical signs of toxicity for up to one week postdose. Any animals with obvious suffering were sacrificed and necropsy was conducted. At termination of study all rats were sacrificed and necropsy conducted. Toxicity study of **1** was previously outsourced to MDS Pharma, Services, Saint-Laurent, Quebec, Canada. This was carried out using 4 male Sprague−Dawley rats (7−9 weeks) per dose. **1** was administered orally at a dose volume of 2 mL/kg. Gross necropsies were performed on all animals exhibiting adverse effects.

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**Supporting Information Available:** SDS-PAGE Coomassie gel and Western blot analysis showing purity of DPP8/V5/His and DPP9/V5/His enzyme preparations as described above. This material is available free of charge via the Internet at http://pubs.acs.org.

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