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Pro-Soft Val-boroPro: A Strategy for Enhancing in Vivo Performance of Boronic Acid Inhibitors of Serine Proteases

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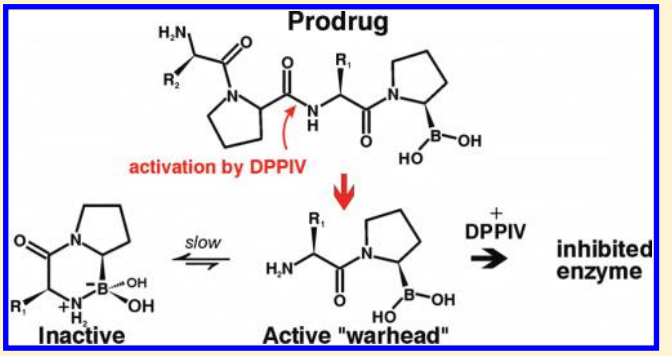
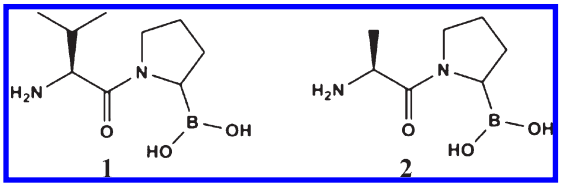
ABSTRACT: Val-boroPro, **1**, is a potent, but relatively nonspecific inhibitor of the prolyl peptidases. It has antihyperglycemic activity from inhibition of DPPIV but also striking anticancer activity and a toxicity for which the mechanisms are unknown. **1** cyclizes at physiological pH, which attenuates its inhibitory potency >100-fold, which is a "soft drug" effect. Here we show that this phenomenon can be exploited to create prodrugs with unique properties and potential for selective in vivo targeting. Enzyme-mediated release delivers **1** to the target in the active form at physiological pH; cyclization attenuates systemic pharmacological effects from subsequent diffusion. This "pro-soft" design is demonstrated with a construct activated by and targeted to DPPIV, including in vivo results showing improved antihyperglycemic activity and reduced toxicity relative to **1**. Pro-soft derivatives of **1** can help to illuminate the mechanisms underlying the three biological activities, or to help localize **1** at a tumor and thereby lead to improved anticancer agents with reduced toxicity. The design concept can also be applied to a variety of other boronic acid inhibitors.



INTRODUCTION

Val-boroPro, (**1**), also known as PT-100[1] or talabostat, and closely related dipeptide boronic acids such as Ala-boroPro (**2**), were first synthesized as inhibitors of dipeptidyl peptidase IV (DPPIV, EC 3.14.5) and were among the first compounds used to interrogate the biological function of DPPIV in vivo and ex vivo.[2,3] Since then, DPPIV has been shown to play a key role in regulating the incretin hormones GLP-1 and GIP and has become a validated target for the treatment of type 2 diabetes.[4,5] Two DPPIV inhibitors, sitagliptin and saxagliptin, have been approved to date by the FDA, vildagliptin has been approved in Europe, and several others are in late stage clinical trials.[6−10]



Although **1** is a potent inhibitor of DPPIV (K_i = 0.18 nM) and exceptionally effective and long-acting in vivo, it was never considered as a clinical candidate for diabetes because it is also quite toxic, especially in Sprague−Dawley rats, where the maximum tolerated dose (MTD) is 0.025 mg/kg.[11] However, early on, **1** was discovered to also have striking anticancer activity in a variety of animal models, even receiving FDA fast track status, but failed to meet its end points in a phase 3 trial of nonsmall cell lung carcinoma for reasons still unclear.[12−14]

The toxicity of **1** may have played a role, as the late-stage cancer patients in the trial could not tolerate more than 800 μg/patient/day, a dose that might have been too low for the anticancer effects.[13] Another possibility, suggested by recent work of Fry and co-workers,[15] is that **1** works well against *early* stage tumors but not *established* tumors. These findings might have rendered **1** of limited interest and value as a cancer drug except that the same group also found **1** to be quite effective in shrinking *established* tumors when combined with a dendritic cell vaccine.

In either case, the anticancer activity of **1** remains of interest but would be of considerably greater interest if it could be separated from the toxicity. A knowledge of the mechanisms would be helpful in assessing whether or not and how this might be achieved, but unfortunately, neither mechanism is well understood. The anticancer activity of **1** appears to be mediated through an immune stimulatory mechanism, but neither the target triggering this activity nor that of the toxicity are known.[1] One certainty is that neither activity is mediated solely through the inhibition of DPPIV, as there are a number of highly potent and selective DPPIV inhibitors that lack both toxicity and anticancer activity.[16,17] Another is that **1** is a relatively nonspecific inhibitor of serine hydrolyses that cleave after proline, inhibiting other members of this group, which include FAP, DPPII, DPP8, DPP9 (usually referred to jointly as DPP8/9 owing to the difficulty in distinguishing between them), and PREP as potently as DPPIV.[11,18,19] These

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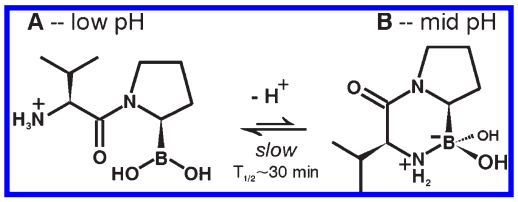
Scheme 1. Cyclization Equilibrium of Val-boroPro



other prolyl peptidases should therefore be considered among the most probable targets for both the anticancer activity and toxicity. FAP inhibition has been reported to yield anticancer effects, but the effects are modest and thus cannot alone account for the profound anticancer activity of **1**.[19,20] Inhibition of DPP8/9 has been implicated in both the anticancer activity[21] and toxicity[16] of **1**. However, their role in these activities is doubtful, as DPP8/9-specific inhibitors exist but have not been reported to trigger immune responses or to demonstrate anticancer activity in vivo, while several groups have challenged the hypothesis that DPP8/9 inhibition causes toxicities.[22,23]

Compound **1** has some unique chemical properties that could be utilized to separate the anticancer activity from the toxicity and to shed light upon the underlying mechanisms. In aqueous solution, **1** undergoes a reversible, pH-dependent equilibration (Scheme 1) between an open chain, enzyme−inhibitory form (**A**) and a cyclic, inactive form (**B**). The open chain structure predominates at low pH, but as the pH is raised and the N-terminal amino group deprotonates, the cyclic structure becomes increasingly favored.[3,24] A localized, unshared electron pair on the P2 amino nitrogen is required for cyclization, as N-terminally alkylated derivatives such as *N*-methyl-Val-boroPro will cyclize, but N-terminally acylated derivatives, such as Ac-Val-boroPro or X_{aa}-Val-boroPro tripeptides, will not. The cyclization reaction is relatively slow for what is essentially a conformational change ($T_{1/2} \sim 30$ min at physiological pH) but consistent with a prolyl peptide bond isomerization.[24]

At physiological pH, the equilibrium favors the cyclic over the open chain form by more than 2 orders of magnitude.[24] This reduces the pharmacological activity of **1** by a factor approaching this amount under equilibrium conditions which would likely obtain following systemic administration. The loss in pharmacological activity with time is characteristic of the action of "soft drugs," drugs designed to deactivate in a predictable and controlled manner to prevent unwanted systemic effects after exerting their therapeutic effects.[25] **1** could therefore be deployed as a soft drug. To do so beneficially would require that it be applied directly to the site of intended action, the modus operandi for all soft drugs. Such application is often difficult, and **1** presents an additional problem: it would need to be applied in a low (≤2) pH preparation to ensure sufficient excess of the open chain species and the low pH could cause unwanted effects.

However, having a free amino group, **1** is also amenable to incorporation into the C-terminus of a longer peptide or other entity, from where it could be released by the action of a selected protease. Such release would deliver **1** to the site of the activating protease in the open-chain form with a conformational purity unmatchable by the most sedulous deployment of **1** directly to the site. The effectiveness of the delivery will, of course, depend on the degree to which the activating protease is localized at the site and the specificity with which the construct is activated by the intended protease. The slow rate of cyclization following release

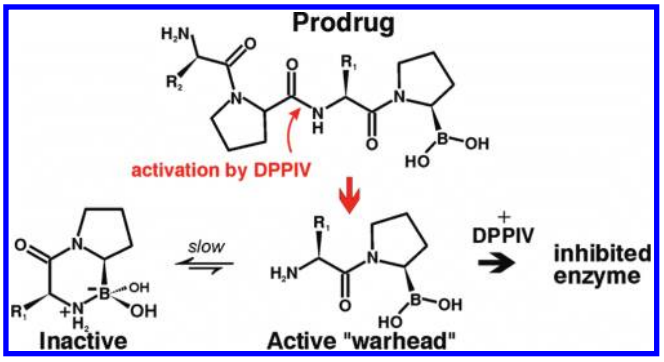


Figure 1. Mechanism of pro-soft inhibition of DPPIV. Once the systemically inactive pro-soft drug encounters the DPPIV target enzyme, the N-terminal dipeptide is cleaved, releasing the linear dipeptide inhibitor in close proximity to its target enzyme. Any inhibitor that diffuses away, however, undergoes a pH-dependent cyclization, thereby attenuating potential systemic activity.

should then allow for a substantial pharmacological effect at the site of release while attenuating effects at more distant sites.

Prodrugs of soft drugs have previously been termed "pro-soft" drugs. Although the pro-soft concept has been discussed previously, there are few working examples and there has been little exploration of their potential.[26] **1** is well suited for testing such potential (Figure 1). With three distinct biological activities and six known targets, a wide variety of pro-soft constructs of **1** can be envisaged. Constructs designed to deliver and localize **1** at targets thought to mediate toxicity or anticancer activity could help to illuminate the underlying mechanisms. Constructs designed to deliver and localize **1** at a tumor could be a way to obtain the full anticancer activity while reducing toxicity, even if the mechanisms underlying toxicity and the anticancer effects are the same.

Here we report the in vitro and in vivo characterization of **3** (Chg-Pro-Val-boroPro), a prodrug of **1**, which is activated by and targeted to DPPIV. This construct was chosen because it should permit convenient and expeditious evaluation of the performance of the pro-soft design in vivo. DPPIV is an established target for the treatment of type 2 diabetes. Inhibition of DPPIV will have antidiabetic activity, a far easier action to measure than anticancer activity. Furthermore, DPPIV inhibition is clearly not responsible for the toxicity of **1**. Thus, a DPPIV-activated construct of **1**, by working to direct **1** toward DPPIV and away from other potential toxicity-inducing targets, should have a better therapeutic index with respect to blood glucose lowering than **1**. The results demonstrate that **3** does indeed have the pro-soft properties outlined above in vitro and in vivo appears to be both safer and more effective as an antihyperglycemic agent than **1**. The significance of these results and their implications for other pro-soft agents based upon **1** and other boronic acid inhibitors targeting other enzymes are discussed.

RESULTS AND DISCUSSION

Effects of Cyclization on Enzyme Inhibition in Vitro. The inhibitory potency of **1** depends on the pH of pre-equilibration prior to addition to standard enzyme inhibition assays buffered at pH 7.4. The dipeptide boronic acid inhibitors, **1** and **2**, and their corresponding prodrugs, **3** and **4** (Sch 2), were preincubated at either pH 2.0 or pH 7.4 and assayed for DPPIV inhibition in vitro. As shown in Figure 2 and Table 1, **1** pre-equilibrated at pH 2.0 yields an IC_{50} value ~300-fold lower than when pre-equilibrated

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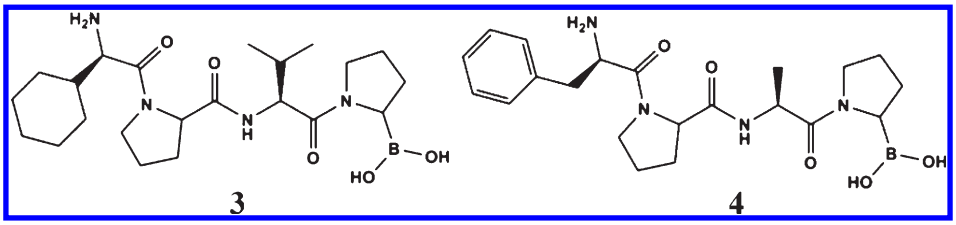
Scheme 2. Pro-Soft Drugs Chg-Pro-Val-boroPro (3) and Phe-Pro-Ala-boroPro (4)



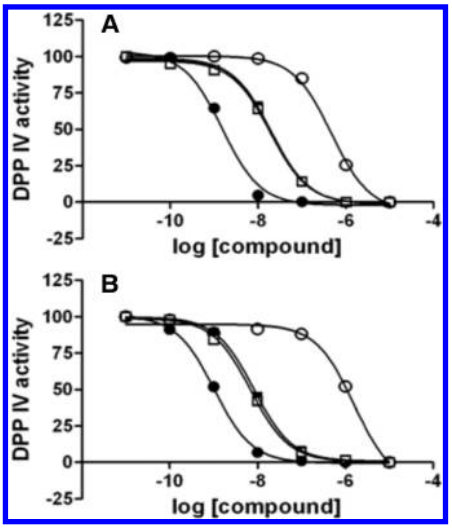


Figure 2. pH independence of smarter protease inhibitors in vitro. (A) Dipeptide boronic acid inhibitor **1** (circles) and its corresponding pro-soft drug **3** (squares). Open for pH 7.4 and closed for pH 2.0. (B) Dipeptide boronic acid inhibitor **2** (circles) and its corresponding pro-soft drug **4** (squares). Open for pH 7.4 and closed for pH 2.0.

Table 1. IC_{50} Values for Dipeptide Boronic Acid Inhibitors 1 and 2 and Corresponding Pro-Soft Drugs 3 and 4 at Various Pre-equilibrium pH Values

compd	IC_{50} (nM) pH 2.0	IC_{50} (nM) pH 7.4
1	1.5	470
2	1.0	1600
3	20	19
4	8.3	7.0

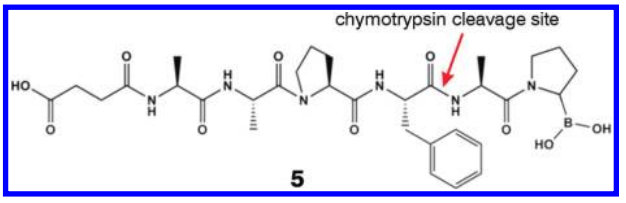
at pH 7.4. The effect is fully reversible; adjusting the pH 7.4 pre-equilibration solution to pH 2.0, and incubating for several hours regenerates the inhibitor potency.

The difference in IC_{50} values between pH 2.0 and 7.4 solutions reflects the different position of the cyclization equilibrium at the two pH values. That this difference can be so readily observed in conventional enzyme assays demonstrates the slowness of the cyclization reaction. It also provides a convenient method for measuring the position of the equilibrium. The closely related inhibitor, **2**, exhibits the same phenomenon but with a greater difference in IC_{50} values (~1600-fold) indicating that its cyclic form is even more favored at pH 7.4 than it is for **1**.

Pro-Soft Drug Activation by DPPIV in Vitro. The design strategy outlined above together with known cyclization rates predict that inhibition mediated by prodrugs releasing **1** or **2** should appear to be pH-independent in standard inhibition assays. Inhibition kinetics of **3** and **4** confirm that this is indeed the case (Figure 2). Pre-equilibration of **3** and **4** at pH 2.0 or pH 7.4 has no effect on the "observed IC_{50}" values, in striking contrast to what is observed with the corresponding dipeptide inhibitors themselves. The pH independence cannot be attributed to **3** and **4** themselves mediating the inhibition. Peptides of boroPro larger than dipeptides, such as **3** and **4**, are not DPPIV inhibitors because they do not contain the requisite free amino group at P2. The inhibition observed for **3** and **4** is due entirely to released **1** and **2**, respectively.

The pH independent inhibition confirms that the inhibitors are released in the same configuration regardless of pre-equilibration pH, as expected. Moreover, that the IC_{50} values of **3** and **4** are more similar to those of **1** and **2** pre-equilibrated at pH 2.0 than at 7.4 fits with the expectation that the inhibitors would be released in the open-chain and enzyme−inhibitory configuration, even at pH 7.4. The observation that the apparent IC_{50} values for the prodrugs are slightly higher than for the corresponding free dipeptide inhibitors preincubated at pH 2.0 can be ascribed largely to the kinetics of activation, not to cyclization. Activation is not instantaneous, and the rate of release slows down as DPPIV becomes inhibited. Both factors will work to attenuate the observed "tetrapeptide IC_{50}s" relative to that of the corresponding dipeptide inhibitors added directly.

Soft-Drug Action Following Activation. Demonstrating soft drug action from postactivation cyclization is problematic in a homogeneous system when the activating enzyme and target enzyme are the same, as is case for the DPPIV-mediated activation of **3** and **4**, because in this case there is no spatial or temporal separation between activation and inhibition in which cyclization can take place and be measured. **5** (Suc-Ala-Ala-Pro-Phe-Ala-boroPro) was therefore designed and synthesized to enable the separation needed to demonstrate the soft drug action. **5** does not itself inhibit DPPIV, nor can it be activated by DPPIV to release the DPPIV inhibitor, **2**. It also does not inhibit chymotrypsin, but it can be cleaved by chymotrypsin after the phenylalanine residue to release **2.** Monitoring the loss of DPPIV inhibitory potency of a solution containing **5** as a function of time following activation by chymotrypsin should provide a demonstration, and measurement, of the soft drug action.



Compound **5** was allowed to preincubate with chymotrypsin for varying lengths of time prior to the addition of DPPIV and measurement of DPPIV inhibitory activity. Prior to the

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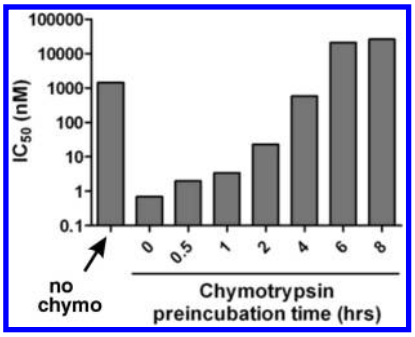

Figure 3. Time course of pH dependent inactivation of a dipeptide boronic acid inhibitor following prodrug activation. IC_{50} values for compound **5** were measured in vitro in the absence of chymotrypsin or following preincubation with chymotrypsin for varying periods of time.

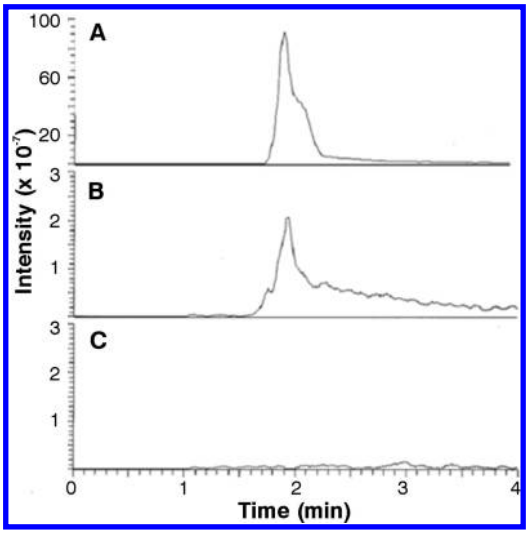

Figure 4. Pro-soft drug cleavage in vitro, monitoring for the dipeptide metabolite Chg-Pro. Activation of the pro-soft drug, **3**, in the presence (B), or absence (C) of purified DPPIV. Purified Chg-Pro was run as a reference (A).

addition of chymotrypsin, the solution containing **5** had little DPPIV inhibitory activity. Immediately following the addition of chymotrypsin, the solution becomes very potently DPPIV inhibitory, yielding an IC_{50} value of 0.7 nM with respect to the amount of **5** contained in the solution. The DPPIV potency of the solution then decreases with time, as expected for cyclization of the DPPIV inhibitor, **2** (Figure 3). This experiment nicely demonstrates the workings of a pro-soft construct in its entirety, from the initial pharmacological inactivity prior to activation, through the hyper-potent phase immediately following activation, to the loss of inhibitory potency with longer postactivation times.

Direct Evidence for DPPIV-Mediated Activation in Vitro and DPPIV Selectivity in Vivo. The release of **1** is difficult to directly monitor and measure in vitro and especially in vivo using mass spectrometry for several reasons, including cyclization, high affinity enzyme binding, and the properties of the major ion. In contrast, Chg-Pro, the other product of DPPIV activation, or the intact prodrug itself, **3**, are relatively easy to directly observe and monitor using mass spectrometry. Figure 4 shows that Chg-Pro is rapidly formed from **3** following incubation with, but not without, DPPIV in vitro, providing direct evidence for the DPPIV-mediated activation of **3**.

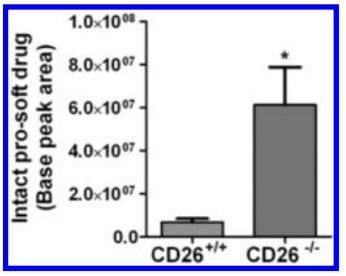

Figure 5. Cleavage of **3** in wild-type ($CD26^{+/+}$) and DPPIV knockout ($CD26^{-/-}$) mice. Levels of intact **3** in serum from wild-type and DPPIV knockout mice previously given an intraperitoneal injection of the molecule were monitored via LC/MS (n = 3). The compound was administered 5 min prior to serum collection. *, $P < 0.05$. Error bars show mean ± SEM.

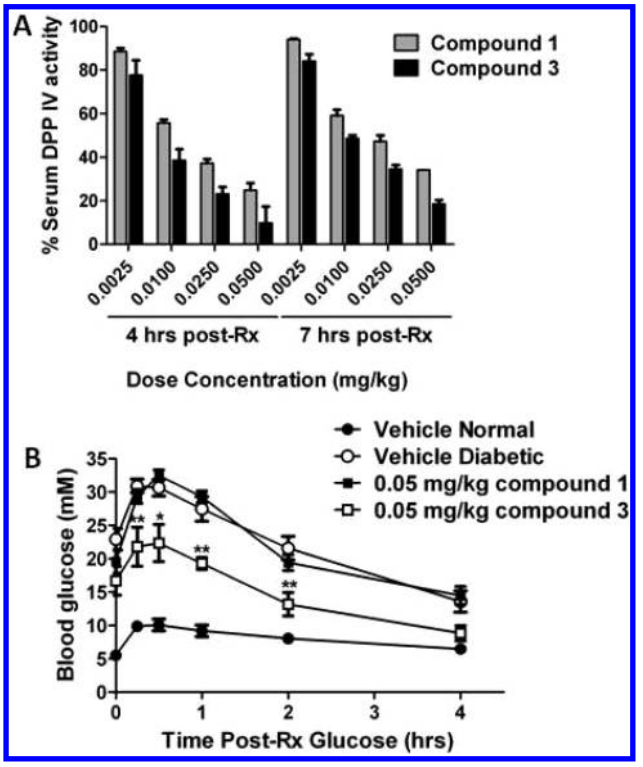

Figure 6. Comparison of the in vivo performance of a dipeptide inhibitor (compound **1**) vs a pro-soft drug (compound **3**) in db/db mice. Serum DPPIV activity both 4 and 7 h following oral administration of 0.0025, 0.01, 0.025, or 0.05 mg/kg of **1** or **3** (n = 2, except for 0.5 mg/kg **1**, where n = 1) (A). Blood glucose levels following an oral glucose tolerance test in vehicle treated wild-type, vehicle treated db/db, and db/db mice administered 0.05 mg/kg **1** or **3** (n = 5) (B). The compounds were administered three hours prior to the oral glucose challenge. *, $P < 0.05$ and **, $P < 0.001$ versus diabetic control. Error bars show mean ± SEM.

Although the above experiment shows that DPPIV is capable of activating **3**, it does not address whether or not other enzymes might also be able to activate this prodrug, a key factor that will affect its performance in vivo. To address this issue, the concentration of **3** was determined in the serum of DPPIV (CD26) wild-type and knockout mice following intraperitoneal injection (Figure 5). Significantly larger amounts of **3** were detected in the serum of knockout mice than in wild-type mice, providing not only direct evidence for DPPIV-mediated activation but also some measure of DPPIV selectively in vivo.

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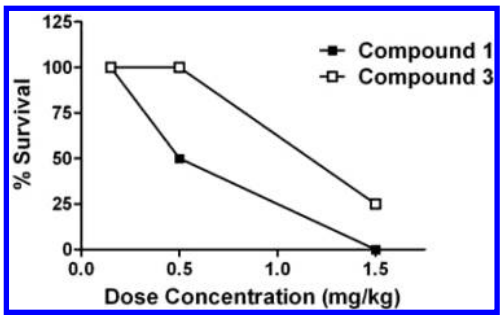

Figure 7. Toxicity of the pro-soft drug (compound **3**) vs the dipeptide inhibitor (compound **1**) in rats. Percent survival after 24 h was assessed in Fischer rats following oral administration of 0.15, 0.5, or 1.5 mg/kg of **1** or **3** ($n = 4$).

In Vivo Performance. To evaluate the in vivo performance of **3**, its ability to inhibit serum DPPIV activity and to lower the area under the curve (AUC) in an oral glucose tolerance test (OGTT) was compared to that of **1** at four different orally administered doses in db/db mice. The doses of **3** are given in mg/kg of contained **1** so as to be directly comparable. While both compounds were quite effective at inhibiting serum DPPIV and both exhibited dose-dependent inhibition over the range examined, **3** was the more effective at every dose and time point tested (Figure 6A). Furthermore, **3** was able to bring about a significant lowering of the AUC following a glucose challenge at 0.05 mg/kg, a dose at which **1** exhibited little or no effect (Figure 6B). Thus, the prodrug of **1**, **3**, produces more effective **1**-mediated inhibition of DPPIV in vivo and more effective antihyperglycemic activity than **1** itself. A 3-fold higher dose of **1** would be required to produce an equivalent effect on the OGTT.

Pro-Soft Drug Toxicity vs 1 Alone. The toxicities of **1** and **3** were compared in a dose escalation study in Fischer rats, the most sensitive species and most reliable indicator of the severe toxicity of **1**. We and others have previously found that in rats **1** has an LD_{50} of ~0.5 mg/kg. Figure 7 confirms the LD_{50} for **1** while showing that the equivalent dose of **3** exhibited no obvious toxicity. The LD_{50} for **3** appears to be approximately 2- to 3-fold higher.

Although **3** is both safer and more effective than **1** as an antihyperglycemic agent, the gains are smaller than might have been hoped for. DPPIV is widely distributed throughout the body and therefore is probably not the optimal target for showcasing the workings of a drug concept designed to focus pharmacological activity at a selected site. A tumor, for example, would provide a better target and a construct that is selectively activated at the tumor should show greater gains in efficacy and safety. Nevertheless, even the modest gains of the magnitude achieved here might be sufficient to make a tumor-selective construct an attractive clinical candidate for cancer. The design and testing of such tumor-specific pro-soft derivatives of **1**, and of other boronic acids that have anticancer activity such as bortezomib, is underway.

CONCLUSIONS

Here we have demonstrated that the pH dependent cyclization reaction intrinsic to **1** confers unique properties on prodrugs of **1**, including a soft drug like activity such that prodrugs of **1** are actually pro-soft drugs. The workings of the pro-soft design is demonstrated in vitro and in vivo with a construct activated by and targeted to DPPIV. The concept outlined here is not limited to constructs of **1** but can be applied to wide variety of other inhibitors and targets. Perhaps the most useful applications will be in tissue-specific targeting. For example, bortezomib is a dipeptide boronic acid approved for the treatment of multiple myeloma.[27] It targets the proteasome, an enzyme complex found in all cells and tissues, and has a very narrow therapeutic window, most likely from toxicities arising from on-target inhibition in off-target normal cells and tissues. This perhaps explains why bortezobib has thus far not shown efficacy against solid tumors. The pro-soft strategy, with its potential for tissue-specific targeting, could help to make proteasome inhibition an effective strategy against solid tumors.

EXPERIMENTAL SECTION

Synthesis of Boronic Acid Dipeptides. Synthesis of all X_{aa}-boroPro dipeptides was performed essentially as described previously, except that the N- and C-terminal protection groups of the N-Boc-(*S*)-X_{aa}-(*R*)-boroPro-(1*S*,2*S*,3*R*,5*S*)-pinanediol ester products were removed simultaneously.[18] Specifically, to a stirred solution of the protected product in CH_2Cl_2 cooled to −78 °C, an equimolar amount of a 1.0 M solution of BCl_3 in CH_2Cl_2 was added. After 1 h, the product was extracted into water and purified on a Supelco Discovery-C18 column, eluting with water (0.1% TFA) and acetonitrile (0.08% TFA). Lyophilization yielded the free dipeptide boronic acids, the identities of which were confirmed by ^{1}H NMR and LC/MS, using the latter to assess a level of relative purity ≥95%.

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. 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 μm) 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, 2−98% B over 10 min and 98%B for the final 10 min.

Valinyl-L-boroproline (1). ^{1}H NMR (D_2O) δ 0.98 (d, $J = 7.0$ Hz, 3H, CH_3CHCH_3), 1.08 (d, $J = 7.0$ 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, $J = 6.5$ Hz, 1H, H_2NCHCO). LC-MS (ESI$^+$) m/z (rel intensity): 393.3 ($[2 \times (M - H_2O) + H]^+$, 60); 197.1 $[M - H_2O + H]^+$, tr = 11.1 min.

Alaninyl-L-boroproline (2). ^{1}H NMR (D_2O) δ 1.48 (d, $J = 7.0$ 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, $J = 7.0$ Hz, 1H, H_2NCHCO). LC-MS (ESI$^+$) m/z (rel intensity): 337.2 $[2 \times (M - H_2O) + H]^+$; 169.0 $[M - H_2O + H]^+$, tr = 5.9 min.

Synthesis of Free Boronic Acid Pro-Soft Drugs. All protected amino acids, with the exception of the boroPro derivative, which was synthesized as described above, were purchased from Novabiochem. Peptide coupling reactions were performed twice per final product, with N-Boc-X_{aa}-OH and H-X_{aa}-OMe used for the N-terminal dipeptide and N-Boc-X_{aa}-OH and the H-boroX_{aa}-(1*S*,2*S*,3*R*,5*S*)-pinanediol ester for the C-terminal dipeptide. To a stirred solution of the N-terminal protected product in a 1:1 solution of water:THF at 0 °C, 5 equiv of LiOH were added. The reaction was quenched after 1 h with 12 N HCl and the product extracted into EtOAc. Evaporation of the solvent yielded the crude N-Boc-$(X_{aa})_2$-OH product. The Boc group of the C-terminal dipeptide was removed as described previously, yielding the H-X_{aa}-boroPro pinanediol ester. The N- and C-terminal dipeptides were coupled as described previously, yielding the crude N-Boc-$(X_{aa})_3$-boroPro pinanediol ester. The protection groups were removed as previously described for the dipeptide inhibitors, yielding the boronic acid pro-soft drugs, the identities of which were confirmed by ^{1}H NMR and LC/MS, using the latter to assess a level of relative purity ≥95%.

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Cyclohexyl(glycinyl)-prolinyl-valinyl-L-boroproline (3). ^{1}H NMR (D_2O) δ 0.98 (6H, CH_3CHCH_3), 1.10−1.80 (11H, $H_2NCHCH_2CH_2CH_2CH_2CH_2$ and CH_2CHB), 1.84−2.29 (9H, CH_2CH_2CHCO, CH_2CH_2CHB, and CH_3CHCH_3), 2.95 (1H, CH_2CHB), 3.52−3.82 (4H, NCH_2CH_2 and $CH_2CH_2CH_2CHB$), 4.15 (1H, H_2NCHCO), 4.38 (1H, $HNCHCO$), 4.53 (1H, $NCHCO$). LC-MS (ESI^+) m/z (rel intensity): 433.3 $[M - H_2O + H]^+$, tr = 12.7 min.

Phenylalaninyl-prolinyl-alaninyl-L-boroproline (4). ^{1}H NMR (D_2O) δ 1.38 (3H, $HNCHCH_3$), 1.69−2.29 (8H, CH_2CH_2CHCO and CH_2CH_2CHB), 3.01 (1H, CH_2CHB), 3.14−3.82 (6H, NCH_2CH_2, $CH_2CH_2CH_2CHB$, and H_2NCHCH_2C), 4.03 (1H, H_2NCHCO), 4.24 (1H, $HNCHCO$), 4.52 (1H, $NCHCO$), 7.38 (5H, aromatic H). LC-MS (ESI^+) m/z (rel intensity): 413.3 $[M - H_2O + H]^+$, tr = 12.1 min.

Synthesis of the Succinylated Boronic Acid Pro-Soft Drug. Following synthesis of the appropriate N-Boc-Ala-Ala-Pro-Phe-Ala-boroPro-(1*S*,2*S*,3*R*,5*S*)-pinanediol ester as using the pro-soft drug synthesis protocol described above, the Boc group was removed as before. The resulting H-Ala-Ala-Pro-Phe-Ala-boroPro pinanediol ester was dissolved in a 5:1 solution of CH_2Cl_2:DMF. This was treated with 1.25 equiv of succinic anhydride and TEA, yielding the Suc-Ala-Ala-Pro-Phe-Ala-boroPro pinanediol ester after 18 h. Removal of the pinanediol group yielded the free final product, the identity of which was confirmed by ^{1}H NMR and LC/MS, using the latter to assess a level of relative purity ≥95%.

Succinyl-alaninyl-alaninyl-prolinyl-phenylalaninyl-alaninyl-L-boroproline (5). ^{1}H NMR (D_2O) δ 1.32 (9H, H_2NCHCH_3 and $HNCHCH_3$), 1.54−2.09 (8H, CH_2CH_2CHCO and CH_2CH_2CHB), 2.53−2.62 (4H, $HOCOCH_2CH_2$), 2.96 (1H, CH_2CHB), 3.13−3.72 (6H, NCH_2CH_2, $CH_2CH_2CH_2CHB$, and H_2NCHCH_2C), 4.24−4.56 (5H, H_2NCHCO, $HNCHCO$, and $NCHCO$), 7.38 (5H, aromatic H). LC-MS (ESI^+) m/z (rel intensity): 637.6 $[M - 2\cdot H_2O + H]^+$, tr = 14.0 min.

In Vitro DPPIV Enzyme Assay. Enzymatic activity of purified DPPIV was measured at either 25 or 37 °C on a Molecular Devices SPECTRAmax 340PC^{384} microtiter plate reader, monitoring the absorbance at 410 nm and using H-Ala-Pro-pNA, purchased from Bachem, as the chromogenic substrate. The reaction mixture contained 0.3 mM substrate, approximately 1 nM DPPIV, 0.1 M HEPES, 0.14 M NaCl buffer, pH 7.4 or 8.0, and a suitable amount of inhibitor or pro-soft drug (ranging between 10^{-5} and 10^{-11} M) in a total volume of 310 μL.

The IC_{50} value is defined as the concentration of inhibitor required to reduce the DPPIV activity by 50% after a 10 min preincubation with the enzyme at 25 or 37 °C prior to addition of the substrate. Inhibitor stock solutions were prepared in either HCl solution, pH 2.0, or buffer, pH 7.4 or 8.0. Stock solutions were diluted with 0.1 M HEPES, 0.14 M NaCl buffer, pH 7.4 or 8.0, as required, immediately prior to the commencement of the experiment.

α-Chymotrypsin/DPPIV Enzyme Assay. The assay was similar to the DPPIV enzyme assay, except that the pro-soft drug was incubated with 0.1 mM α-chymotrypsin, purchased from Sigma, for 0, 0.5, 1, 2, 4, 6, or 24 h prior to the addition of the purified DPPIV enzyme.

Pro-Soft Drug Cleavage by Purified DPPIV in Vitro. The following three reactions were set up, all of which were prepared in 0.1 M HEPES, 0.14 M NaCl buffer, pH 8.0 DPP IV buffer:

Enzyme/dipeptide control: 100 μL of 2 μM DPP IV stock, 100 μL 20 mM Chg-Pro.

No enzyme control: 100 μL of DPP IV buffer, 100 μL 10 mM **3**.

Enzyme/pro-soft drug sample: 100 μL DPP IV stock, 100 μL 10 mM **3**.

The samples were incubated for 10 min at 25 °C, following which they were transferred to a C_{18} column, eluted with 5 mL 2% acetonitrile, and lyophilized. The samples were analyzed by reverse-phase HPLC-MS on a Thermo Finnigan LCQ Duo, scanning an m/z range of 254.5−255.5, which corresponded to the mass of the Chg-Pro dipeptide.

Assay for Pro-Soft Drug Cleavage in Vivo. Three wild-type C57BL/6 ($CD26^{+/+}$) or DPPIV knockout ($CD26^{-/-}$) mice[28] were acclimated for at least one week prior to the study. The mice were given an intraperitoneal injection of 50 μg of **3** in 400 μL PBS. After 5 min, blood samples were collected via cardiac puncture, treated with 10% TCA, and spun down. The resulting supernatant was collected and the samples analyzed by reverse-phase HPLC-MS on a Thermo Finnigan LCQ Duo, quantifying the peaks corresponding to the intact pro-soft drug in the resulting base peak chromatograms.

Serum DPPIV Activity Assay. To 10 μL of the serum samples provided by MDS Pharma Services, 150 μL of 3 mM H-Ala-Pro-pNA Bachem in 0.1 M HEPES, 0.14 M NaCl buffer, pH 8.0 was added. The absorbance at 410 nm was measured on a Molecular Devices SPECTRAmax 340PC^{384} plate reader following a 1 h incubation at 25 °C. Relative DPPIV activity was based on the A_{410} of the diabetic control equaling 100% serum enzyme activity.

Oral Glucose Tolerance Test in Diabetic Mice. The following experiment was performed by MDS Pharma Services, Saint-Laurent, Quebéc, Canada. Briefly, 21 diabetic male mice (C57BL/KS01HSD-Leprdb) and 7 nondiabetic male mice (C57BL/6NHHsd) were acclimated for 3 weeks prior to the study. Following a 4 h fasting period, 7 mice per group were dosed via oral gavage with 2 mL/kg of a 5.8, 23.3, 58.3, or 116.7 μM solution of either **1** or **3**. This is equivalent to dosing each animal with 0.0025, 0.01, 0.025, or 0.05 mg/kg of free inhibitor. As controls, 7 diabetic and 7 nondiabetic mice were treated with vehicle (H_2O) alone. Three hours following dose administration, a single oral dose (1 g/kg) of glucose (Dextrose USP, Abbott Laboratories) was administered. At predose, 15, 30, 60, 120, and 240 min post-Rx glucose, blood samples were collected from 5 mice/group for blood glucose testing on a calibrated hand-held glucometer, and serum samples were collected from the remaining 2 mice/group at 60 and 240 min post-Rx glucose.

Toxicity Study in Fisher Rats. The following experiment was performed by the Tufts Division of Laboratory Animal Medicine. Briefly, 24 male Fischer rats (F344) were acclimated for 1 week prior to the study. Four rats per group were dosed by oral gavage with 2 mL/kg of a 0.35, 1.17, or 3.5 mM solution of either **1** or **3**. This is equivalent to dosing each animal with 0.15, 0.5, or 1.5 mg/kg of free inhibitor. The animals were monitored over a 24 h period, during which time the mortality rate was recorded.

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ABBREVIATIONS USED

GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; DPPIV, dipeptidyl peptidase IV; DPP8, dipeptidyl peptidase 8; DPP9, dipeptidyl peptidase 9; DPPII, dipeptidyl peptidase II; FAP, fibroblast activation protein; PREP, prolyl oligopeptidase; Chg, cyclohexyl(glycine); MTD, maximum tolerated dose; K_i, inhibition constant; IC_{50}, median inhibition concentration

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