

Review

Prostate-specific antigen: an overlooked candidate for the targeted treatment and selective imaging of prostate cancer

Aaron M. LeBeau^{1,a,*}, Maya Kostova², Charles S. Craik³ and Samuel R. Denmeade^{1,2}

¹Department of Pharmacology and Molecular Science, The Johns Hopkins University School of Medicine, 75 N. Wolfe St., Baltimore, MD 2131, USA

²The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, 1650 Orleans St., Baltimore, MD 21231, USA

³Department of Pharmaceutical Chemistry, University of California, San Francisco, 600 16th St., San Francisco, CA 94158, USA

*Corresponding author
e-mail: Aaron.Lebeau@ucsf.edu

Abstract

The role of prostate-specific antigen (PSA) or kallikrein-related peptidase 3 (KLK3) as a biomarker for prostate cancer is well known; however, the precise physiological role of its serine protease activity in prostate cancer remains a mystery. PSA is produced at high levels by both androgen-dependent and -independent prostate cancers. Studies have documented high levels of active PSA in the milieu surrounding osseous and soft tissue metastases. This evidence, coupled with growing experimental evidence, suggests that PSA plays an important role in the pathobiology of prostate cancer. These observations support the development of PSA-selective inhibitors as useful tools for the targeted treatment and imaging of prostate cancer. Here, we review the research that has been conducted to date on developing selective inhibitors for PSA. The different approaches used to determine PSA substrate specificity and for creating inhibitors are discussed. In addition, the unique active site characteristics of PSA and how these motifs aided our research in developing PSA targeted agents are highlighted.

Keywords: human kallikrein-related peptidase 3; inhibition; prostate cancer; prostate-specific antigen; serine protease; small molecule.

Introduction

Prostate cancer is uniformly lethal once it has escaped the confines of the prostate gland, resulting in the death of over 25 000 American men each year (Jemal et al., 2007). Although we have learned a great deal about the biochemistry and genetics of prostate cancer over the past decade, there are two critical needs associated with the treatment of metastatic prostate cancer that have yet to be met. First, all men undergoing androgen ablation therapy eventually relapse and no longer respond to androgen ablation, no matter how completely given (Crawford et al., 1989; Laufer et al., 2000). At this point in the disease process there is an urgent need for more effective non-hormonal therapies for patients with metastatic disease. Second, the clinical development of novel therapies is limited by the inability to image adequately the therapeutic response of prostate cancer metastases in patients. In men failing androgen ablative therapy, prostate cancer preferentially metastasizes to the bone resulting in the formation of predominantly osteoblastic lesions (O'Keefe and Guise, 2003; Roodman, 2004). Therapeutic response in the bone is difficult to evaluate with current 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 evaluate better the therapeutic efficacy of new treatments. Several prostate cancer targets have been identified (e.g., androgen receptor, pTEN, etc.) and novel therapies directed at these targets are currently under development (Sarker et al., 2009; Vis and Schröder, 2009). One target for prostate cancer that has been overlooked is prostate-specific antigen (PSA) or human kallikrein-related peptidase 3 (KLK3). Prostate cancer cells, like normal prostate secretory-luminal epithelial cells, produce very high levels of the PSA (Lilja, 1985; Watt et al., 1986). PSA is a chymotrypsin-like kallikrein that is used extensively as a biomarker to screen for prostate cancer, to detect recurrence following local therapies, and to follow response to systemic therapies for metastatic disease (Lilja, 1985; Watt et al., 1986; Denmeade and Isaacs, 2004). PSA is aptly named, in that it is produced at high levels exclusively by prostate cells and is not produced in significant amounts by any other normal tissue in men (Lilja et al., 2000).

Since the initial discovery of PSA, thousands of papers have been written about its role as a biomarker for prostate cancer. The functional role for PSA in the normal prostate and in prostate cancer is not known and has not been fully addressed in the literature. Thus, PSA is an enigmatic pro-

^aPresent address: Department of Pharmaceutical Chemistry, University of California, San Francisco, 600 16th St., San Francisco, CA 94158, USA.

tease; it is well known, yet poorly understood. Recently, several studies have implicated a role for PSA in the pathobiology of prostate cancer. Accumulating evidence suggests that PSA is more than a tumor biomarker and can function as a salient player in prostate cancer growth, invasion, and metastasis. To understand the role of PSA in prostate cancer, the field would be greatly aided by the development of a small-molecule inhibitor for the enzymatic activity of PSA. The selective inhibition of PSA could have important biological effects that would allow for the interrogation of the mechanism of action of PSA in promoting prostate cancer. In addition to defining the role of PSA in prostate cancer, small-molecule inhibitors of the enzymatic activity of PSA could hold promise as therapeutic agents against prostate cancer local growth, progression, and/or metastasis. Such PSA inhibitors could also find a potential use as imaging agents owing to the presence of high levels of enzymatically active PSA in the peritumoral fluid surrounding prostate cancers. In this review, we hope to shed some light on this enigma through an overview of current data addressing the role of PSA in prostate cancer pathobiology. In addition, we will review research efforts to develop inhibitors for PSA that could represent new tools to study, treat, and/or image prostate cancer.

PSA in prostate cancer

Several possible mechanisms whereby PSA can contribute to biological responses have been identified (Yousef and Diamandis, 2003). In *in vitro* studies, active PSA has been shown to directly cleave or release from binding proteins cytokines that are involved in growth stimulation and inflammation. PSA can cleave insulin-like growth factor binding protein-3 (IGFBP-3) resulting in local release of IGF-1 (Williams et al., 2007a). The roles of IGF-1 and IGFBP-3 in prostate cancer are controversial. Several studies have demonstrated an association between IGF-1 plasma levels and prostate cancer, whereas an equal amount has found that there are no increases in cleaved IGFBP-3 or IGF-1 in the plasma of men with prostate cancer when compared to healthy individuals (Cohen et al., 1992; Chan et al., 1998; Stattin et al., 2001; Koistinen et al., 2002). Despite this contradictory evidence *in vivo*, IGF-1 is known to have important effects *in vitro*. IGF-1 can have mitogenic and antiapoptotic effects on normal and transformed prostate epithelial cells as well as an effect on stromal-epithelial cells interactions (Iwamura et al., 1993; Culich et al., 1995). Additionally, in primary cultures of prostatic epithelial cells, PSA was found to activate specifically the small latent form of TGF β 2 suggesting that PSA can directly stimulate a variety of cellular processes through the release of active TGF β 2 stored within the matrix of the prostatic stroma (Dallas et al., 2005).

Unlike most solid tumor types, a hallmark of prostate cancer is the almost universal development of osteoblastic bone metastases in men failing androgen ablative therapy (O'Keefe and Guise, 2003; Roodman, 2004). The mecha-

nism whereby PSA can stimulate bone adhesion or osteoblast growth and/or differentiation has not been clearly defined. As described above, active PSA can release cytokines involved in bone turnover such as IGF-1 and TGF β 2. For example, PSA cleavage of IGFBP-3 can release IGF-1 which can promote osteoblastic activity in bone. PSA activation of the small latent form of TGF β 2 in the bone microenvironment can also potentially contribute to the formation of osteoblastic lesions (Kanety et al., 1993). Previously, it had been demonstrated that PSA could hydrolyze PTHrP, an important mediator of osteolytic bone metastases, and inactivate PTHrP-stimulated cAMP accumulation in mouse osteoblasts (Iwamura et al., 1996; Dallas et al., 2005). PTHrP 1–141 can be cleaved by PSA to release 22 and 23 amino acid fragments of PTHrP (Cramer et al., 1996). PTHrP stimulates osteoblastic production of the osteoclastic factor RANKL, but PTHrP has been shown also to inhibit production of the osteoblastic factor endothelin-1 (Lawrence, 2001). This suggests PSA could alter the bone metastatic phenotype from osteoclastic to osteoblastic through PTHrP degradation.

PSA is known to interact or metabolize several factors involved in the production of osteoblastic lesions. For example, PSA can degrade extracellular matrix components fibronectin and laminin (Lilja et al., 2000). PSA-specific mAbs have been shown to block proteolysis of extracellular matrix components and decrease invasion of PSA-producing LNCaP cells in Matrigel invasion assays (Webber et al., 1995). Using phage display, Romanov et al. identified peptides with homology to the C-terminus of PSA that attenuated binding of a prostate cancer cell line to bone marrow endothelial cells (BMECs) (Romanov et al., 2004). In this study, PSA antibodies and exogenous enzymatically active PSA attenuated binding of prostate cancer cells to BMECs (Romanov et al., 2004). These results suggest a role for active PSA in preferential adhesion of prostate cancer cells to bone. Additionally, Gygi et al. (2002) demonstrated that exogenous PSA could stimulate modest proliferation of human osteosarcoma cells. Using this same osteosarcoma cell line, Nadiminty et al. showed that PSA-expressing SaOS-2 cells markedly upregulated expression of genes associated with osteoblast differentiation including *runx-2* and osteocalcin (Nadiminty et al., 2006).

To add further mystery and complexity to the role PSA plays in prostate cancer development, numerous studies have documented the antiangiogenic properties of PSA. PSA has been shown to inhibit angiogenesis both *in vitro* and *in vivo* (Fortier et al., 1999, 2003). In early findings, Fortier et al. noted that purified PSA inhibited human umbilical vein endothelial cell (HUVEC) proliferation, migration, and invasion. Additionally, PSA inhibited endothelial cell response to the angiogenic stimulators fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor. Later the same group demonstrated *in vivo* using a murine model that PSA inhibited FGF mediated angiogenesis in a Matrigel plug assay (Fortier et al., 2003). In an attempt to exploit the antiangiogenic properties of enzymatically active PSA, Wu et al. used phage display to discover PSA-activating peptides (Wu et al., 2000). Selected peptides from phage libraries were

expressed as glutathione-S-transferase (GST) fusion peptides and tested for activity. The best peptide stimulated PSA activity 5-fold over the control and was highly selective for PSA when compared with proteases possessing similar specificity. Such peptides also inhibited HUVEC growth and are currently undergoing structural refinement to gain favorable pharmacokinetics and dynamics *in vivo* (Koistinen et al., 2008a; Mattsson et al., 2009).

Peptide substrates for PSA

Although the studies referenced above have identified a series of putative protein substrates for PSA, several studies have evaluated PSA substrate requirements using small peptide substrates. Initially, a variety of chymotrypsin substrates containing tyrosine in the P1 position were used to assay the enzymatic activity of PSA. These substrates, however, had relatively high K_m values (1–2 mM), low k_{cat} values for PSA, and poor specificity (i.e., 10 000-fold more rapidly cleaved by chymotrypsin) (Watt et al., 1986; Lilja et al., 1989; Kurkela et al., 1995). The discovery that PSA could cleave the gel-forming proteins semenogelins I and II in human semen led Lilja and colleagues to map PSA-specific cleavage sites within these proteins. As expected, PSA cleaved after tyrosine and leucine residues (Lilja, 1985; Lilja et al., 1989; Malm et al., 2000). Somewhat surprising was the finding that out of the nearly 30 peptides detailed in the cleavage map, nearly 40% of the peptides contained a glutamine residue in the P1, making PSA the only known serine protease with this substrate specificity *in vivo*.

With the goal of developing PSA-activated peptide prodrugs, Denmeade and colleagues used the semenogelin cleavage map to define selective PSA substrates (Denmeade et al., 1997). In this study, peptides of seven amino acids representing the sequence proximal to the PSA cleavage site (P7–P1) were synthesized with 7-amino-4-methylcoumarin (AMC) attached to the C-terminus of the P1 amino acid in the P-1 position (Denmeade et al., 1997). This method yielded PSA substrates with improved K_m and k_{cat} values and a high degree of specificity. Those substrates containing tyrosine in the P1 position were hydrolyzed efficiently by PSA, but the proteolysis rates were 10–20 times higher with chymotrypsin and elastase. Substrates with glutamine in the P1 position were less efficiently hydrolyzed, but demonstrated better specificity for PSA. The P1 glutamine containing peptide EHSSKLQ-AMC was chosen from among the other sequences for additional characterization based on its PSA activity, its specificity for PSA, and its stability in different sera. This sequence was used to generate substrates of varying lengths that were analyzed for PSA activity and specificity. It was found that PSA could only cleave substrates that were four amino acids or greater in length. From these studies the six amino acid substrate HSSKLQ-AMC ($K_m=470 \mu\text{M}$, $k_{cat}/K_m=23 \text{ M}^{-1} \text{ s}^{-1}$) was evaluated further based on a combination of efficiency and selectivity of hydrolysis by PSA. This PSA substrate sequence has been used to measure PSA activity in a variety of tissues (Den-

meade et al., 1997). In addition, it has been used to generate novel PSA activated prodrugs and protoxins for the treatment of prostate cancer and benign prostatic hyperplasia (BPH) (Denmeade et al., 1998, 2003).

Other groups have sought to define further the substrate specificity of PSA using combinatorial methods and by refining sequences from naturally occurring substrates. Coombs et al. (1998) investigated the sequence characteristics of the substrate prime site binding pockets. Optimized semenogelin peptides and phage display were used to indentify PSA cleavable substrates containing P4 to P2' residues. Five rounds of phage display yielded sequences that were rich in hydrophobic residues populating the P1 and P2 positions, with tyrosine and leucine being the most common P1 residues. When the residues contributed by the phage linker region were removed to prevent any bias towards serine, glycine, or alanine, the resulting consensus sequence was: P5 (R, L>X), P4 (S>A), P3 (S>A, R, T), P2 (Y>X), P1 (Y>L), P1' (S, T, A>Q), P2' (S>A, R), P3' (A, S). The hydrolysis rates of peptides selected from phage display were determined for PSA and chymotrypsin. In each case the k_{cat}/K_m value was found to be greater for chymotrypsin, often significantly so ($2.6 \text{ M}^{-1} \text{ s}^{-1}$ for PSA versus $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). The substrates discovered were excellent PSA substrates, but even better substrates for chymotrypsin. This was most probably as a result of the fact that peptides identified by this method primarily contained Y or L in the P1 site. Taking advantage of the available crystal structure data showing that serine proteases as a group interact most strongly with positions P3–P3', Yang et al. developed synthetic hexapeptide substrates for PSA using single position mini-libraries (Yang et al., 1999). P1' and P2 mini-libraries were constructed based on a selected semenogelin cleavage peptide with tyrosine as the constant P1 residue. In this limited study, serine was found to be the preferred P1' residue and phenylalanine the preferred P2 residue. The enzyme kinetics of the optimized substrate Mca-QFYSSNK(ϵ -Dnp) were measured for PSA and were found to have a K_m value of $77 \mu\text{M}$, but a low k_{cat}/K_m value of $39 \text{ M}^{-1} \text{ s}^{-1}$. Finally, Debela and colleagues recently determined the non-prime side specificity of several kallikreins, including PSA, using a positional scanning synthetic combinatorial library of tetrapeptide substrates (Debela et al., 2006). Using this method the order of preferred PSA P1 residues was surprisingly found to be Met>Nle=Ala>Tyr=Arg.

Structural features of the S1 pocket define PSA substrate specificity

The development of potent and selective PSA substrates and inhibitors requires a detailed understanding of the structure of the catalytic site of PSA. Particular attention should be focused on the S1 pocket of the protease, which in large part determines the specificity. Sequence alignment of PSA with all of the human kallikreins and bovine α -chymotrypsin (Figure 1A) reveals that the region spanning the S1 pocket

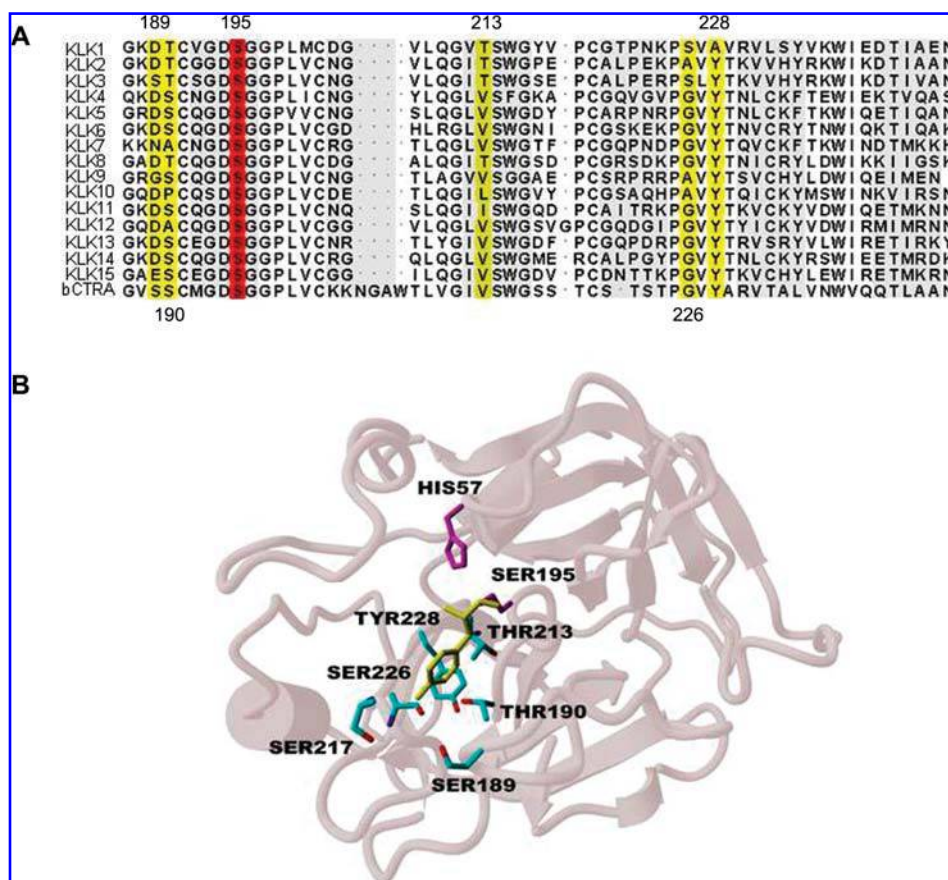


Figure 1 Sequence alignment of human kallikreins with bovine α -chymotrypsin (A) and structure of the S1 specificity pocket of PSA (B).

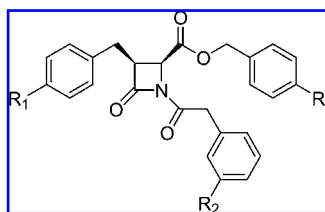
(A) Amino acid sequence alignment of the 15 human kallikreins and bovine α -chymotrypsin. Only the residues spanning the S1 pocket of serine proteases are presented. The residues highlighted in yellow are critical for the P1 specificities of respective protease. The catalytic Ser195 residue is highlighted in red. (B) Ribbon representation of the S1 specificity pocket of prostate-specific antigen. The protease residues lining the pocket are shown in cyan, whereas the catalytic histidine and serine residues are shown in magenta. The S1 tyrosine residue of peptide substrate KGISSQY is shown in yellow.

contains several highly conserved residues that are critical for the structural integrity of the pocket and indirectly responsible for the maintenance of enzymatic activity. Specifically, residues such as Cys¹⁹¹, Cys²²⁰, Pro²²⁵, and Ser²¹⁴ form crucial elements of the architecture surrounding the S1 pocket (Singh et al., 2009). Conversely, the variable regions of residues 183–190 and 221–224 within a given serine protease dictate the unique specificity of substrates and inhibitors for the individual protease.

For all serine proteases, substrate and inhibitor recognition is mainly governed by the binding of the P1 amino acid residue to the S1 pocket of the enzyme (Figure 1A). The amino acid residue at the bottom of the S1 pocket is therefore the key determinant for the P1 preference. For the majority of the kallikreins, this residue is aspartic acid. Thus, the majority of kallikreins exhibit trypsin-like specificity, cleaving after basic amino acids such as arginine and lysine. In contrast, for PSA the residue at the bottom of the pocket is serine (i.e., Ser¹⁸⁹). Chymotrypsin also has serine in this position and thus shares some substrate specificity with PSA, cleaving after tyrosine and phenylalanine. Two other kalli-

kreins also have a unique (i.e., non-aspartic acid) amino acid in the S1 pocket with KLK7 having asparagine and KLK9 having glycine at this position.

The Ser¹⁸⁹ residue in the PSA S1 pocket is flanked by the polar side chains of Ser²²⁶ and Thr¹⁹⁰ (Figure 1B). The alignment of the polar hydroxyl moieties of these residues at the bottom of the pocket results in a cavity that is polar at the bottom, but hydrophobic on the sides owing to the aliphatic parts of the side chains forming the walls of the cavity. As shown in Figure 1B, the aromatic ring of a P1 tyrosine residue is optimally sandwiched between the hydrophobic cavity walls while making a hydrogen bond with the backbone carbonyl of the Ser²¹⁷ residue via its hydroxyl moiety. Thus, a residue such as tyrosine with a medium-sized hydrophobic side chain and a polar phenolic group is ideal for binding at the S1 pocket of PSA. Other medium-sized hydrophobic side chains such as leucine and norleucine can also interact favorably with this pocket. The other residues, Thr¹⁹⁰, Thr²¹³, and Ser²²⁶, in the vicinity of the 189-position in the PSA protein are also responsible for the subtle variations in the polarity and hydrophobic character of the S1 binding site. These res-

Table 1 Structures and IC₅₀ values of the β-lactam-based PSA inhibitors developed by Adlington et al. (1997, 2001).

Compound	R ₁	R ₂	R ₃	IC ₅₀ (μM)
1	H	CO ₂ Bn	CO ₂ H	8.98±0.1
2	H	<i>p</i> -CO ₂ CH ₂ -C ₆ H ₄ -CO ₂ H	CO ₂ H	5.84±0.9
3	H	H	CO ₂ H	24.3±3.9
4	H	CO ₂ H	CH ₂ NH ₂ -TFA	1.34±0.1
5	H	CO ₂ Bn	H	1.43±0.2
6^a	OH	CO ₂ Bn	H	0.348±0.1
7^b	OH	CO ₂ Bn	H	0.226±0.01

^aRacemic mixture of compounds.^bHomochiral 3S4S enantiomer.

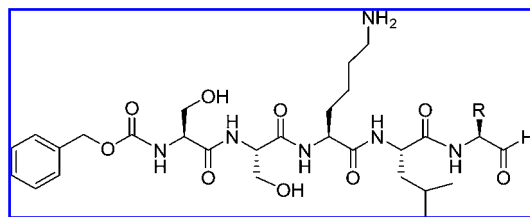
idues produce a pocket that is truly unique in its binding characteristics.

These insights into the S1 pocket of PSA came initially from models built using the specificity pocket of other similar tissue kallikreins (Adlington et al., 2001; Singh et al., 2008). These models were later modified once a partial crystal structure for PSA was solved (Menez et al., 2008). The first PSA inhibitors reported in the literature used such a homology model derived from porcine kallikrein to design and characterize a series of monocyclic β-lactam compounds (Adlington et al., 2001). Prior to this study, it had been shown that β-lactams were efficient and specific inhibitors of several serine proteases, particularly human leukocyte elastase (Knight et al., 1992; Green et al., 1995). The first generation of monocyclic β-lactam inhibitors for PSA began with a lead compound **1** (IC₅₀=8.98 μM) developed by Adlington et al. (2001) (Table 1). This group then used the homology model to aid in the development of more potent PSA inhibitors **1–7** (Adlington et al., 1997). Using a low energy conformation of the azetidinone compound **1**, initial modeling studies suggested that the (3S, 4S) enantiomer should be used as the template for further SAR studies. With this template the importance of binding of the three side chains appended to the 2-azetidinone was investigated in the active site pocket of PSA homology model. The best inhibitor from these studies was the enantiomerically pure inhibitor **7** which had an IC₅₀ value of 0.226 nM for PSA (Adlington et al., 2001). The specificity of this compound and others in the class versus chymotrypsin and other proteases was, unfortunately, not reported in this study.

To develop potent and selective β-lactam based inhibitors for PSA, our laboratory investigated the mechanism behind PSA inhibition by β-lactams and tested newly synthesized β-lactams for candidate inhibitors (Singh et al., 2008). It has been suggested that β-lactams inhibit PSA in a time-depend-

ent manner with a 1:1 stoichiometry. To investigate the time-dependent nature of β-lactam inhibition, time course assays the enzymatic reaction was allowed to run for 1 h, after which a representative β-lactam **1** (IC₅₀=8.98 μM) was added at a 1 mM final concentration and the assay was allowed to proceed for another hour. The inhibitory effect was not immediate and not observed until 24 min after the addition of the inhibitor, at which time the inhibition was complete. Based on these results, it was concluded that the β-lactams react with PSA in a time-dependent manner, competing with substrate, until a majority of PSA is converted into an irreversible inactive state. Inhibitor wash-out studies and MALDI mass spectrometry analysis indicated that a stable covalent complex formed between the β-lactam and the active site serine residue in PSA (Singh et al., 2008).

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 inhibit PSA effectively (Huang et al., 2001). The best inhibitor from this study had a K_i value of 0.5 μM and a *k*_{inact}/K_i value of 32 000 M⁻¹ s⁻¹. To elucidate the role PSA in angiogenesis, Koistinen and colleagues screened a library of nearly 50 000 compounds to discover inhibitors of PSA (Koistinen et al., 2008b). Out of the compounds screened only two were identified that inhibited PSA in a dose-dependent manner. The active compounds were either benzoxazinone or triazole derivatives. Focused mini-libraries of compounds similar in structure to the initial compounds were constructed resulting in the discovery of several compounds with low micromolar and nanomolar IC₅₀ values for PSA. It was found that the benzoxazinone compounds were non-competitive inhibitors and the triazoles were competitive inhibitors of PSA. The most effective inhibitors were tested in a HUVEC angiogenesis model. Two of the compounds were found to abrogate the

Table 2 The chemical structures, K_i values, and GOLD scores of the PSA inhibitors used in this study.

Compound	R	K_i (μM)	GOLD score (PSA)
8	$\text{CH}_2\text{CH}_2\text{CONH}_2$	45.21 ± 3.89	33.45
9	$\text{CH}_2\text{CH}_2\text{CON}(\text{CH}_3)_2$	2.53 ± 0.13	30.66
10	$\text{CH}_2\text{CON}(\text{CH}_3)_2$	13.09 ± 0.88	19.82
11	CH_2NHCHO	0.91 ± 0.06	29.54
12	$\text{CH}_2\text{NHCOCH}_3$	3.91 ± 0.35	29.56
13	$\text{CH}_2\text{NHCOCH}_2\text{CH}_3$	9.84 ± 0.33	34.13
14	$\text{CH}_2\text{NHCOCH}(\text{CH}_3)_2$	13.28 ± 0.93	33.25
15	$\text{CH}_2\text{NHCO}(\text{C}_6\text{H}_5)$	>25	27.55
16	$\text{CH}_2\text{CH}_2\text{SCH}_3$	3.84 ± 0.21	34.77
17	$\text{CH}_2\text{CH}_2\text{SOCH}_3$	7.25 ± 0.44	38.27
18	$\text{CH}_2\text{CH}_2\text{CN}$	8.14 ± 0.29	27.47
19	$\text{CH}(\text{CH}_3)_2$	>100	16.16
20	$\text{CH}_2\text{CH}(\text{CH}_3)_2$	6.51 ± 0.25	26.37
21	$\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	11.24 ± 0.26	30.93
22	(C_6H_5)	>100	22.92
23	$\text{CH}_2(\text{C}_6\text{H}_5)$	0.57 ± 0.04	38.84
24	$\text{CH}_2(\text{C}_6\text{H}_5)\text{OH}$	0.37 ± 0.02	39.66

antiangiogenic function of PSA as noted by the increased tube area and the formation of tubular connections in the HUVEC model (Koistinen et al., 2008b).

Peptide-based PSA inhibitors

Peptide-based inhibitors have the advantage of ease of synthesis over the other small molecule classes described above. Peptides also provide a convenient platform for the addition of therapeutic and imaging moieties. On this basis, we used an iterative approach to generate a series of inhibitors. For screening purposes, peptide aldehydes were initially generated based on ease of synthesis (LeBeau et al., 2008, 2009a,b). Subsequently, boronic acid-based inhibitors were generated from promising peptide aldehydes.

As the recognition of glutamine in the P1 position is relatively unique to PSA, the first set of studies was designed to evaluate the specificity of glutamine containing peptide aldehyde inhibitors using the peptide substrate SSKLQ as a starting template. The evaluation of a P1 glutamine peptide aldehyde inhibitor is problematic, as glutamine aldehydes exist primarily in the cyclic hemiaminal form. Although NMR analysis confirmed that the full length glutamine peptide aldehyde **8** was in equilibrium between a cyclic hemiaminal and an aldehyde, it could still act as a modest inhibitor of PSA with a K_i value of $45.21 \mu\text{M}$ (Table 2). Although cyclization does occur and is thermodynamically favored, the side chain is still potent and able to form favor-

able interactions with the bottom of the S1 pocket. Therefore, this result suggested that a non-cyclizable glutamine aldehyde derivative would be an even more potent and viable PSA inhibitor. The non-cyclizable γ -*N,N*-dimethyl glutamine derivative **9** was a more potent inhibitor with a nearly 20-fold lower K_i value of $2.53 \mu\text{M}$ (LeBeau et al., 2009a). PSA inhibitor **9** still possessed the desired predicted motif, a side chain with two hydrophobic methylenes, an amide carbonyl and a nitrogen with a lone pair of electrons that were in the vicinity of other polar residues and could act as hydrogen bond acceptors. A shorter side chain **10** resulted in a less potent inhibitor with a K_i value of $13.09 \mu\text{M}$ for PSA (Table 2).

Isostere replacement glutamine derivative peptide aldehydes (**11–15**) were tested and a pronounced increase in the ability to inhibit PSA was observed with some of the compounds (LeBeau et al., 2009a). The best compound of this series, **11** the *N*-formyl derivative, had a K_i value of $0.909 \mu\text{M}$ for PSA. The rest of the compounds in the series all possessed K_i values of $>1 \mu\text{M}$. Compound **12**, with the larger acetyl group on the γ amine, had a higher K_i value of $3.91 \mu\text{M}$. The K_i values for the compounds (**13–15**) steadily increased as the acyl chain increased in size and branching, leading to unfavorable steric clashes with the S1 pocket wall. Based on positional scanning substrate studies documenting the ability of PSA to cleave after methionine residues (Debla et al., 2006), peptide aldehydes containing a P1 methionine **16** and methionine sulfoxide **17** were generated and also found to inhibit PSA, with **16** having a lower K_i value of $3.84 \mu\text{M}$ and **17** possessing a nearly 2-fold higher K_i value of $7.25 \mu\text{M}$ (LeBeau et al., 2009a) (Table 2).

Peptide aldehydes possessing hydrophobic P1 aldehydes containing natural and unnatural amino acids were next tested for their ability to inhibit PSA (LeBeau et al., 2009a; see Table 2). Compound **19**, the full length valine peptide aldehyde, was not an inhibitor of PSA with a $K_i > 100 \mu\text{M}$. By increasing the side chain of the valine by one carbon to the leucine peptide aldehyde **20**, the K_i value decreased to $6.51 \mu\text{M}$, emphasizing that although β -branching is not well tolerated, γ -branching is allowed. The norleucine peptide aldehyde **21**, containing the longest hydrocarbon side chain out of the series, worked as an inhibitor, but with a higher K_i value of $11.24 \mu\text{M}$. The P1 phenylglycine peptide aldehyde derivative **22** did not inhibit PSA with a K_i value > 100 . Moving from **22** to the phenylalanine peptide aldehyde **23**, a marked decrease in the K_i is observed yielding a submicromolar K_i value of $0.57 \mu\text{M}$. The substitution of a phenolic side chain as in the tyrosine peptide aldehyde **24** produced an even more potent PSA inhibitor with a K_i value of $0.37 \mu\text{M}$. An explanation for this observation is that the tyrosine side chain makes sufficient hydrophobic interactions with the walls of the S1 pocket and its hydroxyl group is able to interact in a favorable manner with the polar residues at the bottom of the pocket.

To demonstrate the unique substrate specificity of PSA versus chymotrypsin, the best P1 peptide aldehyde inhibitors were tested against chymotrypsin (LeBeau et al., 2009a). The full length glutamine aldehyde **8**, although not the best

Table 3 The specificity of six peptide aldehydes for PSA versus chymotrypsin and the corresponding GOLD scores for each protease.

Compound	PSA K_i (μM) ^a	Chymotrypsin K_i (μM) ^a	Chy/PSA ratio ^b	PSA Gold score	Chymotrypsin Gold score
8	45.21±3.89	>1000	>22	33.45	16.53
9	2.53±0.13	>100	>40	30.66	14.27
11	0.91±0.06	>100	>110	29.54	19.25
16	3.84±0.21	>100	>26	34.77	23.07
23	0.57±0.04	0.62±0.05	1.08	38.84	28.38
24	0.37±0.02	0.29±0.03	0.78	39.66	28.86

^aValue reported is for n=3 (\pm SE).

^bPSA selectivity in terms of (Chymotrypsin K_i)/(PSA K_i).

inhibitor but one that demonstrated the unique proteolytic activity of PSA, was tested against chymotrypsin and showed no ability to inhibit chymotrypsin with a K_i value for chymotrypsin >1000 μM (Table 3). Likewise, the non-cyclizable γ -*N,N*-dimethyl glutamine derivative **9** did not inhibit chymotrypsin to any degree nor did the glutamine isostere **11**. Compounds **23** and **24** were almost equally as potent for PSA and chymotrypsin as both compounds were peptide aldehydes of canonical hydrophobic P1 residues. Having established a positive correlation between GOLD score and the inhibitory potency of the peptide aldehyde inhibitors, we set out to use the same methodology for elucidating the difference in potency of six peptide aldehyde inhibitors that were simultaneously tested against both PSA and chymotrypsin. Table 3 presents the respective K_i values and the GOLD docking scores of these compounds when their P1 side chain was docked in the S1 pocket of either protease. Remarkably, the difference in the potency of **8**, **9**, **11**, and **16** against PSA versus chymotrypsin was consistent with the respective differences in GOLD scores, particularly for compounds **8**, **9**, and **11**. Similarly, **23** and **24** were equally potent against both PSA and chymotrypsin, and also possessed the highest GOLD scores.

The P1 residues of the most specific peptide aldehyde inhibitors **8**, **9**, **11**, and **16** could not easily be converted into boronic acids; thus, our next study consisted of evaluating peptide aldehydes with fixed P1 side chains whose structures could be translated into boronic acids. We decided to fix the P1 position as leucine and norleucine owing to the ease of synthesizing resin-bound leucine/norleucine peptide aldehydes and the short synthesis required to make boronic acid derivatives. The effect on substituting different amino acids in the P2 position and the peptide length was investigated (Table 4). The data from the P2 focused library provided evidence that PSA prefers hydrophobic residues in the P2 position (LeBeau et al., 2009b). Both negatively and positively charged residues were not tolerated. It was observed that residues containing β -branched side chains, i.e., isoleucine, valine, and threonine, were not effective inhibitors. On the inhibitor length, it was discovered the inhibitor had to be at least four residues in length for effective inhibition with the P5 residue representing a variable position. Based on these findings, the inhibitor Z-SSKLL-H ($K_i=6.5$ μM) was selected as the best inhibitor chosen for further study.

Boronic acid-based PSA inhibitors

The ultimate goal of these studies was to identify a candidate PSA inhibitor that could be used to target therapeutic agents or modified to create imaging agents. Although peptide aldehydes are useful screening tools, they would not be useful in complex biological matrixes. They are reactive with both cysteine and serine proteases and the aldehyde functionality can react with numerous nucleophiles in a biological milieu. To overcome this, our best aldehydes inhibitors would in turn be synthesized into boronic acid derivatives. Peptide boronic acids react only with nucleophilic serine residues and are potent serine proteases inhibitors owing to the Lewis acid nature of the electron deficient boron (Bone et al., 1987). They are also stable at physiological pH and in complex biological environments.

A strategy using peptidyl boronic acids was pursued using the sequence preferences discovered in the primary peptide aldehyde screen (LeBeau et al., 2008). A dramatic increase in inhibitory ability was observed when the sequences went from peptide aldehydes to boronic acids. For example, the K_i value of Z-SSKL(boro)L (**57**) was 65 nM which was a 100-fold improvement in K_i compared with the corresponding aldehyde (Table 4). D-isomer amino acids were incorporated into the sequence of our lead inhibitor Z-SSKL(boro)L (**58–64**) to determine the relative importance of each amino acid residue. It was found that incorporating one or multiple D-amino acids into the P2–P4 positions was highly deleterious to inhibitor function, whereas D-amino acid substitution at the P5 position was more tolerated as it only increased the K_i \sim 2-fold (LeBeau et al., 2008).

Next, the inhibitor Z-SSKL(boro)L was tested for specificity against a panel of proteases consisting of chymotrypsin, trypsin, DPP-4, elastase, and the non-serine proteases cathepsins B and D (LeBeau et al., 2008). Although chymotrypsin is capable of cleaving after leucine residues, the K_i value for inhibiting chymotrypsin was 60-fold higher than for PSA. Modest inhibition of elastase was observed, whereas the compounds did not inhibit trypsin or DPP-4 at all. No inhibition of either cathepsins B or D was observed over a range of concentrations, consistent with the fact that boronic acids alone are not effective inhibitors of cysteine or aspartyl protease.

Table 4 Peptide aldehyde and boronic acid inhibitors of PSA.

Compound		K_i (μM)	Compound		K_i (μM)
Leucine aldehydes			Peptidyl boronic acids		
25	Z-SSKLL-H	6.51±0.25	50	Z-Q(boro)L	265.15±18.39
26	Z-SSKFL-H	11.9±0.9	51	Z-K(boro)L	353.5±23.68
27	Z-SSKYL-H	13.09±0.91	52	Z-SSK(boro)L	18.49±1.48
28	Z-SSKML-H	13.7±1.2	53	Z-SSK(boro)Nle	34.21±0.83
29	Z-SSKQL-H	21.79±0.51	54	Z-KL(boro)L	5.98±0.24
30	Z-SSKIL-H	37.43±2.43	55	Z-KL(boro)Nle	23.57±0.47
31	Z-SSKKL-H	57.85±1.14	56	Z-SSKL(boro)Nle	0.398±0.020
32	Z-SSKVL-H	70.71±1.77	57	Z-SSKL(boro)L	0.065±0.005
33	Z-SSKTL-H	>100	D-isomer amino acids		
34	Z-SSKAL-H	>100	58	Z-SSKL(boro)L	0.149±0.009
35	Z-SSKF(Br)L-H	>500	59	Z-SSKL(boro)L	21.48±2.28
36	Z-SSKLD-H	>1000	60	Z-SSKL(boro)L	28.67±1.32
37	Z-SKLL-H	42.43±2.13	61	Z-SSKL(boro)L	75.76±5.21
38	Z-SSKL-H	>1000	62	Z-SSKL(boro)L	32.21±1.73
39	Z-KLL-H	>100	63	Z-SSKL(boro)L	227.27±11.61
40	Z-YL-H	>500	64	Z-SSKL(boro)L	439.94±19.04
41	Z-KL-H	>1000	Lysine derivatives		
42	Z-QL-H	>1000	65	Z-SSL(boro)L	3.86±0.29
43	Z-SSKLL-H	>1000	66	Z-SSK(Ac)L(boro)L	0.294±0.026
Norleucine aldehydes					
44	Z-SSKLNle-H	11.24±0.26			
45	Z-KLNle-H	414.1±22.49			
46	Z-KNle-H	>1000			
47	Z-QNle-H	>1000			
48	Z-SSKNle-H	>1000			
49	Z-SSKLNle-H	>1000			

Residues containing D-isomer amino acids are underlined.

The specificity of our inhibitor for PSA over chymotrypsin can be explained by observing the role of the P3 lysine residue in binding to PSA. In our model of the PSA catalytic site interaction with Z-SSKL(boro)L, the P3 residue serves as a lid to the specificity pocket and docks at a location just above the P1 binding site in the specificity pocket (Singh et al., 2008; LeBeau et al., 2009b; Figure 2). In this model, we observed that the P3 lysine residue of the inhibitor interacts with a carboxyl group of the Glu²⁰⁸ side chain in the protease active site. The distance between the two groups (3.5 Å) is close enough for the formation of a salt bridge, thus leading to favorable binding interactions between the protease and the inhibitor. As noted earlier, omission of the lysine residue (**65**) or replacing it with its mirror image, abrogated this important interaction and yielded poor inhibitors. The presence of an acidic residue at position 208 is not common in chymotrypsin family proteases.

Modeling studies further documented that the P4 pocket, located in the lower groove area, is mainly formed by His¹⁶⁴, Pro¹⁶⁵, Gln¹⁶⁶, and Trp²⁰⁵ residues (Singh et al., 2008, 2009; Figure 2). The P4 binding pocket of PSA is bounded by the kallikrein loop from above but loop residues are not involved in any important energetic interactions. The Gln¹⁶⁶ side chain is ideally placed to form a hydrogen bond with the hydroxyl of P4 serine (Singh et al., 2008, 2009). The presence of Gln¹⁶⁶ at this position is unique for PSA and not common in other serine proteases from the kallikrein family. The importance of this interaction with S4 was demonstrated by

marked increase in K_i for inhibitors lacking P4. Finally, the P5 side chain was found to dock in the lower groove area but it is exposed to the solvent and there are no specific interactions with the protease residue. This suggests that P5 and additional N-terminal amino acid residues should not provide a significant contribution towards the overall binding affinity of inhibitor molecule.

Subsequent to these studies and after completing an extensive aldehyde screen (LeBeau et al., 2009b), the refined sequence of the best PSA inhibitor was Z-SSQn(boro)L with glutamine in the P3 position and norleucine now in the P2

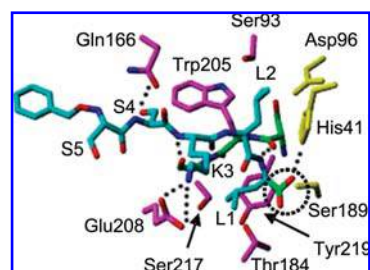


Figure 2 Interactions of the Z-S⁵S⁴K³L²(boro)L¹ inhibitor with the key residues in the catalytic pocket.

Catalytic triad residues are shown in yellow, the protease residues in magenta, and the backbone of 204–206 residues in green. Dotted lines represent hydrogen bonds, and the dotted circle pinpoints the exact location of the boronic acid moiety.

position. This new inhibitor, with an increased serum half-life over **57**, is the most potent described to date for PSA with a $K_i=25$ nM, although it is somewhat less specific with a K_i value of 211 nM for chymotrypsin. This inhibitor was subsequently coupled at the amino terminus to a single amino acid chelating group capable of chelating ^{99m}Tc without any impact on K_i for PSA inhibition (LeBeau et al., 2009b). Further studies are underway to determine the ability of such chelates to image PSA producing human prostate cancer xenografts *in vivo*.

Conclusions

PSA represents an excellent target for prostate cancer therapy and imaging. As prostate cancer progresses, the glandular architecture of the prostate becomes compromised allowing PSA in the tissue to leak into the circulation where it is found in high concentrations, thus becoming the basis for the PSA test. Low PSA concentrations in prostatic tissue are often associated with an increase in malignancy grade and tumor stage (Grande et al., 2000; Stege et al., 2000). PSA is produced at high levels by both androgen dependent and castrate resistant prostate cancer cells. Autopsy studies have demonstrated the homogeneity of PSA production in both bony and soft tissue metastases with the majority of cells in metastatic sites producing PSA (Roudier et al., 2003). In addition, PSA is aptly named as it is highly specific and not produced to any measurable degree by any other tissues in the adult male except by normal and malignant prostate epithelial cells (Lilja et al., 2000). As reviewed here and elsewhere, accumulating evidence suggests that this high level of PSA production could play a role in the growth and progression of prostate cancer (Williams et al., 2007a). These combined results provide the background rationale to support the further development of PSA activated therapies. In this regard, clinical studies have been performed using a PSA-activated peptide doxorubicin prodrug. This prodrug was not developed further owing to poor specificity of the peptide and non-specific activation within the blood (Wong et al., 2001). However, a PSA-activated prodrug consisting of the more specific HSSKLQ peptide coupled to an analog of the highly potent natural product thapsigargin is also under development (Denmeade et al., 2003). The bacterial toxin proaerolysin has also been modified to a form that requires PSA for activation and this protoxin (Williams et al., 2007b), termed PRX302, has completed phase I studies as intraprostatic therapy for men with locally recurrent prostate cancer and phase I and phase II as therapy for BPH. Finally, the potent PSA inhibitors described here are also under study as potential targeted therapeutic agents and are also being modified to generate selective imaging agents for prostate cancer (LeBeau et al., 2009b).

Acknowledgments

The authors would like to acknowledge Daniel Hostetter, Amanda Vlastic, and Paco Rodriguez for their careful proofreading of the

manuscript and for numerous stimulating discussions related to the topic. A.M. LeBeau gratefully acknowledges the financial support of the US Department of Defense Postdoctoral Prostate Cancer Training Award PC094386.

References

- Adlington, R.M., Baldwin, J.E., Chen, B., Cooper, S.L., McCoull, W.M., Pritchard, G.E., Howe, T.J., Becker, G.W., Hermann, R.B., McNulty, A.M., et al. (1997). Design and synthesis of novel monocyclic β -lactam inhibitors of prostate-specific antigen. *Bioorg. Med. Chem. Lett.* **7**, 1689–1694.
- Adlington, R.M., Baldwin, J.E., Becker, G.W., Chen, B., Cheng, L., Cooper, S.L., Hermann, R.B., Howe, T.J., McCoull, W., McNulty, A.M., et al. (2001). Design, synthesis, and proposed active site binding analysis of monocyclic 2-azetidinone inhibitors of prostate specific antigen. *J. Med. Chem.* **44**, 1491–1508.
- Bone, R., Shenvi, A.B., Kettner, C.A., and Agard, D.A. (1987). Serine protease mechanism: structure of an inhibitory complex of alpha-lytic protease and a tightly bound peptide boronic acid. *Biochemistry* **26**, 7609–7614.
- Chan, J.M., Stampfer, M.J., Giovannucci, E., Gann, P.H., Ma, J., Wilkinson, P., Hennekens, C.H., and Pollak, M. (1998). Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **279**, 563–566.
- Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992). Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. *J. Clin. Endocrinol. Metab.* **75**, 1046–1053.
- Coombs, G.S., Bergstrom, R.C., Pellequer, J.L., Baker, S.I., Navre, M., Smith, M.M., Tainer, J.A., Madison, E.L., and Corey, D.R. (1998). Substrate specificity of prostate-specific antigen. *Chem. Biol.* **5**, 475–488.
- Cramer, S.D., Chen, Z., and Peehl, D.M. (1996). Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. *J. Urol.* **15**, 526–531.
- Crawford, E.D., Eisenberger, M.A., McLeod, D.G., Spaulding, J.T., Benson, R., Dorr, F.A., Blumenstein, B.A., Davis, M.A., and Goodman, P.J. (1989). A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. *N. Engl. J. Med.* **321**, 419–424.
- Culig, Z., Hobisch, A., Cronauer, M.V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., Klocker, H. (1995). Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Eur. Urol.* **27** (Suppl.), 45–47.
- Dallas, S.L., Zhao, S., Cramer, S.D., Chen, Z., Peehl, D.M., and Bonewald, L.F. (2005). Preferential production of latent transforming growth factor beta-2 by primary prostatic epithelial cells and its activation by prostate-specific antigen. *J. Cell. Physiol.* **202**, 361–370.
- Debela, M., Magdolen, V., Schechter, N., Valachova, M., Lottspeich, F., Craik, C.S., Choe, Y., Bode, W., and Goettig, P. (2006). Specificity profiling of seven human tissue kallikreins reveals individual subsite preferences. *J. Biol. Chem.* **281**, 25678–25688.
- Denmeade, S.R. and Isaacs, J.T. (2004). The role of prostate-specific antigen in the clinical evaluation of prostate cancer. *BJU Int.* **1** (Suppl.), 10–15.
- Denmeade, S.R., Lou, W., Malm, J., Lovgren, J., Lilja, H., and Isaacs, J.T. (1997). Specific and efficient peptide substrates for

- assaying the proteolytic activity of prostate-specific antigen. *Cancer Res.* 57, 4924–4930.
- Denmeade, S.R., Nagy, A., Gao, J., Lilja, H., Schally, A., and Isaacs, J.T. (1998). Enzymatic-activation of a doxorubicin-peptide pro-drug by prostate-specific antigen. *Cancer Res.* 58, 2537–2540.
- Denmeade, S.R., Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B., and Isaacs, J.T. (2003). Prostate-specific antigen (PSA) activated thapsigargin prodrug as targeted therapy for prostate cancer. *J. Natl. Cancer Inst.* 95, 990–1000.
- Fortier, A.H., Nelson, B.J., Grella, D.K., and Holaday, J.W. (1999). Antiangiogenic activity of prostate-specific antigen. *J. Natl. Cancer Inst.* 91, 1635–1640.
- Fortier, A.H., Holaday, J.W., Liang, H., Dey, C., Grella, D.K., Holland-Linn, J., Vu, H., Plum, S.M., and Nelson, B.J. (2003). Recombinant prostate-specific antigen inhibits angiogenesis *in vitro* and *in vivo*. *Prostate* 56, 212–219.
- Grande, M., Carlström, K., Lundh Rozell, B., Stege, R., and Pousette, Å. (2000). Prognostic value of serial tissue prostate-specific antigen measurements during different hormonal treatments in prostate cancer patients. *Clin. Cancer Res.* 6, 1790–1795.
- Green, B.G., Chabin, R., Mills, S., Underwood, D.J., Shah, S.K., Kuo, D., Gale, P., Maycock, A.L., Liesch, J., Burgey, C.S., et al. (1995). Mechanism of inhibition of human leucocyte elastase by beta-lactams. Stability, reactivation kinetics, and products of beta-lactam-derived E-I complexes. *Biochemistry* 44, 14331–14343.
- Gygi, C.M., Leibovitch, I.Y., Adlington, R., Baldwin, J.E., Chen, B., McCoull, W., Pritchard, G.J., Becker, G.W., Dixon, E.P., Little, S.P., et al. (2002). Prostate-specific antigen (PSA)-mediated proliferation, androgenic regulation and inhibitory effects of LY312340 in HOS-TE85 (TE85) human osteosarcoma cells. *Anticancer Res.* 5, 2725–2732.
- Huang, A., Knoell, C., Fey, G., Hazegh-Azam, M., Tashjian, A., Hedstrom, L., and Abeles, R.H. (2001). Modulation of recombinant human prostate-specific antigen: activation by hofmeister salts and inhibition by azapeptides. *Biochemistry* 40, 11734–11741.
- Iwamura, M., Sluss, P.M., Casamento, J.B., and Cockett, A.T. (1993). Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. *Prostate* 22, 243–252.
- Iwamura, M., Hellman, J., Cockett, A.T., Lilja, H., and Gershagen, S. (1996). Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology* 48, 317–325.
- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M.J. (2007). Cancer statistics. *CA Cancer J. Clin.* 57, 43–66.
- Kanety, H., Madjar, Y., Dagan, Y., Levi, J., Papa, M.Z., Pariente, C., Goldwasser, B., and Karasik, A. (1993). Serum insulin-like growth factor-binding protein-2 (IGFBP-2) is increased and IGFBP-3 is decreased in patients with prostate cancer: correlation with serum prostate-specific antigen. *J. Clin. Endocrinol. Metab.* 77, 229–233.
- Knight, W.B., Green, B.G., Chabin, R.M., Gale, P., Maycock, A.L., Weston, H., Kuo, D.W., Westler, W.M., Dorn, C.P., and Finke, P.E. (1992). Specificity, stability, and potency of monocyclic β -lactam inhibitors of human leukocyte elastase. *Biochemistry* 35, 8160–8170.
- Koistinen, H., Paju, A., Koistinen, R., Finne, P., Lövgren, J., Wu, P., Seppälä, M., and Stenman, U.H. (2002). Prostate-specific antigen and other prostate-derived proteases cleave IGFBP-3, but prostate cancer is not associated with proteolytically cleaved circulating IGFBP-3. *Prostate* 50, 112–118.
- Koistinen, H., Närvänen, A., Pakkala, M., Hekim, C., Mattsson, J.M., Laakkonen, P., and Stenman, U.H. (2008a). Development of peptides specifically modulating the activity of KLK2 and KLK3. *Biol. Chem.* 389, 633–642.
- Koistinen, H., Wohlfart, G., Mattsson, J.M., Wu, P., Lahdenpera, J., and Stenman, U.H. (2008b). Novel small molecule inhibitors for prostate-specific antigen. *Prostate* 68, 1143–1151.
- Kurkela, R., Herrala, A., Henu, P., Nal, H., and Vihko, P. (1995). Expression of active, secreted human prostate-specific antigen by recombinant baculovirus-infected insect cells on a pilot scale. *Biotechnology* 13, 1230–1234.
- Laufer, M., Denmeade, S.R., Sinibaldi, V.J., Carducci, M.A., and Eisenberger, M.A. (2000). Complete androgen blockade for prostate cancer: what went wrong? *J. Urol.* 164, 3–9.
- Lawrence, D.A. (2001). Latent-TGF- β : an overview. *Mol. Cell. Biochem.* 219, 163–170.
- LeBeau, A.M., Singh, P., Isaacs, J.T., and Denmeade, S.R. (2008). Potent and selective peptidyl boronic acid inhibitors of the serine protease prostate-specific antigen. *Chem. Biol.* 15, 665–674.
- LeBeau, A.M., Singh, P., Isaacs, J.T., and Denmeade, S.R. (2009a). Prostate-specific antigen is a 'chymotrypsin-like' serine protease with unique P1 substrate specificity. *Biochemistry* 15, 3490–3496.
- LeBeau, A.M., Banerjee, S.R., Pomper, M.G., Mease, R.C., and Denmeade, S.R. (2009b). Optimization of peptide-based inhibitors of prostate-specific antigen (PSA) as targeted imaging agents for prostate cancer. *Bioorg. Med. Chem.* 17, 4888–4893.
- Lilja, H. (1985). A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J. Clin. Invest.* 76, 1899–1903.
- Lilja, H., Abrahamsson, P.-A., and Lundwall, A. (1989). Semenogelin, the predominant protein in human semen. *J. Biol. Chem.* 264, 1894–1900.
- Lilja, H., Piironen, T.P., Rittenhouse, H.G., Mikolajczyk, S.D., and Slawin, K.M. (2000). Prostate-specific antigen. In: *Comprehensive Textbook of Genitourinary Oncology*, N.J. Vogelzang, W.U. Shipley, P.T. Scardino and D.S. Coffey, eds. (Philadelphia, PA, USA: Lippincott Williams and Wilkins), pp. 638–650.
- Malm, J., Hellman, J., Hogg, P., and Lilja, H. (2000). Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by Zn^{2+} , a tight-binding inhibitor. *Prostate* 45, 132–139.
- Mattsson, J.M., Laakkonen, P., Stenman, U.H., and Koistinen, H. (2009). Antiangiogenic properties of prostate-specific antigen. *Scand. J. Clin. Lab. Invest.* 69, 447–451.
- Menez, R., Michel, S., Muller, B.H., Bossus, M., Ducancel, F., Jolivet-Reynaud, C., and Stura, E.A. (2008). Crystal structure of a ternary complex between human prostate-specific antigen, its substrate acyl intermediate and an activating antibody. *J. Mol. Biol.* 376, 1021–1033.
- Nadiminty, N., Lou, W., Lee, S.O., Mehraein-Ghomi, F., Kirk, J.S., Conroy, J.M., Zhang, H., and Gao, A.C. (2006). Prostate-specific antigen modulates genes involved in bone remodeling and induces osteoblast differentiation of human osteosarcoma cell line SaOS-2. *Clin. Cancer Res.* 12, 1420–1430.
- O'Keefe, R.J. and Guise, T.A. (2003). Molecular mechanisms of bone metastasis and therapeutic implications. *Clin. Orthop. Relat. Res.* 415, S100–S104.
- Romanov, V.I., Whyard, T., Adler, H.L., Waltzer, W.C., and Zucker, S. (2004). Prostate cancer cell adhesion to bone marrow endothelium: the role of prostate-specific antigen. *Cancer Res.* 64, 2083–2089.
- Roodman, G.D. (2004). Mechanisms of bone metastasis. *N. Engl. J. Med.* 350, 1655–1664.

- Roudier, M.P., True, L.D., Higano, C.S., Vessella, H., Ellis, W., Lange, P., and Vessella, R.L. (2003). Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. *Hum. Pathol.* *34*, 646–653.
- Sarker, D., Reid, A.H., Yap, T.A., and de Bono, J.S. (2009). Targeting the PI3K/AKT pathway for the treatment of prostate cancer. *Clin. Cancer Res.* *15*, 4799–4805.
- Singh, P., Williams, S.A., Shah, M.H., Lectka, T., Pritchard, G.J., Isaacs, J.T., and Denmeade, S.R. (2008). Mechanistic insights into the inhibition of prostate specific antigen by β -lactam class compounds. *Proteins* *77*, 984–993.
- Singh, P., LeBeau, A.M., Lilja, H., Denmeade, S.R., and Isaacs, J.T. (2009). Molecular insights into substrate specificity of prostate specific antigen through structural modeling. *Proteins* *4*, 984–993.
- Stattin, P., Stenman, U.H., Riboli, E., Hallmans, G., and Kaaks, R. (2001). Ratios of IGF-I, IGF binding protein-3, and prostate-specific antigen in prostate cancer detection. *J. Clin. Endocrinol. Metab.* *86*, 5745–5748.
- Stege, R., Grande, M., Carlström, K., Tribukait, B., and Pousette, Å. (2000). Prognostic significance of tissue prostate-specific antigen in endocrine-treated prostate carcinomas. *Clin. Cancer Res.* *6*, 160–165.
- Vis, A.N. and Schröder, F.H. (2009). Key targets of hormonal treatment of prostate cancer. Part 2: the androgen receptor and 5- α reductase. *BJU Int.* *104*, 1191–1197.
- Watt, K.W.K., Lee, P.J., M'Timkulu, T., Chan, W.P., and Loor, R. (1986). Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc. Natl. Acad. Sci. USA* *83*, 3166–3170.
- Webber, M.M., Waghray, A., and Bello, D. (1995). Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion. *Clin. Cancer Res.* *1*, 1089–1094.
- Williams, S.A., Singh, P., Isaacs, J.T., and Denmeade, S.R. (2007a). Does PSA play a role as a promoting agent during the initiation and/or progression of prostate cancer? *Prostate* *67*, 312–329.
- Williams, S.A., Merchant, R.F., Garrett-Mayer, E., Isaacs, J.T., Buckley, J.T., and Denmeade, S.R. (2007b). A prostate-specific antigen-activated channel forming toxin as therapy for prostatic disease. *J. Natl. Cancer Inst.* *99*, 376–385.
- Wong, B.K., DeFeo-Jones, D., Jones, R.E., Garsky, V.M., Feng, D.M., Oliff, A., Chiba, M., Ellis, J.D., and Lin, J.H. (2001). PSA-specific and non-PSA-specific conversion of a PSA-targeted peptide conjugate of doxorubicin to its active metabolites. *Drug Metab. Dispos.* *29*, 313–318.
- Wu, P., Leinonen, J., Koivunen, E., Lankinen, H., and Stenman, U.H. (2000). Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. *Eur. J. Biochem.* *267*, 6212–6220.
- Yang, C.F., Porter, E.S., Boths, J., Kanyi, D., Hsieh, M., and Cooperman, B.S. (1999). Design of synthetic hexapeptide substrates for prostate-specific antigen using single-position minilibraries. *J. Pept. Res.* *54*, 444–448.
- Yousef, G.M. and Diamandis, E.P. (2003). An overview of the kallikrein gene families in humans and other species: emerging candidate tumour markers. *Clin. Biochem.* *36*, 443–452.

Received November 18, 2009; accepted January 18, 2010