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Structural Optimization, Biological Evaluation and Application of Peptidomimetic Prostate Specific Antigen Inhibitors

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Abstract

Prostate-Specific Antigen (PSA) is a serine protease produced at high levels by normal and malignant prostate epithelial cells that is used extensively as a biomarker in the clinical management of prostate cancer. To better understand PSA's role in prostate cancer progression we prepared a library of peptidyl boronic acid based inhibitors. To enhance selectivity for PSA vs. other serine proteases, we modified the P1 site of the inhibitors to incorporate a bromopropylglycine group. This allowed the inhibitors to participate in halogen bond formation with the serine found at the bottom of the specificity pocket. The best of these Ahx-FSQn(boro)Bpg had PSA K_i of 72 nM and chymotrypsin K_i of 580 nM. *In vivo* studies using PSA-producing xenografts demonstrated that candidate inhibitors had minimal effect on growth but significantly altered serum levels of PSA. Biodistribution of ¹²⁵I labeled peptides showed low levels of uptake into tumors compared to other normal tissues.

Keywords

Prostate cancer; PSA inhibitors; boronic acids; peptidyl-boronic acid; peptidomimetics; serine protease inhibition; chymotrypsin inhibition

INTRODUCTION

A hallmark of prostate cancer is the production of the prostate tissue differentiation protein Prostate-Specific Antigen (PSA).^{1,2} PSA is aptly named in that its expression is highly restricted to normal and malignant prostate cells in men.³ Although controversy exists over the use of PSA as a screening tool for prostate cancer, it continues to be a useful biomarker for the detection of cancer recurrence after localized therapy and as a marker of therapeutic response in the metastatic setting.⁴ PSA is found in high $\mu\text{g/mL}$ to mg/mL amounts in the

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extracellular fluid where it exists in an enzymatically active state.⁵ Upon entering the circulation, PSA is rapidly inhibited by the protease inhibitors α_2 -macroglobulin and α_1 -antichymotrypsin that are present in molar excess in the serum.^{6,7} This functional and quantitative difference in PSA compartmentalization has resulted in strategies to target PSA's enzymatic activity for prodrug/protoxin activity^{8,9} and to the design of antibodies directed at PSA's catalytic site for potential use as imaging agents¹⁰.

Due to the presence of the characteristic His-Asp-Ser catalytic triad, PSA is classified as a serine protease. For all serine proteases, substrate and inhibitor recognition is mainly governed by the binding of the P1 residue to the S1 pocket of the enzyme. PSA (i.e. KLK3) is also one of 15 members of the kallikrein family of proteases.³ Most of the other kallikreins have trypsin-like proteolytic activity due to the presence of an aspartic acid residue at the bottom of the S1 specificity pocket.^{3,4} In contrast, PSA is considered a chymotrypsin-like protease due to the presence of a serine at the bottom of the S1 pocket (i.e. Ser189).¹¹ However, while PSA has some similarity to chymotrypsin in its preference for hydrophobic amino acids at the P1 position, PSA also displays enzymatic properties that differentiate it from chymotrypsin and other serine proteases. In particular, PSA demonstrates preference for glutamine in the P1 position that is a unique characteristic compared to chymotrypsin¹², which has no ability to cleave after glutamine. Chymotrypsin is also not inhibited appreciably by peptide aldehyde inhibitors containing glutamine or glutamine analogs in the P1 position.¹¹

Previously, using docking studies based on crystal structure data we documented important differences in the physical and electrostatic characteristics of the S1 pocket of these two serine proteases that explain this differential preference for P1 glutamine on the part of PSA.¹¹⁻¹⁴ In the case of PSA, the presence of polar chains provides a noticeable polar character to its shallow bottomed pocket. In contrast, the S1 cavity of chymotrypsin is less polar and more spacious. A comparative analysis of the binding modes of the peptide aldehyde inhibitor SSKLQ-CHO docked in the S1 pocket of PSA and chymotrypsin respectively revealed drastic differences in orientation of the glutamine side chain that underlies the marked difference in K_i values for this inhibitor for these two proteases (PSA $K_i = 45 \pm 4 \mu\text{M}$ vs. chymotrypsin $K_i < 1000 \mu\text{M}$).¹¹

These modeling studies suggested that a P1 glutamine based PSA inhibitor would be the most selective for PSA vs. other chymotrypsin-like proteases. Previously, we generated boronic acid analogs of leucine and norleucine and determined that the substitution of boronic acid for aldehyde as inhibitory group greatly decreased the K_i for PSA inhibition. Boronic acid was selected as the inhibitory moiety based on selectivity for serine protease inhibition and due to the fact that a boronic acid based inhibitor bortezomib has achieved FDA approval as cancer therapy. However, while boronic acid analogs of these P1 preferred amino acids can be generated, the synthesis of a boronic acid analog of glutamine is not feasible.¹⁴ Previous studies demonstrated that an aldehyde analog of methionine in the P1 position produced an inhibitor with a K_i of $3.8 \mu\text{M}$ for PSA inhibition but a K_i of $>100 \mu\text{M}$ for chymotrypsin.¹¹ Based on this previous data, in this study we describe our efforts to produce more potent and potentially more selective PSA inhibitors by generating a series of peptidyl based inhibitors containing a boronic acid analog of 3-bromopropylglycine (Bpg) in the P1 position. Bpg resembles Met and Gln as they have very similar side chain lengths. Like Met, the side chain of Bpg terminates in a bulky electron rich atom that can form hydrogen bonds. We compared the ability of Bpg inhibitors vs. inhibitors containing a boronic acid analog of phenylalanine in the P1 position to inhibit both PSA and chymotrypsin. Selective PSA inhibitors were further evaluated *in vitro* for ability to effect the growth of PSA-producing vs. non-producing prostate cancer cell lines. Further optimization of selected inhibitors was achieved through modification of the P5 position of

the peptide to enhance circulating half-life. These inhibitors were labeled with ^{125}I to assess biodistribution *in vivo* and to evaluate their potential for imaging of prostate cancers. Finally, we assessed the effect of the lead inhibitor on the *in vivo* growth and PSA production of human prostate cancer xenografts.

RESULTS

For the synthesis of the (boro)phenylalanine containing peptidomimetics, the building block (*R*)-borophenylalanine-(1*S*,2*S*,3*R*,5*S*)-(+)-2,3-pinane-1,2-diol ester **5** was synthesized (Scheme 1) using modified literature precedence originally developed by Matteson and Winssinger.^{15,16} The (boro)bromopropylglycine analogs were prepared using commercial (1*R*)-1-amino-4-bromobutan-1-ylboronic acid (1*S*,2*S*,3*R*,5*S*)-(+)-2,3-pinane-1,2-diol ester **6** (Scheme 2). All peptides, containing only *L*-amino acids, were synthesized using Fmoc solid phase peptide synthesis. All peptidyl boronic acid inhibitors were prepared by amide bond formation between the fully protected peptide and the pinane-1,2-diol protected boronic acid (Scheme 2). Consequently, the side chains of the peptide were deprotected and in majority of the sequences this was followed by boronic acid deprotection (Scheme 2). For compounds **21** and **23** (Scheme 4) the ester protection of the acid was not deprotected and is abbreviated as boro-Pin. Table 1 lists the inhibitor sequences synthesized for these studies. All final products were purified by HPLC and characterized by MS (Table 1). The first set of peptides have their N-terminus capped by Cbz and were prepared to determine whether peptides that included boronic acid analog of Phe **12** or Bpg **13** maintained the ability to inhibit PSA's enzymatic activity (Table 2). In the subsequent peptide inhibitors, aminohexanoic acid (Ahx) was incorporated at the N-terminus of the peptide to serve as a linker whereby other groups such as chelating groups or radiohalogenated prosthetic groups could be attached.

The binding efficiency of protease substrates and inhibitors is highly dependent on the amino acid residues binding to the corresponding pockets within the vicinity of the catalytic site as illustrated in Scheme 3. The binding specificity is highly dependent on the amino acid residue present at the P1 position. The structural models of the PSA active site suggest a hydrophobic character for the sides of the specificity pocket and a polar character for the bottom of the pocket¹³, where the polar hydroxyl of Ser189 is flanked by the polar hydroxyl side chains of Ser226 and Thr190.¹¹ PSA-Modeling studies suggested that amino acids with medium length hydrophobic side chains such as leucine, norleucine or phenylalanine or those with hydrophobic side chains terminating in a polar group such as glutamine, methionine and tyrosine would be preferred by PSA in the P1 position. However, while aldehyde-based peptide inhibitors with hydrophobic aliphatic side chains (Leu, Nle) or aromatic side chains (Phe, Tyr) in the P1 amino acids could inhibit PSA, these compounds were even more potent chymotrypsin inhibitors. In contrast, aldehyde inhibitors containing glutamine or methionine in the P1 position were highly specific for PSA and were not effective at inhibiting chymotrypsin.

Thus, to produce a more potent and specific peptidyl PSA inhibitor, we synthesized and evaluated (boro)Bpg based inhibitors. The bromopropylglycine boronic acid was selected because it possesses a medium length hydrophobic chain as that found in residues like Leu, Nle, Met, and Gln but the presence of the bromine also allows for interactions with the polar groups present within of the bottom of the S1 pocket of PSA (Scheme 3). A similar strategy has been used to prepare thrombin¹⁷ and proteasome inhibitors.^{18–21} The presence of bromine at the end of the side chain of P1 allows for halogen bond formation (electrostatic interaction between a halogen and an oxygen), which provides a unique base for inhibitor-enzyme recognition. Also, halogen-oxygen interactions can play significant role in intermolecular binding.²² An example in drug design and halogen bonding is the binding of

inhibitor IDD 594 (5-fluoro-2-(4-bromo-2-fluoro-benzylthiocarbamoyl)-phenoxyacetic acid) to human aldose reductase.²³ In our case of protease-inhibitor interactions, the polarized bromine can interact with the carbonyl from the peptide backbone and/or hydroxyl groups of the Thr and the two Ser residues located at the bottom of the S1 pocket.

In addition, we synthesized (boro)Phe-based inhibitors to compare them with the (boro)Bpg-based inhibitors with respect to the potency and the specificity. The most important differences in the binding modes of (boro)Bpg and (boro)Phe inhibitors lie in their interaction with the S1 specificity pocket. The bromopropyl group is longer and reaches deeper into the S1 pocket compared to phenylalanine. The additional halogen interaction with the enzyme backbone facilitates tighter binding between the enzyme and the inhibitor. Prior modeling studies with aldehyde inhibitors contrasted the differences in the S1 pockets of PSA and chymotrypsin. As we predicted based on the results from these studies, (boro)Phe inhibitor **12** was 9-fold better at inhibiting chymotrypsin and (boro)Bpg inhibitor **13** was ~8-fold better at inhibiting PSA (Table 2).

Previously we used an iterative approach that combined analysis of peptidyl aldehyde inhibitor libraries with molecular modeling studies. Based on these studies, it was determined that a peptide sequence length of at least four amino acids was required for PSA inhibition. From a series of 27 natural and unnatural amino acids, we determined that hydrophobic amino acids were best in the P2 position with norleucine being the most preferred. In the P3 position, glutamine and lysine were equally preferred. Since the presence of lysine makes the peptide more susceptible to cleavage by trypsin-like proteases present in the serum, for our extended studies glutamine was selected as a preferred amino acid for the P3 position. In addition, having Lys along with Ahx in the peptide sequence could present more elaborate synthetic routes when it comes to selective radiolabeling or other N-terminal modifications. Finally, modeling studies suggested a preference for serine or alanine in the P4 position. Therefore, based on these studies we prepared peptide inhibitors **15–25** with the sequence X-SQn(boro)Bpg or X-SQn(boro)F and characterized their ability to inhibit PSA vs. chymotrypsin (Table 2).

Effects of P5 modification on PSA inhibition

Previous modeling studies indicated that amino acids in P5 position and beyond in the PSA inhibitor sequence were outside of PSA's catalytic site and were solvent exposed. This suggested that hydrophobic amino acids and bulky adducts such as chelating groups, could be added to the amino terminus of the peptide without affecting the ability of the compound to act as a PSA inhibitor (Figure 1). In addition, preliminary *in vivo* studies suggested that inhibitors such as **14** had a remarkably short serum half-life due to rapid renal clearance. Thus, in an attempt to improve the half-life, inhibitors **19, 20** and **24, 25** were generated that contained bulky hydrophobic amino acids in the P5 position. In addition, an aminohexanoic (Ahx) group was placed at the N-terminus to serve as a linker to chelating groups (e.g. NOTA, DOTA) or radiolabeled prosthetic groups (SIB, SFB, etc). The addition of this Ahx group did not affect PSA inhibition to a significant degree. Analysis of the K_i for the (boro)Bpg inhibitors demonstrated that in some cases deletion as in **16** or substitution as in **25** of P5 Ser had a deleterious effect on PSA inhibition, whereas in other cases the effect on K_i was minimal as in **18** and **20**. From this entire group, **20** was the most selective PSA inhibitor with an 8-fold lower K_i for PSA vs. chymotrypsin. In contrast to the specificity conveyed by the (boro)Bpg, all of the (boro)Phe inhibitors were much better (i.e. **19** – 450 fold) chymotrypsin inhibitors. In fact, the inhibitor **19** with a K_i of 135 picomolar, is one of the most potent chymotrypsin inhibitors ever described (Table 2).

PSA inhibitors affect PSA blood levels

PSA is secreted in an enzymatically active form and accumulates to high levels in the extracellular fluid surrounding prostate cancer cells. A fraction of this PSA enters the circulation where it is rapidly inactivated due to the formation of covalent complexes with the serum protease inhibitors alpha-2-macroglobulin (A₂M) and alpha-1-antichymotrypsin (ACT).^{6,7} To assess whether the formation of these complexes could be inhibited, PSA was incubated with either A₂M or ACT in aqueous buffer in the presence or absence of **20** (Figure 2A). Western blot analysis demonstrated that **20** completely blocked the ability of PSA to bind to both of these serum protease inhibitors. Subsequently, we evaluated the effect of the PSA inhibitor **20** on serum PSA levels generated by PSA-producing human prostate cancer xenografts in nude mice. First we determined that the PSA inhibitor **20** had no effect on the standard ELISA used to measure PSA levels in humans (Figure 2B). Using different antibodies, this assay can measure free PSA, which corresponds to the fraction of PSA in the blood that is unbound to protease inhibitors because it lacks enzymatic activity and total PSA, which corresponds to the sum of the free PSA plus the amount of PSA bound to ACT. The fraction of PSA bound to A₂M cannot be measured due to lack of antibody that specifically recognizes this complex. In this experiment mice received three 5-day courses of **20** at 10 mg/kg and then blood was obtained for free and total PSA measurement. Mice treated with **20** had an approximately 40% lower level of total PSA/gram of tumor and a 23% lower level of free PSA/gram of tumor compared to control mice (Figure 2C, D). These results suggest that the inhibitor is able to block PSA complex formation with ACT and alter PSA clearance in treated animals.

To be able to predict the inhibitor's behavior *in vivo*, we next assessed whether **20** maintained the ability to inhibit PSA in buffer, cell culture media and plasma (Table 3). Enzyme-inhibitor interactions are generally measured in buffer, where we observed the lowest K_i value for Ahx-FSQn(boro)Bpg. When the assay was performed in RPMI with no additives, there was no change in the K_i value. However, when the assay was performed in serum containing media we saw a linear decrease in the inhibition potential of the agent (Figure 3). The study demonstrated that in the presence of serum containing RPMI the K_i for PSA inhibition increased ~10-fold. These results suggest that the inhibitor may not bind PSA efficiently in plasma due to non-specific protein binding.

In vivo study

Based on these results suggesting **20** can bind to PSA *in vivo*, we next evaluated whether this inhibitor could affect the growth of PSA-producing LNCaP human prostate cancer xenografts growing subcutaneously in nude mice. Based on prior studies with peptidyl boronic acid inhibitors in this setting, we selected a daily dose of 10 mg/kg administered intravenously for 5 consecutive days. This regimen was well tolerated and induced no significant weight loss in treated animals. PSA inhibitor treated animals experienced a delay in tumor growth over the first two cycles of therapy and had smaller tumors at the end of the treatment period, although the difference did not achieve statistical significance (Figure 4).

Addition of 4-iodobenzoate and ¹²⁵I labeling

An important advantage of our peptide-based inhibitors is that they can be readily modified to incorporate imaging moieties such as radionuclide-bearing groups. To assess circulating half-life and biodistribution select inhibitors were radiolabeled via the addition of 4-[¹²⁵I]iodobenzoate to the Ahx linker. To introduce this label, containing *N*-hydroxysuccinimidyl-4-[¹²⁵I]iodobenzoate (SIB) was prepared as previously described by Dekker et al.²⁴ (Scheme 4). This [¹²⁵I]SIB prosthetic group was previously developed for radioiodination of proteins and peptides.^{25,26} Unlabeled SIB-peptides were prepared as chromatographic references and also as a comparison with the non-iodinated analogs in the

enzyme kinetic studies. The coupling of SIB to Ahx as in **22**, slightly decreased the K_i for PSA and increased the K_i for Chymotrypsin (Table 2).

In initial studies we generated [125 I]SIB-Ahx-SSQn(boro)Bpg, which when injected intraperitoneally (i.p.) into mice had an extremely short circulating half-life of 12 minutes (data not shown). Subsequently, in an effort to improve circulating half-life we generated the [125 I] labeled version of Ahx-NaphSQn(boro)F. This compound had a longer circulating half-life of ~2.7 h following i.p. injection, (Figure 5A). Further, we performed a biodistribution study in mice bearing PSA-producing LNCaP tumors. Mice were injected with [125 I]SIB-Ahx-NaphSQn(boro)F and at 2, 4 and 6 h individual mice were sacrificed and their tissues (liver, kidney, heart, brain, skeletal muscle, and tumor) were collected, weighed and analyzed using gamma-counter (Figure 5B). [125 I]SIB-Ahx-FSQn(boro)Bpg **26** and [125 I]SIB-Ahx-FSQn(boro-Pin)Bpg **27** were also used for biodistribution studies (Figure 5C). For these three inhibitors, the majority of activity was detected within the liver and kidney by 2 h. Tumor levels at each time point and dose were very similar to those in the heart and skeletal muscle. Highest amount of activity detected in the tumors was around 3 to 4 h postinjection.

DISCUSSION

The goal of these studies was to identify a specific and potent PSA inhibitor that could be used to investigate the role of PSA in the pathobiology of prostate cancer.⁴ Such a role is suggested by the continued production of PSA throughout the course of the disease, including production by men with poorly differentiated cancers that have become highly resistant to multiple forms of androgen ablative therapy. PSA is able to proteolytically modify growth regulatory proteins that are important in cancer growth and survival. These include Insulin Growth Factor Binding Proteins (IGFBP) 2, 3, and 5,²⁷ PTH-related protein,^{28,29} latent TGF- β 2,³⁰ and extracellular matrix components fibronectin, and laminin³¹. Using siRNA to knockdown PSA production, Williams et al.³² demonstrated the importance of PSA production in the *in vitro* and *in vivo* growth of human prostate cancer cell lines. These combined results suggest that the enzymatic activity of PSA may also represent a potential therapeutic target in prostate cancer and further support the development of selective PSA inhibitors to determine the significance of PSA's enzymatic activity in the progression and growth of prostate cancer.

A further goal of these studies would be to identify a peptidyl PSA inhibitor as an imaging agent in patients with prostate cancer. The potential use of a PSA inhibitor for imaging prostate cancer is particularly attractive because prostate cancer preferentially metastasizes to the bone resulting in the formation of predominantly osteoblastic lesions.^{33,34} Therapeutic response in the bone is difficult to evaluate with current bone scan imaging modalities. Thus, there is a compelling need for better imaging modalities for prostate cancer that can be used to detect disease recurrence at an earlier time point and to better evaluate the therapeutic efficacy of new treatments. In this regard, Ulmert et al.³⁵ recently reported on the development of ^{89}Zr labeled monoclonal anti-PSA antibody that selectively binds to the catalytic site of PSA. Positron emission tomography (PET) imaging demonstrated that this ^{89}Zr antibody accumulated in tumors with a peak activity of 19.6 % ID/g at 24 hours postinjection.

In our studies we have focused on peptidyl boronic acid based approach to PSA inhibition. We have pursued this strategy because peptides provide a platform for easy introduction of imaging moieties and therapeutic agents. Other strategies for PSA inhibition have also been described, the first PSA inhibitors reported in the literature used a homology model derived from porcine kallikrein to design and characterize a series of monocyclic β -lactam

compounds.^{36,37} Prior to this study, it had been shown that β -lactams were efficient and specific inhibitors of several serine proteases, especially human leukocyte elastase.^{38,39} The best inhibitor from the studies of Adlington et al. had an IC_{50} value of 226 nM for PSA.^{36,37} However, the specificity and stability of this compound and others in the class versus chymotrypsin and other proteases was not reported. Other attempts have been made to find small-molecule inhibitors for PSA using compounds other than β -lactams. Azapeptides, that can inhibit both cysteine and serine proteases, have been developed and used to effectively inhibit PSA with K_i as low as 500 nM.⁴⁰ To elucidate the role of PSA in angiogenesis, Koistinen et al. screened a library of nearly 50,000 compounds to discover inhibitors of PSA.⁴¹ Out of the compounds screened only three were identified that inhibited PSA at sub μ M concentrations. The active compounds were either benzoxazinone or triazole derivatives, which inhibited PSA with IC_{50} values of 300–900 nM and had 10–30 fold specificity vs. chymotrypsin.

In this work we have developed a small library of peptidomimetics containing peptidyl boronic acid, which shows an improved potency for PSA (<100 nM) and specificity over chymotrypsin (up to 10 fold). These results suggest a potential use of these inhibitors as targeted therapeutics or imaging agents. Systemic administration of inhibitors at doses of 10 mg/kg for 5 consecutive days/week $\times$ 3 resulted in tumor growth changes. PSA inhibitor **20** was able to block complex formation between PSA and serum protease inhibitors A_2M and ACT and did not affect measurement of PSA by ELISA assay. PSA inhibitor treatment altered PSA blood levels, reducing the total PSA/gram by 40% and the free PSA/gram by 23% while increasing the Free/Total PSA ratio by ~25%. These lowering of the total PSA levels appear consistent with the PSA inhibitor disrupting PSA binding to ACT thus decreasing the amount of PSA-ACT complex that typically makes up about 75% of the total PSA measured in men with prostate cancer. The reasons for the reduction in free PSA levels in this model is less clear as free PSA in the circulation has been demonstrated to be enzymatically inactive due to a combination of incomplete processing of the proPSA zymogen and proteolytic processing.⁴² Potential explanation for this observed decrease in free PSA may be that the inhibitor changes the kinetics of PSA clearance upon binding to PSA or may decrease production of PSA by prostate cancers by some undefined mechanism. While these changes in serum PSA levels suggest that the inhibitor is reaching the target intratumorally, *in vivo* tumor growth assays demonstrated that the PSA inhibitor has a non-significant, minimal effect on the inhibition of growth of subcutaneously implanted human prostate cancer xenografts. This lack of effect may be due to the short biological half-life of the drug as there is insufficient exposure to completely inhibit PSA, which is continuously being secreted into the tumor microenvironment by prostate cancer cells. Alternatively, the lack of effect may be a contextual one: PSA may play no role in the growth of subcutaneously implanted prostate cancer xenografts, as prostate cancer typically forms osteoblastic bone metastases. The mechanism whereby PSA may stimulate bone adhesion or osteoblast growth and/or differentiation has not been clearly defined. However, PSA can release cytokines involved in bone turnover such as IGF-1 and TGF- β 2.^{27,30} PSA also hydrolyzes PTHrP, an important mediator of osteolytic bone metastases, and inactivates PTHrP-stimulated cAMP accumulation in mouse osteoblasts.^{28,29} This suggests PSA could alter the bone metastatic phenotype from osteoclastic to osteoblastic through PTHrP degradation. Therefore, in future studies we plan to assess the effect of PSA inhibitors on growth in potentially more relevant models of prostate cancer bone metastases.

CONCLUSION

One of our strategies in validating PSA as a ‘druggable’ target is to inhibit its protease activity by creating PSA specific enzyme inhibitors. By introducing new structural differences at P5 and P1 we prepared boronic acid peptidates for targeted inhibition of PSA.

These boronic acid peptidates have K_i values in the nanomolar range and rank among the best inhibitors of PSA reported so far. The best of these inhibitors achieved 8-fold selectivity vs. chymotrypsin. In addition, we demonstrated that the inhibitors could block PSA complex formation with serum protease inhibitors. *In vivo* studies showed that inhibition of PSA resulted in decrease of the total PSA, as well as the free PSA. The inhibitors did not significantly inhibit the growth of subcutaneous PSA-producing xenografts and further study is needed to determine the microenvironmental context in which PSA activity is most relevant. In addition, these studies suggest that, while these PSA inhibitors will not be useful as imaging agents without further optimization, the inhibitors are useful research tools to help in the elucidation of PSA's role in the growth and progression of prostate cancer.

EXPERIMENTAL SECTION

Chemistry

All amino acids used in this study are of *L*-form. All Fmoc-amino acids and 2-chlorotrityl chloride resin used were purchased from AnaSpec (Fremont, CA). Benzylboronic acid was purchased from Alfa Aesar (Ward Hill, MA) and (1*R*)-1-amino-4-bromobutan-1-ylboronic acid (1*S*,2*S*,3*R*,5*S*)-(+)-2,3-pinandiol ester was purchased from BoroChem SAS (Caen, France). Fmoc-(*R*)-3-amino-4-(2-naphthyl)-butyric acid was used for the synthesis of Naph-containing analogs and was purchased from PepTech Corp. (Burlington, MA). All other reagents and solvents were purchased from Sigma-Aldrich (USA) and used as received. All reactions were carried out under anhydrous conditions, nitrogen atmosphere and dry solvents, unless otherwise noted. Reactions were monitored by thin layer chromatography (TLC) carried out on 250 μ m silica gel plates (60 F₂₅₄). All organic extracts were dried over MgSO₄ and solvents were removed with a rotary evaporator, followed by high vacuum oil pump pressure. The products were characterized by ¹H and ¹³C NMR on a 400 MHz Bruker Avance NMR Spectrometer. Mass spectra were measured on a Bruker 3000 Esquire Mass Spectrometer equipped with ESI. The HPLC used for the analysis and purification was equipped with photodiode array detector and purchased from Waters (Milford, MA). The final products were purified on a semi-prep HPLC column from Phenomenex: Luna C18, particle size 10 μ m, 250 $\times$ 10 mm. The purity of all synthesized peptides was determined by analytical HPLC to be >95%.

[¹²⁵I]NaI was purchased from MP Biomedicals (Solon, OH). All radiolabeled products were purified on reverse phase radio-HPLC using Waters Delta 600 pump, Waters 2487 UV/Vis dual wavelength absorbance detector and Beta-RAM radio-HPLC detector, equipped with sodium iodide cell for soft gammas (I-125) from IN/US Systems (Brandon, FL). The HPLC column was purchased from Phenomenex: Luna C18, particle size 5 μ m, 150 $\times$ 4.60 mm. Solid-phase C18 extraction cartridges (Sep-Pak Plus) were purchased from Waters. Radioactivity was measured in CRC-10R Capintec dose calibrator (Ramsey, NJ). The CT/SPECT scanning was performed in our small animal imaging center using X-SPECT imager (Gamma Medica, Northridge, CA). All animal studies performed were in full compliance with our institutional guidelines related to conducting experiments using animals. The radioactivity of the harvested tissue was measured with automated Gamma 5500 counter (Beckman, Irvine, CA). The percent injected dose per gram of tissue (%ID/gram) was calculated based on the number of counts measured for 0.01 mL of the dosing solution.

Biology

Chymotrypsin (from bovine pancreas) and PSA (from human seminal fluid) were purchased from CalBiochem (San Diego, CA). The fluorescent PSA substrate, Mu-SRKSQQYAMC (Morpholinocarbonyl-Ser-Arg-Lys-Gln-Gln-Tyr-aminomethylcoumarin), was selected as the PSA substrate based on a previous study¹² and was custom synthesized by California

Peptides (Napa Valley, CA). The chymotrypsin substrate Suc-AAPF-AMC was purchased from Sigma-Aldrich. All kinetic assays were performed on a Beckman DTX-880 multimode detector plate reader.

Enzyme assays

Peptidyl boronic acids were dissolved in DMSO at 20 mM. Stock solutions could be stored at -20°C and were stable for one year. More diluted solutions were prepared as necessary. The activity of the inhibitors was determined by monitoring the change of fluorescence of AMC substrate (ex. 370 nm; em. 465 nm) due to hydrolysis by PSA. Product formation was followed over 30 min. The K_m for the substrate Mu-SRKSQQY-AMC was previously determined to be 140 μM .¹⁴ Kinetic measurements were done in triplicate and run at 37°C in PSA buffer (50 mM TRIS, 100 mM NaCl, pH 7.8). Reactions were performed on 96-well plate where the final concentration of PSA was 2.5 $\mu\text{g/mL}$ (2 nM active PSA) and the substrate was 300 μM . The assay mixture contained 100 μL of buffer, peptidyl boronic acid in DMSO (20 μL), PSA solution (20 μL) and Mu-SRKSQQY-AMC in buffer (60 μL). The following concentrations of inhibitors were employed for the assay: 25, 12.5, 6.25, 3.1, 1.56, 0.78, 0.39, 0.19, 0.098 and 0.049 μM .

Selectivity of the compounds was determined by screening the products against chymotrypsin. Chymotrypsin assays were performed by monitoring the fluorescence of Suc-AAPF-AMC substrate at the above described conditions. The K_m value of chymotrypsin for the substrate Suc-AAPF-AMC was determined to be 14 μM . Reactions were performed on 96-well plate where the final concentration of chymotrypsin was 2 nM and the substrate was 25 μM . The assay mixture contained peptidyl boronic acid in DMSO (20 μL), Suc-AAPF-AMC in PSA buffer (90 μL) and chymotrypsin solution (90 μL). The chymotrypsin substrate solution was added last to each assay solution and the kinetic data was collected immediately.

The inhibition constant (K_i) of each compound was determined by solving the equation: $K_i = 1/\text{slope} \times 1/(1+[S]/K_m)$, where the slope is obtained from a linear plot of $V_o/V_i - 1$ vs. $[I]$. The reciprocal of the slope is also equal to the $K_{i(\text{app})}$, apparent inhibition constant. $[S]$ = substrate concentration, $[I]$ = inhibitor concentration, K_m = Michaelis constant, V_o = rate of substrate hydrolysis in the absence of inhibitor, and V_i = rate in the presence of inhibitor. The K_i values are reported in Table 3.

Biodistribution studies

Biodistribution studies were conducted to evaluate the uptake of [^{125}I]SIB-Ahx-NaphSQn(boro)F, [^{125}I]SIB-Ahx-FSQn(boro)Bpg and [^{125}I]SIB-Ahx-FSQn(boro-Pin)Bpg in LNCaP human prostate cancer xenograft models. Mice received between 230 and 580 μCi , of the selected radiolabeled inhibitor in 200 μL sterile saline through intravenous tailvein injection. The animals ($n = 2$ per group) were euthanized between 2 to 6 h postinjection and blood was immediately harvested by cardiac puncture. These time points were selected based on biodistribution assays previously performed in our lab. The tumors along with liver, kidney, spleen, heart, lung, and skeletal muscle were harvested, weighed and analyzed using gamma counter for accumulation of ^{125}I radioactivity. Count data were background and decay corrected and the tissue uptake for each sample was calculated as counts/mass.

In vivo assay

In vivo studies were performed by using PSA-producing LNCaP human prostate cancer xenografts as previously described.⁸ Nude male mice ($n = 10$ per group) bearing subcutaneous tumors were treated once daily for three cycles of 5 days with doses of 10 mg/

kg. The compound of interest was reconstituted in sterile saline and given by intravenous injections. Tumors were measured by caliper and their volume was determined by the formula: $\text{Vol(cc)} = L \times W \times H \times 0.5326$. Body weight and tumor sizes were measured twice per week. Serum levels of free and total PSA were measured 24 h after the last dose of the inhibitor.

Synthesis

Peptide synthesis—All peptides were individually prepared by solid-phase synthesis using AAPPTec Apex 396 40-well peptide synthesizer. The products were obtained using standard Fmoc protocol, starting with preloaded Fmoc-Nle-OH onto a 2-CITrt resin. The amino acids (0.1 mmol) were coupled for 1 h using double coupling conditions. HOBt (5 eq.) served as activating reagent; 5 eq. of Fmoc-protected amino acids were used with 0.5 M DIC in NMP. Once completed, the peptide on the resin was washed consecutively with NMP, methanol and DCM. When necessary the peptides were capped with Cbz (0.4 M) in presence of DIEA (4 eq.). In other instances, Ahx was added to the N-terminus of the sequence to serve as a handle containing free amino group. The final sequences were washed with DCM and cleaved from the resin with 1% TFA in DCM. On cleavage, the peptides were diluted with ethyl acetate and after removal of the solvent the peptides were purified by semi-prep RP-HPLC using a Phenomenex Luna 250 × 10 mm, C18 column, followed by lyophilization for 24 h. The purity of all synthesized peptides was determined by HPLC to be >95%. The peptides masses were confirmed by mass spectrometry and were used for further coupling.

Pinanediol boronic acid ester—The synthesis of (+)-pinanediol protected benzyl boronic acid, (boro-Pin)Phe (**5**) was performed as described^{43–46} (Scheme 1). The synthesis of (+)-pinanediol protected bromopropylglycine boronic acid, (boro-Pin)Bpg or (1*R*)-1-amino-4-bromobutan-1-ylboronic acid (1*S*,2*S*,3*R*,5*S*)-(+)-2,3-pinanediol ester **6** has been previously described, as well.^{16,47} However, both starting materials are now commercially available and can be purchased from BoroChem SAS, France. For preparation of (boro)Phe analogs the following procedure modifications were performed and the products were obtained as TFA salts.

(+)-Pinanediol phenylmethyl-1-boronate (2)—To a solution of 1.0 g (7.36 mmol) benzyl boronic acid **1** in 80 mL of diethyl ether were added 1 eq. of (+)-pinanediol (1.25 g) and 3.0 g of MgSO₄. The solution was stirred for 2–3 days and the product formation was followed by NMR. The reaction mixture was filtered out and the organic layer was washed with saturated solution of NaHCO₃ (20 mL × 2) and brine (20 mL × 2). After drying the organic layer was concentrated under low pressure and kept under high vacuum overnight to yield 85% colorless oil. The product was used for the next step without purification. ¹H NMR (CDCl₃): δ 0.85 (s, 3H), 1.07 (d, *J* = 11.0 Hz, 1H), 1.28 (s, 3H), 1.39 (s, 3H), 1.9 (m, 2H), 2.05 (m, 1H), 2.2 (m, 1H), 2.28 (m, 1H), 2.35 (s, 2H), 4.29 (dd, *J* = 8.9, 2.1 Hz, 1H), 7.16–7.34 (m, 5H); ¹³C NMR (CDCl₃): δ 23.95, 26.50, 28.31, 28.68, 36.22, 38.36, 39.48, 51.31, 78.34, 85.89, 110.64, 124.84, 128.47, 129.13, 138.86; MS (ESI) *m/z* calcd for C₁₇H₂₃BO₂ 270.18, found 288.0 [M+H+H₂O]⁺, 293.1 [M+Na]⁺.

(+)-Pinanediol (S)-1-chloro-2-phenylethaneboronate (3)—In a three-neck flask equipped with thermometer, a solution of 10 mL of freshly distilled THF and 1.3 mL of DCM was cooled to –100 °C (in ethanol/liquid nitrogen bath) under argon atmosphere. Chilled at –78 °C *n*-BuLi (8.6 mmol; 2.5 M in hexane) was added via syringe by running it down the cold wall of the reaction flask over a period of 15 min. White precipitate of LiCHCl₂ was formed and after 10 min of stirring, cold solution of **2** (0.38 g, 1.4 mmol) in 2 mL of dry THF was added. After 10 min of stirring at –100 °C, 8 mL of ZnCl₂ solution (0.5

M in THF) were added. The solution was stirred overnight and the temperature was allowed to rise to room temperature. The reaction mixture was evaporated and re-dissolved in hexane. The organic phase was washed with sat. NH_4Cl , water, and brine, then dried and concentrated to give about 80% crude product as colorless oil. The product was used for the next step without purification. ^1H NMR (CDCl_3): δ 0.84 (s, 3H), 1.08 (d, J = 11.2 Hz, 1H), 1.28 (s, 3H), 1.36 (s, 3H), 1.90 (m, 2H), 2.08 (m, 1H), 2.19 (m, 1H), 2.35 (m, 1H), 3.11 (dd, J = 13.7, 8.6 Hz, 1H), 3.23 (dd, J = 13.9, 7.9 Hz, 1H), 3.68 (dd, J = 8.5, 7.8 Hz, 1H), 4.46 (dd, J = 11.3, 2.0 Hz, 1H), 7.20–7.35 (m, 5H); ^{13}C NMR (CDCl_3): δ 23.95, 26.22, 27.02, 28.33, 29.71, 35.15, 38.24, 39.35, 40.45, 51.22, 78.56, 86.79, 126.72, 126.75, 128.35, 129.20, 129.24, 138.42. MS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{24}\text{BClO}_2$ 318.16, found 283.0 $[\text{M}-\text{Cl}+\text{H}]^+$.

(+)-Pinanediol (*R*)-1-(*N*-bis(trimethylsilyl)amino)-2-phenylethane-1-boronate (4)

—An oven dried flask containing 5 mL of freshly distilled THF was cooled to -78°C and flushed with argon. After addition of 7.2 mL (1.25 eq.) of lithium bis(trimethylsilyl)amide, $\text{LiN}(\text{SiMe}_3)_2$ (1 M solution in THF) the contents of the flask were stirred for 10 min. Crude boronic ester **3** (1.8 g, 5.6 mmol) was dissolved in 10 mL of THF and added dropwise. The reaction mixture was stirred overnight and allowed to warm to room temperature. The resulting solution was concentrated under reduced pressure, reconstituted in 25 mL of DCM and filtered through Celite. The organic layer was dried with MgSO_4 and evaporated to give 2.5 g of crude product as light brown oil. ^1H NMR (CDCl_3): δ 0.09 (s, 18H), 0.83 (s, 3H), 0.97 (d, J = 10.6 Hz, 1H), 1.27 (s, 3H), 1.38 (s, 3H), 1.82 (m, 2H), 2.01 (m, 1H), 2.12 (m, 1H), 2.30 (m, 1H), 2.67 (m, 1H), 2.74 (dd, J = 13.2, 7.2 Hz, 1H), 3.05 (m, 1H), 4.30 (dd, J = 8.6, 2.1 Hz, 1H), 7.20–7.32 (m, 5H). MS (ESI) m/z calcd for $\text{C}_{24}\text{H}_{43}\text{BNO}_2\text{Si}_2$ 444.29, found 444.1 $[\text{M}+\text{H}]^+$.

(+)-Pinanediol (*R*)-1-amino-2-phenylethane-1-boronate trifluoroacetate salt (5)

—0.5 g of the crude **4** (1.1 mmol) were dissolved in 10 mL of ethyl ether and cooled down to 0°C . To the mixture was added TFA (0.25 mL, 3.3 mmol) and the reaction was allowed to stir for 3 h. The TFA salt crashed out of solution and was filtered. The final product was washed three times with cold ethyl ether resulting in white powder with 73% yield (0.34 g). ^1H NMR (methanol- d_4): δ 0.89 (s, 3H), 1.11 (d, J = 10.8 Hz, 1H), 1.34 (s, 3H), 1.42 (s, 3H), 1.93 (m, 2H), 2.06 (m, 1H), 2.26 (m, 1H), 2.43 (m, 1H), 3.03 (dd, J = 14.0, 8.1 Hz, 1H), 3.11 (dd, J = 14.2, 6.4 Hz, 1H), 3.25 (dd, J = 8.0, 6.5 Hz, 1H), 4.50 (d, J = 8.2 Hz, 1H), 7.30–7.37 (m, 5H); ^{13}C NMR (methanol- d_4): δ 24.25, 27.37, 27.49, 28.81, 35.92, 36.46, 39.38, 40.77, 52.40, 80.33, 89.14, 128.58, 130.00, 130.35, 137.88; MS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{27}\text{BNO}_2^+$ 300.21, found 300.1 $[\text{M}]^+$.

General method for synthesis of peptidyl boronic acids (12–25)—The desired N-terminus protected peptide Boc-Ahx- or Z- (1 mmol) was dissolved in 50 mL of CH_2Cl_2 and the solution was cooled in ice-bath. HOBt (1.5 mmol, 0.229 g) and EDC (1.5 mmol, 0.288 g) were added to the mixture and stirred for 15 min. Boronic acid pinanediol ester (1 eq.) was added and after 10 min of stirring at 0°C *N*-methylmorpholine (2 mmol, 0.23 mL) was added. The solution was stirred overnight and the temperature was allowed to warm slowly to room temperature. The reaction mixture was washed with water $\times$ 2, and brine $\times$ 2. The organic layer was dried with MgSO_4 and evaporated to dryness to yield crude protected peptide in 95–97% yield. The resulting white powder was then stirred with 95% TFA in water to remove all of the peptide side chain protecting groups. After 1 h of stirring at room temperature the reaction was concentrated in *vacuo* and resulting residue was resuspended in a 1:1 mixture of ethyl ether/water (40 mL). Phenyl boronic acid (0.305 g, 2.5 mmol) was then added to remove the (+)-pinanediol protecting group. The deprotection was allowed to go overnight at room temperature. The aqueous layer containing the fully deprotected

peptidyl boronic acid was washed with ethyl ether (30 mL, 3 ×) and lyophilized. The product was purified by HPLC and the resulting white powder was characterized by MS. The purity of the product was determined by HPLC to be >95%.

(4-Iodo-benzoylamino)-Ahx-FSQn(boro)Bpg (22)—To a solution of **20** (0.0047 g, 0.006 mmol) in 0.3 mL of dry methanol was added 1.2 eq. of *N*-hydroxysuccinimidyl-4-iodobenzoate²⁴ (0.0025 g, 0.007 mmol), followed by 3 eq. of triethylamine (0.0025 mL, 0.018 mmol). The mixture was stirred for 30 min at room temperature and purified on analytical RP-HPLC column (Phenomenex: Luna C18, particle size 5 μm, 150 × 4.60 mm), using gradient of 30–100% eluent B over 16 min at 1.25 mL/min, then gradient of 0–70% eluent A over 12 min (buffer A: 0.1% TFA in 5% acetonitrile; eluent B: 0.1% TFA in acetonitrile), product eluted at 16 min. The purity of the product was determined by HPLC to be >95%. MS (ESI) *m/z* calcd for C₄₀H₅₈BBrIN₇O₁₀ 1013.26, found 1037.8 [M+Na]⁺.

(4-Iodo-benzoylamino)-Ahx-FSQn(boro-Pin)Bpg (23)—was prepared from **21** using the same procedure as in **22**. The product eluted at 18 min. The purity of the product was determined by HPLC to be >95%. MS (ESI) *m/z* calcd for C₅₀H₇₂BBrIN₇O₁₀ 1147.37, found 1147.8 [M+H]⁺, 1171.7 [M+Na]⁺.

General method for preparation of radiolabeled peptidyl boronic acids, such as 26 and 27—[¹²⁵I]SIB was prepared according to a procedure suggested by Dekker et al. and purified by radio-HPLC (retention time: iodobenzoic acid - 10.57 min, SIB - 12.54 min, and Sn-precursor - 24.40 min). Radiolabeling of the peptidyl boronic acids using [¹²⁵I]SIB was performed as suggested by Chen et al.⁴⁸ To a solution of 0.005 g (or about 0.007 mmol) of peptidyl boronic acid (**20**) or peptidyl boronic pinanediol ester (**21**) in 0.1 mL of DMF was added 0.002 mL of triethylamine, followed by [¹²⁵I]*N*-hydroxysuccinimidyl-4-iodobenzoate (0.100 mL, 0.5–5 μCi). After stirring at room temperature for 40 min, 0.005 mL of TFA was added, the mixture was subjected to HPLC purification (Phenomenex: Luna C18, 5 μm, 150 × 4.60 mm), using gradient of 30–100% eluent B over 16 min at 1.25 mL/min, then gradient of 0–70% eluent A over 12 min (buffer A: 0.1% TFA in 5% acetonitrile; eluent B: 0.1% TFA in acetonitrile). The purity of the product was determined by HPLC to be >95%. The [¹²⁵I]-peptidyl boronic acid containing fraction was neutralized with 1 M NaHCO₃, concentrated under vacuum and reconstituted in PBS (pH 7.4). The solution was passed through a 0.22 μm syringe filter into a sterile vial.

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ABBREVIATIONS

Abbreviations for common amino acids are in accordance with the recommendations of IUPAC. Other abbreviations:

Ahx	6-aminohexanoic acid
Boc	tert-butoxycarbonyl
DIC	diisopropylcarbodiimide
DIEA	N, N-Diisopropylethylamine
DMAP	4-(dimethylamino)pyridine

EDC	N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide
Fmoc	fluorenylmethoxycarbonyl
Z or Cbz	benzyloxycarbonyl protecting group for N-terminus
GBTU	O-benzotriazole, N', N', N', N'-tetramethyluronium hexafluorophosphate
HOBt	N-hydroxybenzotriazole
Mu	Morpholinocarbonyl
NMP	N-methylpyrrolidone
Bpg	3-bromopropylglycine
Naph	(2-Naphthyl)- β -homoalanine; Aminoboronic acid or (boro)aa is where the CO ₂ H of the equivalent amino acid is replaced by B(OH) ₂ ; H is used to designate an unprotected amino function
Pin	pinanediol protecting group
PSA	prostate-specific antigen
KLK3	kallikrein-related peptidase 3
IGFBP	insulin-like growth factor binding protein
TGF-β2	transforming growth factor-beta 2
PTHrP	parathyroid hormone-related protein
A₂M	alpha-2-macroglobulin
ACT	alpha-1-antichymotrypsin
cAMP	cyclic adenosine monophosphate
IDD	Institute for Diabetes Discovery

REFERENCES

1. Watt KW, Lee PJ, M'Timkulu T, Chan WP, Loo R. Human prostate-specific antigen: Structural and functional similarity with serine proteases. *Proc. Natl. Acad. Sci. USA*. 1986; 83:3166–3170. [PubMed: 2422647]
2. Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J. Clin. Invest.* 1985; 76:1899–1903. [PubMed: 3902893]
3. Yousef GM, Diamandis EP. An overview of the kallikrein gene families in humans and other species: emerging candidate tumor markers. *Clin. Biochem.* 2003; 36:443–452. [PubMed: 12951170]
4. Williams SA, Singh P, Isaacs JT, Denmeade SR. Does PSA play a role as a promoting agent during the initiation and/or progression of prostate cancer? *Prostate*. 2007; 67:312–329. [PubMed: 17143882]
5. Denmeade SR, Sokoll LJ, Chan DW, Khan SR, Isaacs JT. Concentration of enzymatically active Prostate-Specific Antigen (PSA) in the extracellular fluid of primary human prostate cancers and human prostatic cancer xenograft models. *Prostate*. 2001; 48:1–6. [PubMed: 11391681]
6. Lilja H, Christensson A, Dahlen U, Matikainen MT, Nilsson O, Pettersson K, Lovgren T. Prostate-specific antigen in serum occurs predominantly in complex with α 1-antichymotrypsin. *Clin. Chem.* 1991; 37:1618–1625. [PubMed: 1716536]
7. Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O. A complex between prostate specific antigen and α 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: Assay of the complex improves clinical sensitivity for cancer. *Cancer Res.* 1991; 51:222–226. [PubMed: 1703033]

8. Denmeade SR, Jakobsen C, Janssen S, Khan SR, Lilja H, Christensen SB, Isaacs JT. Prostate-specific antigen (PSA) activated thapsigargin prodrug as targeted therapy for prostate cancer. *Natl. Cancer Inst.* 2003; 95:990–1000.
9. Williams SA, Merchant RF, Garrett-Mayer E, Isaacs JT, Buckley JT, Denmeade SR. A prostate-specific antigen-activated channel-forming toxin as therapy for prostatic disease. *J. Natl. Cancer Inst.* 2007; 99:376–385. [PubMed: 17341729]
10. Ulmert D, Evans MJ, Holland JP, Rice SL, Wongvipat J, Pettersson K, Abrahamsson PA, Scardino PT, Larson SM, Lilja H, Lewis JS, Sawyers CL. Imaging androgen receptor signaling with a radiotracer targeting free prostate-specific antigen. *Cancer Discov.* 2012; 2:320–327. [PubMed: 22576209]
11. LeBeau AM, Singh P, Isaacs JT, Denmeade SR. Prostate-specific antigen is a ‘chymotrypsin-like’ serine protease with unique P1 substrate specificity. *Biochemistry.* 2009; 48:3490–3496. [PubMed: 19281249]
12. Denmeade SR, Lou W, Malm J, Lovgren J, Lilja H, Isaacs JT. Specific and efficient peptide substrates for assaying the proteolytic activity of prostate specific antigen. *Cancer Res.* 1997; 57:4924–4930. [PubMed: 9354459]
13. Singh P, LeBeau AM, Lilja H, Denmeade SR, Isaacs JT. Molecular insights into substrate specificity of prostate specific antigen through structural modeling. *Proteins.* 2009; 77:984–993. [PubMed: 19705489]
14. LeBeau AM, Singh P, Isaacs JT, Denmeade SR. Potent and selective peptidyl boronic acid inhibitors of the serine protease prostate-specific antigen. *Chem. Biol.* 2008; 15:665–674. [PubMed: 18635003]
15. Matteson DS, Michnick TJ, Willett RD, Patterson CD. [(1R)-1-Acetamido-3-(methylthio)propyl]boronic acid and the x-ray structure of its ethylene glycol ester. *Organometallics.* 1989; 8:726–729.
16. Debaene F, Da Silva JA, Pianowski Z, Duran FJ, Winssinger N. Expanding the scope of PNA-encoded libraries: divergent synthesis of libraries targeting cysteine, serine and metalloproteases as well as tyrosine phosphatases. *Tetrahedron.* 2007; 63:6577–6586.
17. Elgendy S, Patel, Green D, Goodwin CA, Scully MF, Husman W, Skordalakes E, Kakkar VV, Deadman JJ. Design of a novel class of bifunctional thrombin inhibitors, synthesised by the first application of peptide boronates of solid phase chemistry. *Tetrahedron Lett.* 1997; 38:3305–3308.
18. Zhu Y, Zhao X, Zhu X, Wu G, Li Y, Ma Y, Yuan Y, Yang J, Hu Y, Ai L, Gao Q. Design, synthesis, biological evaluation, and structure-activity relationship (SAR) discussion of dipeptidyl boronate proteasome inhibitors, part I: comprehensive understanding of the SAR of alpha-amino acid boronates. *J. Med. Chem.* 2009; 52:4192–4199. [PubMed: 19537716]
19. Zhu Y, Yao S, Xu B, Ge Z, Cui J, Cheng T, Li R. Design, synthesis and biological evaluation of tripeptide boronic acid proteasome inhibitors. *Bioorg. Med. Chem.* 2009; 17:6851–6861. [PubMed: 19747832]
20. Watanabe T, Abe H, Momose I, Takahashi Y, Ikeda D, Akamatsu Y. Structure-activity relationship of boronic acid derivatives of tyropeptin: proteasome inhibitors. *Bioorg. Med. Chem. Lett.* 2010; 20:5839–5842. [PubMed: 20727746]
21. Watanabe T, Momose I, Abe M, Abe H, Sawa R, Umezawa Y, Ikeda D, Takahashi Y, Akamatsu Y. Synthesis of boronic acid derivatives of tyropeptin: proteasome inhibitors. *Bioorg. Med. Chem. Lett.* 2009; 19:2343–2345. [PubMed: 19307118]
22. Auffinger P, Hays FA, Westhof E, Ho PS. Halogen bonds in biological molecules. *Proc. Natl. Acad. Sci.* 2004; 101:16789–16794. [PubMed: 15557000]
23. Howard EI, Sanishvili R, Cachau RE, Mitschler A, Chevrier B, Barth P, Lamour V, Van Zandt M, Sibley E, Bon C, Moras TR, Joachimiak A, Podjarny AD. Ultrahigh resolution drug design I: Details of interactions in human aldose reductase-inhibitor complex at 0.66 Å. *Proteins Struct Funct Genet.* 2004; 55:792–804. [PubMed: 15146478]
24. Dekker B, Keen H, Shaw D, Disley L, Hastings D, Hadfield J, Reader A, Allan D, Julyan P, Watson A, Zweit J. Functional comparison of annexin V analogues labeled indirectly and directly with iodine-124. *Nucl. Med. Biol.* 2005; 32:403–413. [PubMed: 15878510]

25. Nanus DM, Milowsky MI, Kostakoglu L, Smith-Jones PM, Vallabahajosula S, Goldsmith SJ, Bander NH. Clinical use of monoclonal antibody HuJ591 therapy: targeting prostate specific membrane antigen. *J. Urol.* 2003; 170:S84–S88. discussion S88–89. [PubMed: 14610416]
26. Smith-Jones PM, Vallabahajosula S, Navarro V, Bastidas D, Goldsmith SJ, Bander NH. Radiolabeled monoclonal antibodies specific to the extracellular domain of prostate-specific membrane antigen: preclinical studies in nude mice bearing LNCaP human prostate tumor. *J. Nucl. Med.* 2003; 44:610–617. [PubMed: 12679407]
27. Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. *J. Clin. Endocrinol. Metab.* 1992; 75:1046–1053. [PubMed: 1383255]
28. Iwamura M, Hellman J, Cockett AT, Lilja H, Gershagen S. Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology.* 1996; 48:317–325. [PubMed: 8753751]
29. Cramer SD, Chen Z, Peehl DM. Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. *J. Urol.* 1996; 156:526–531. [PubMed: 8683730]
30. Dallas SL, Zhao S, Cramer SD, Chen Z, Peehl DM, ; Bonewald LF. Preferential production of latent transforming growth factor-2 by primary prostatic epithelial cells and its activation by prostate-specific antigen. *J. Cell. Physiol.* 2005; 202:361–370. [PubMed: 15389580]
31. Lilja, H.; Piironen, TP.; Rittenhouse, HG.; Mikolajczyk, SD.; Slawin, KM. Comprehensive textbook of genitourinary oncology. Philadelphia: Lippincott Williams and Wilkins Publishers; 2000. p. 638-650.
32. Williams SA, Jelinek CA, Litvinov I, Cotter RJ, Isaacs JT, Denmeade SR. Enzymatically active prostate-specific antigen promotes growth of human prostate cancers. *Prostate.* 2011; 71:1595–1607. [PubMed: 21394741]
33. O’Keefe RJ, Guise TA. Molecular mechanisms of bone metastasis and therapeutic implications. *Clin. Orthop. Relat. Res.* 2003; 415:S100–104. [PubMed: 14600598]
34. Roodman GD. Mechanisms of bone metastasis. *N. Engl. J. Med.* 2004; 350:1655–1664. [PubMed: 15084698]
35. Ulmert D, Evans MJ, Holland JP, Rice SL, Wongvipat J, Pettersson K, Abrahamsson PA, Scardino PT, Larson SM, Lilja H, Lewis JS, Sawyers CL. Imaging androgen receptor signaling with a radiotracer targeting free prostate-specific antigen. *Cancer Discov.* 2012; 2:320–327. [PubMed: 22576209]
36. Adlington RM, Baldwin JE, Becker GW, Chen B, Cheng L, Cooper SL, Hermann RB, Howe TJ, McCoull W, McNulty AM, Neubauer BL, Pritchard GJ. Design, synthesis, and proposed active site binding analysis of monocyclic 2-azetidinone inhibitors of prostate specific antigen. *J. Med. Chem.* 2001; 44:1491–1508. [PubMed: 11334560]
37. Adlington RM, Baldwin JE, Chen B, Cooper SL, McCoull W, Pritchard GJ, Howe TJ, Becker GW, Hermann RB, McNulty AM, Neubauer BL. Design and synthesis of novel monocyclic β -lactam inhibitors of prostate-specific antigen. *Bioorg. Med. Chem. Lett.* 1997; 7:1689–1694.
38. Knight WB, Green BG, Chabin RM, Gale P, Maycock AL, Weston H, Kuo DW, Westler WM, Dorn CP, Finke PE. Specificity, stability, and potency of monocyclic β -lactam inhibitors of human leukocyte elastase. *Biochemistry.* 1992; 35:8160–8170. [PubMed: 1525156]
39. Green BG, Chabin R, Mills S, Underwood DJ, Shah SK, Kuo D, Gale P, Maycock AL, Liesch J, Burgey CS, Doherty JB, Dorn CP, Finke PE, Hagmann WK, Hale JJ, MacCoss M, Westler WM, Knight WB. Mechanism of inhibition of human leucocyte elastase by beta-lactams Stability, reactivation kinetics, and products of β -lactam-derived E-I complexes. *Biochemistry.* 1995; 44:14331–14243. [PubMed: 7578037]
40. Huang A, Knoell C, Fey G, Hazegh-Azam M, Tashjian A, Hedstrom L, Abeles RH. Modulation of recombinant human prostate-specific antigen: activation by hofmeister salts and inhibition by azapeptides. *Biochemistry.* 2001; 40:11734–11741. [PubMed: 11570874]
41. Koistinen H, Wohlfart G, Mattsson JM, Wu P, Lahdenpera J, Stenman UH. Novel small molecule inhibitors for prostate-specific antigen. *Prostate.* 2008; 68:1143–1151. [PubMed: 18500692]

42. Mikolajczyk AD, Marks LS, Partin AW, Rittenhouse HG. Free prostate-specific antigen is becoming more complex. *Urology*. 2002; 59:797–802. [PubMed: 12031356]
43. Matteson DS, Sadhu KM, Lienhard GE. R-1-Acetamido-2-phenylethaneboronic acid. A specific transition-state analog for chymotrypsin. *J. Am. Chem. Soc.* 1981; 103:5241–5242.
44. Matteson DS, Sadhu KM. Synthesis of 1-amino-2-phenylethane-1-boronic acid derivatives. *Organometallics*. 1984; 3:614–618.
45. Matteson DS, Jesthi PK, Sadhu KM. Synthesis and properties of pinanediol α -amido boronic esters. *Organometallics*. 1984; 3:1284–1288.
46. Hansen KK, Grosch B, Greiveldinger-Poenaru S, Bartlett PA. Synthesis and evaluation of macrocyclic transition state analog inhibitors for α -chymotrypsin. *J. Org. Chem.* 2003; 68:8465–470. [PubMed: 14575472]
47. Wityak J, Earl, Richard A, Abelman MM, Bethel YB, Fisher BN, Kauffman GS, Kettner CA, Ma P, McMillan JL, Mersinger LJ, Pesti J, Pierce ME, Rankin FW, Chorvat RJ, Confalone PN. *J. Org. Chem.* 1995; 60:3717–3722.
48. Chen Y, Foss CA, Byun Y, Nimmagadda S, Pullambhatla M, Fox JJ, Castanares M, Lupold SE, Babich JW, Mease RC, Pomper MG. Radiohalogenated prostate-specific membrane antigen (PSMA)-based ureas as imaging agents for prostate cancer. *J. Med. Chem.* 2008; 51:7933–7943. [PubMed: 19053825]

Figure 1.
Structure of peptide boronic acids with hydrophobic amino acid substituents in the P5 position.

Figure 2. PSA inhibitor blocks PSA binding to serum protease inhibitors and alters PSA blood levels. (A) Western blot analysis of inhibition of PSA complex formation with A2M and ACT. FP = control non-PSA containing female plasma; All mixtures were incubated for overnight at room temperature. (B) PSA measurements using Hybritech assay of 100 ng/mL PSA in RPMI + 1% bovine serum albumin + 1 μ M **6** or 1 μ M Z-SSKn-OH peptide + **6** or 1 μ M **20**. (C) Free and Total PSA levels in serum of mice bearing PSA-producing LNCaP prostate cancer xenografts either untreated or receiving 3 courses of **20** at dose of 10 mg/kg for 5

consecutive days/week. **(D)** Free and Total PSA levels per gram of tumor and ratio of Free/Total PSA in mice treated as in (C).

Figure 3.
Evaluation of the activity of inhibitor **20** in presence of bovine serum in the media

Figure 4.

In vivo evaluation of **20**, Ahx-FSQn(boro)Bpg. Mice bearing subcutaneous PSA-producing LNCaP prostate cancer xenografts were either untreated (Tumor control) or received 3 courses of **20** at dose of 10 mg/kg for 5 consecutive days/week (treatment interval indicated by gray bars).

A.

B.

C.

Figure 5.

Evaluation of ^{125}I labeled PSA inhibitor *in vivo*. (A) Blood clearance of ^{125}I SIB-Ahx-NaphSQn(boro)F from mice blood measured over time. Data shows counts per second normalized to starting counts obtained in plasma as 2 h. Four animals were sacrificed at each time point. (B) Biodistribution of ^{125}I SIB-Ahx-NaphSQn(boro)F, shown as percent of injected dose per gram (%ID/gram). Two mice were sacrificed per time point. Tissues were harvested at 2, 4 and 6 h, weighed and counted in gamma counter. Mice had PSA expressing LNCaP xenografts in bilateral flanks. (C) Biodistribution of ^{125}I SIB-Ahx-FSQn(boro)Bpg **26** and ^{125}I SIB-Ahx-FSQn(boro-Pin)Bpg **27**, shown as percent of injected dose per gram (%ID/gram). Each drug was injected into two mice and one animal was sacrificed per time point. Tissues were harvested at 2 and 4 h, weighed and counted in gamma counter. Mice had single PSA expressing LNCaP xenografts.

(a) (+)-pinanediol, Et_2O , MgSO_4 , rt, 85%; (b) LiCHCl_2 , ZnCl_2 , THF, -100°C , 80%; (c) $\text{LiN}(\text{SiMe}_3)_2$, THF, -78°C ; (d) TFA, Et_2O , 0°C , 73%.

Scheme 1.

Synthesis of pinanediol protected phenylalanine boronic acid⁴⁵, (boro-Pin)Phe.

Scheme 2.

Schematic illustration of solid phase peptide synthesis and subsequent conjugation to boronic acids; (boro)Phe and (boro)Bpg are used as examples.

Scheme 3.

PSA active site interactions with amino acids in P1 – P5 positions of peptide boronic acid **20**. Substrate binding pockets on the protease are labeled as S.

(a) *N*-hydroxysuccinimidyl-4-iodobenzoate, triethylamine, MeOH, rt, 30min; (b) [^{125}I]*N*-hydroxysuccinimidyl-4-iodobenzoate, triethylamine, DMF, rt, 40min.

Scheme 4.

Peptide radiolabeling with [^{125}I]SIB and preparation of cold reference.

Table 1

HPLC (semi-prep) retention times and observed m/z ratios by ESI-MS

Inhibitor	R _t , min	Exact Mass Calculated	m/z, [M+H] ⁺	m/z, [M+H] ⁺
12 Z-SSKn(boro)F	25.6 ^a	714.38	697.0	n.o.
13 Z-SSKn(boro)Bpg	24.2 ^a	744.29	729.0	n.o.
14 Ahx-SSQn(boro)L	21.9 ^b	659.40	642.1	660.0
15 Ahx-SQn(boro)F	20.9 ^a	606.35	589.4	607.4
16 Ahx-SQn(boro)Bpg	19.0 ^a	636.27	620.9	638.9
17 Ahx-ASQn(boro)F	23.1 ^b	677.39	660.0	678.0
18 Ahx-ASQn(boro)Bpg	20.7 ^a	707.30	691.9	709.7
19 Ahx-FSQn(boro)F	24.5 ^a	753.42	736.6	754.5
20 Ahx-FSQn(boro)Bpg	22.9 ^a	783.33	768.5	786.5
21 Ahx-FSQn(boro-Pin)Bpg	30.2 ^a	917.44	n.d.	917.9
22 SIB-Ahx-FSQn(boro)Bpg	32.4 ^a	1013.26	n.d.	1015.8
23 SIB-Ahx-FSQn(boro-Pin)Bpg	33.5 ^a	1147.37	n.d.	1147.8
24 Ahx-NaphSQn(boro)F	24.6 ^a	817.45	800.0	818.0
25 Ahx-NaphSQn(boro)Bpg	24.9 ^a	847.37	831.9	849.8

n.d. - not detected

^a HPLC method: 85% A for 5min; gradient of 15–90% B over 25min at 5mL/min; 90% B for 5min

^b HPLC method: 90% A for 5min; gradient of 10–95% B over 25min at 5mL/min; 95% B for 5min

Eluent A = 0.1% TFA in 5% MeOH/95% Water

Eluent B = 0.1% TFA in MeOH

Note: In some cases molecular ion peaks corresponding to [M-(OH)₂+H]⁺ and/or [M+Na]⁺ were detected, as well (data not shown).

Table 2Comparison of the inhibition constant K_i for PSA vs. Chymotrypsin

	Inhibitor	K_i (nM) PSA inhibition	K_i (nM) Chymotrypsin inhibition	Specificity, relative to PSA inhibition
12	Z-SSKn(boro)F	164.503	18.108	0.11
13	Z-SSKn(boro)Bpg	24.228	196.882	8.13
14	Ahx-SSQn(boro)L	43.053	137.837	3.20
15	Ahx-SQn(boro)F	22.01	3.765	0.17
16	Ahx-SQn(boro)Bpg	302.096	250.575	0.83
17	Ahx-ASQn(boro)F	310.286	36.22	0.12
18	Ahx-ASQn(boro)Bpg	52.11	165.921	3.18
19	Ahx-FSQn(boro)F	61.484	0.135	0.002
20	Ahx-FSQn(boro)Bpg	72.288	580.724	8.03
21	Ahx-FSQn(boro-Pin)Bpg	73.327	496.3	6.77
22	SIB-Ahx- FSQn(boro)Bpg	64.409	652.8	10.14
24	Ahx-NaphSQn(boro)F	228.782	7.214	0.03
25	Ahx-NaphSQn(boro)Bpg	102.463	505.676	4.94

Table 3Comparison of the PSA inhibition constant of **20** in different media

Inhibitor Ahx-FSQn(boro)Bpg screened in different media	K _i (nM)
PSA buffer (pH 7.8)	72
RPMI (no additives)	75
RPMI (+1% FBS)	142
RPMI (+10% FBS +1% Pen/Strep +1% Gln)	699
Female plasma	604

PSA buffer – 50 mM TRIS.HCl, 100 mM NaCl

RPMI – RPMI-1640 Medium: cell culture media (Gibco: Invitrogen, Grand Island, NY)

FBS – Fetal bovine serum

Pen/Strep – Penicillin/streptomycin

Gln – *L*-Glutamine