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Mechanistic insights into the inhibition of prostate specific antigen by β-lactam class compounds

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ABSTRACT

Prostate Specific Antigen (PSA) is a biomarker used in the diagnosis of prostate cancer and to monitor therapeutic response. However, its precise role in prostate carcinogenesis and metastasis remains largely unknown. A number of studies arguing in the favor of an active role of PSA in prostate cancer development and progression have implicated this serine protease in the release and activation of growth factors such as insulin-like growth factor 1 (IGF1) through cleavage of insulin like growth factor binding protein 3 and Transforming Growth Factor β (TGF-β) through cleavage of Latent TGF-β. In contrast, other studies suggest that PSA activity might hinder tumor development and progression. In light of these contradictory findings, efficient inhibitors of PSA are needed for exploring its biological role in tumor development and metastasis. Towards the goal of developing potent inhibitors of PSA, we have explored the molecular mechanism of a series of β-lactam based compounds on binding to PSA using activity assays, matrix assisted laser desorption ionization with a time-of-flight mass spectrometry, and GOLD docking methodology. The mass spectrometry experiments and the activity assays confirmed the time-dependent and covalent nature of β-lactam binding. To gain insights on the reaction intermediates at the molecular level, we docked β-lactam inhibitors to a homology modeled PSA using the GOLD docking program in noncovalent and covalent binding modes. The docking studies elucidated the molecular details of the early noncovalent Michaelis complex, the acyl-enzyme covalent complex, and the nature of conformational reorganization required for the long term stability of the covalent complex. Additionally, the molecular basis for the effect of stereochemistry of the lactam ring on the inhibitory potency was elucidated through docking of β-lactam enantiomers. As a validation of our docking methodology, two novel enantiomers were synthesized and evaluated for their inhibitory potency using fluorogenic substrate based activity assays. Additionally, cis enantiomers of eight β-lactam compounds reported in a previous study were docked and their GOLD scores and binding modes were analyzed in order to assess the general applicability of our docking results. The close agreement of our docking results with the experimental data validates the mechanistic insights revealed through the docking studies and paves the way for the design and development of potent and specific inhibitors of PSA.

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Key words: docking; homology model; covalent inhibitors; serine proteases; GOLD; MALDI-TOF.

INTRODUCTION

Prostate specific antigen (PSA) is a serine protease with chymotrypsin-like activity that is a member of the kallikrein family of proteases.[1] PSA is produced and secreted by the prostate gland and its known physiologic function is to cleave the seminal proteins semenogelin I and II which causes the liquefaction of coagulated seminal fluid, thus potentially aiding the fertilization process.[2,3] Since its discovery in seminal fluid in 1971,[4]

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serological screening of PSA has become an important tool for prostate cancer diagnosis and prognosis.

In both local prostatic adenocarcinoma and metastatic disease, PSA levels are elevated in the blood. Elevated expression of PSA in both hormone dependent and hormone-refractory prostate cancer has led to suggestions that it may play an important functional role in disease initiation and progression.[5,6] Several lines of evidence support this claim. First, PSA has been shown to promote the release of insulin-like-growth-factor 1 (IGF1) through the cleavage of its binding partner IGFBP3 (IGF1 binding protein 3).[7] IGF1 is a mitogen for prostate stroma and epithelial cells and its increased level can promote tumor growth. Also, PSA has been implicated in the activation of latent transforming growth factor-β, facilitating tumor-cell proliferation.[8] Finally, PSA, in its active form, can cause epithelial cell damage and promote the degradation of basal membrane. Contrasting these findings are several studies indicating that PSA may play a negative role in carcinogenesis and tumor progression through inhibition of endothelial-cell proliferation and invasion. Also, PSA may inhibit the angiogenic process through the digestion of plasminogen, resulting in the production of anti-angiogenic peptides.[9] In light of these contradictory findings, a full understanding of the functional role of PSA in prostate cancer is required. This will require not only the development of better prostate cancer models but also the rational design and development of highly efficacious and specific inhibitors of PSA. A potent small molecule inhibitor of PSA will not only be useful as a research tool in probing the biological role of PSA but will also provide therapeutic benefit if PSA is implicated in prostate cancer initiation and/or progression.

Unfortunately, the development of efficient inhibitors of PSA is hindered by a lack of understanding on the molecular basis for inhibition and insufficient knowledge of the structure–activity relationships of PSA inhibitors. In addition, crystal structure information on PSA is currently not available. Recently, Adlington *et al.* designed[10] and synthesized β-lactam analogs which showed promising inhibitory activity against PSA. Additionally, compounds from the family of 3-benzyl-azetidine-2-one[11] and 1,3-diazetidine-1,4-diones[12] have been shown to possess inhibitory activity against the chymotrypsin-like serine proteases chymase. Though active against PSA, these β-lactam compounds are not highly efficacious and may possess off-target inhibitory activity. Nonspecific effects can severely limit the development of these compounds as therapeutic drugs as serine proteases are involved in a broad spectrum of cellular functions and off-target activity could lead to undesirable side-effects.

The general mechanism of serine protease inhibition by the β-lactams has been studied in the past using elastase as a model.[13,14] To obtain mechanistic insights into the inhibition of PSA by β-lactams, this elastase model can be used as a framework for elucidating the key intermediates along the inhibitory reaction path. Figure 1 depicts the key features of β-lactam binding at the catalytic site of PSA using a representative compound (**1**) from the β-lactam family. At first, the β-lactam molecule initiates noncovalent interactions in the vicinity of the catalytic triad residues, SER189 and HIS41 [Fig. 1(A)]. In this noncovalent Michaelis complex, the C-3 sidechain of compound **1** is bound at the S-1 specificity pocket while the C-2 carbonyl oxygen orients towards the oxyanion hole of the protease. The hydroxyl oxygen of the SER189 residue is in a favorable position to mount a nucleophilic attack on the lactam ring. Subsequently, via a transition state, this complex leads to an intermediate state [Fig. 1(B)], whereupon the β-lactam ring opens up and a covalent bond is formed between the SER189 and

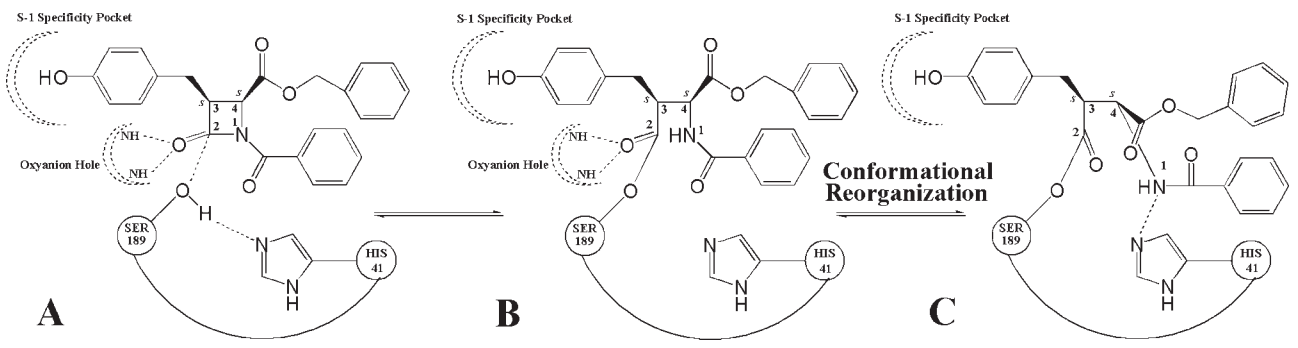

Figure 1

*Schematic delineating the key conformational states during the binding of β-lactam compound **1** (3S, 4S enantiomer) at the catalytic pocket of PSA. Transition states along the reaction path are not shown here. Only the catalytic triad residues, HIS41 and SER189, are depicted. The straight dotted lines represent the presence of hydrogen bonds. (**A**) Initial noncovalent Michaelis complex. (**B**) Intermediate covalent complex. (**C**) Conformational reorganization resulting in a stable acyl-enzyme complex.*

the C-2 carbon of the lactam ring. This intermediate covalent complex is different from the one formed during peptide substrate hydrolysis by PSA. In the case of peptide substrates, the fragment on the C-terminal side of the cleaved bond containing the free amine dissociates from the binding side. Subsequently, a reactive water molecule enters into the binding site and attacks the nascent acyl-enzyme bond resulting in the dissociation of the N-terminal side of the cleaved bond, returning the protease to its ground state. In the case of the β-lactam inhibitors, appropriate conformational reorganization upon ring opening leads to a stable acyl-enzyme, which confers long term inhibition of the enzyme [Fig. 1(C)].

To develop efficient inhibitors of PSA, it is important to study the inhibition mechanism of β-lactams and the molecular details of the intermediate conformational states as outlined in Figure 1. Towards this end, we performed a series of activity assays in conjunction with MALDI-TOF (matrix assisted laser desorption ionization with a time-of-flight) Mass Spectrometry experiments to confirm the covalent nature of β-lactam binding to the PSA catalytic site. Subsequently, we conducted GOLD docking of a representative β-lactam compound in both noncovalent and covalent mode to illustrate the key energetic interactions taking place during the noncovalent Michaelis complex and acyl-enzyme formation, respectively. Additionally, we docked cis enantiomers of two β-lactam compounds to gain insights on the effect of the stereochemistry on inhibitory potency. As a validation of the docking results, two novel enantiomers were synthesized and tested for their inhibition potency using a fluorogenic substrate based assay. Finally, cis enantiomers of eight β-lactam compounds with known IC_{50} values[10] were docked in both noncovalent and covalent binding modes. The GOLD scores and binding modes of these compounds were analyzed to validate the general applicability of our docking findings.

MATERIALS AND METHODS

Activity assays

The protocol for evaluating the inhibitory activity of the lactam compounds was based on a slight modification of the protocol published previously[15] by our group. In each well of a half volume 96-well plate, the candidate inhibitor was added to a solution that included PSA at a concentration of 5 μg/mL (Calbiochem) and a HSSKLQ-7-amino-4-trifluoromethyl-coumarin (AFC) fluorogenic substrate at a concentration of 40 μ*M* (Calbiochem) in PSA activity buffer (50 m*M* Tris, 100 m*M* NaCl, pH to 7.8, 10% DMSO) for a total volume of 100 μL. For the kinetic assay, readings (excitation 400 nm, emission 505 nm) were taken every 3 min over 2 h. As a positive control for PSA inhibition, a PSA-specific inhibitory mouse monoclonal antibody, mAb 5A10 (generously donated by Dr. Hans Lilja from the Memorial Sloan Kettering Institute), was used at a concentration of 10 μg/mL.

To assess the time-dependence of β-lactam inhibition, PSA at a concentration of 5 μg/mL was added to the activity buffer containing fluorogenic substrate at 40 μ*M* and readings were taken every 3 min over 1 h. At the end of 1 h, the inhibitor was added at a final concentration of 100 μ*M* and readings were resumed immediately and continued every 3 min for 2 more hours.

Inhibitor wash-out study

PSA at a concentration of 5 μg/mL was incubated overnight with 100 μ*M* of compound **1** in activity buffer. Upon overnight incubation, the reaction mixture was loaded onto a 10 kDa cut-off Amicon filter and centrifuged at 5000 rpm for 30 min to completely remove the reaction buffer. A sufficient amount of fresh buffer without the inhibitor was added to the concentrated PSA to bring the PSA concentration to the original value of 5 μg/mL. The inhibited PSA with the fresh buffer in the filter was centrifuged once again to remove the buffer and fresh buffer was added again. Subsequently, the enzymatic activity of PSA was measured using the activity assays described in the previous section. As a positive control, the activity assays were performed with the active PSA with the same concentration.

Mass spectrometry analysis

MALDI-TOF mass spectrometry experiments were performed using a Voyager DE-STR (Applied Biosystems) with the help of the AB Mass Spectrometry/Proteomics core facility at Johns Hopkins School of Medicine. The MALDI spectra were acquired within the 5–40 kDa mass range. The matrix used was sinapinic acid (10 mg/mL) in 80% acetonitrile, 20% H_2O and 0.1% trifluoroacetic acid. Compound **1** was added to PSA (0.5 μg/μL) containing buffer (50 m*M* Tris, 100 m*M* NaCl, pH 7.8) with the final concentration of 100 μ*M* and incubated for 1 h. Samples containing PSA with or without the compound **1** were cleaned using a Zip tip C18. The spectra collected on the clean samples were analyzed using Data Explorer v4.0 package.

Homology modeling

The homology model was built using the MOE program suite supplied by CCG, Montreal. The sequence of PSA (P07288) was obtained from the SWISS-PROT protein knowledgebase. The porcine kallikrein (PDB code: 2PKA) bound with the inhibitor benzimidine was used as a main template to build the homology model. The peptide sequence of porcine kallikrein has a 59% homology with human PSA sequence, which is suitable for building a reliable structural model. Compared with

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other available *Apo* or inactive conformations of homologous kallikreins, this inhibitor bound conformation is ideal for building a model which can be used for inhibitor design. Since crystal structure of porcine kallikrein lacks some residues in the kallikrein loop region of PSA, we used Human Tissue kallikrein HK1 (PDB code: 1SPJ) as second template to model the conformation of the kallikrein loop. This crystal structure has its kallikrein loop in a semiopen configuration which leaves the catalytic pocket accessible to both substrates and inhibitors. The resulting loop structure in our homology model is physically more accurate compared to previous homology models,[16,17] which were developed using a single template and a less reliable de novo loop design to model the kallikrein loop conformation.

The quality of the model was evaluated by constructing the Ramachandran plot of the protein residues. All residues except one arginine located in kallikrein loop had their φ and ψ torsion angles in the allowed region. The bond lengths and angles of all the residues were confirmed to be same as their known values and there were no outliers. As typical of serine proteases, the tertiary structure of PSA consists of two domains of six-stranded anti-parallel β-barrels. The interface region of these two domains formed a crevice containing the catalytic site and the kallikrein loop, where a patch of three Arginine residues were located forming a highly positively charged region.

Noncovalent and covalent docking

The GOLD v3.0 docking program was used for docking inhibitors to the catalytic site of PSA in both covalent and noncovalent modes. The GOLD program uses a genetic docking algorithm for docking flexible ligands into protein binding sites. This program has been shown to produce accurate results for many protein–ligand systems and is specially suited for our purpose since it has the capability of docking inhibitors in the covalent binding modes. During noncovalent docking, compounds were free to adopt any conformation with the exception of one distance constraint which guided the carbonyl oxygen of the lactam ring to orient towards the oxyanion hole. This constraint allows the GOLD program to search for conformational space compatible with the previous experimental observations. The covalent docking was performed by specifying a covalent bond constraint between the electrophilic carbon of the lactam ring and the hydroxyl sidechain of the S189 residue. The binding pocket was defined as a 25 Å radius sphere from the catalytic SER189 residue. For each compound, 20 independent runs were performed resulting in 20 different docking poses. For every independent run, 100,000 operations were performed (N_{op} = 100,000). These docking poses were ranked based on their GOLD free energy score. To remove any personal bias, the top scoring conformation for each compound was used for subsequent analysis.

While GOLD methodology has been extensively applied and validated for the noncovalent protein–ligand complexes,[18,19] its application to the covalent interactions has not been fully explored. As a validation of covalent docking through GOLD, we tested the methodology on two covalent complexes, that is, trypsin bound to a β-lactam compound (PDB: 1RXP) and porcine elastase in complex with a tosyl β-lactam compound (PDB: 1BTU). Both of these ligands were extracted from the catalytic site and redocked in a covalent binding mode. The crystallographic water molecules that hydrogen bond with the ligands were kept in the binding pocket during the docking process. The root mean square difference between the top docking pose and original crystallographic geometry was 1.42 and 0.66 Å for trypsin and elastase complexes, respectively. These values were an improvement on the 2 Å cutoff used in previous studies to define a successful validation.[18] This successful validation provides sufficient confidence in drawing meaningful conclusions from the covalent docking studies.

Chemical synthesis

The chemical synthesis of the two β-lactam compounds discussed in this report was performed using a previously published methodology based on bifunctional catalyst systems.[20,21] This methodology results in the production of β-lactams in high yield with efficient stereoselectivity.

RESULTS AND DISCUSSION

β lactams inhibit PSA in a time-dependent fashion

A number of studies in the past have shown that the β-lactam class of compounds possess inhibitory activity against a multitude of serine protease targets such as human elastases, chymases, β-lactamsases,[22] thrombin,[23] and cytomegalovirus proteases.[24] Additionally, a recent study[10] described the design and synthesis of novel β-lactam compounds with inhibitory activity against PSA. In this study, a nonspecific chymotrypsin substrate (Meo-Suc-Arg-Pro-Tyr-p-NA.HCl) was used to evaluate inhibition of PSA by these ß-lactam compounds. To confirm that the inhibitory potency of these compounds is not dependent on the nature of the particular substrate used, we performed the activity assays using a more specific and efficient PSA substrate (HSSKLQ-AFC) previously developed in our lab.[15] As a representative of the β-lactam class of inhibitors, compound **1** (Fig. 2, inset) was used in these activity assays with concentration ranging from 1 μ*M*–1 m*M*. The resulting kinetic response curve is presented in Figure 2. On the basis of the initial slopes of the enzymatic reaction, the IC_{50} value (concentration of the compound which results in the 50% inhi-

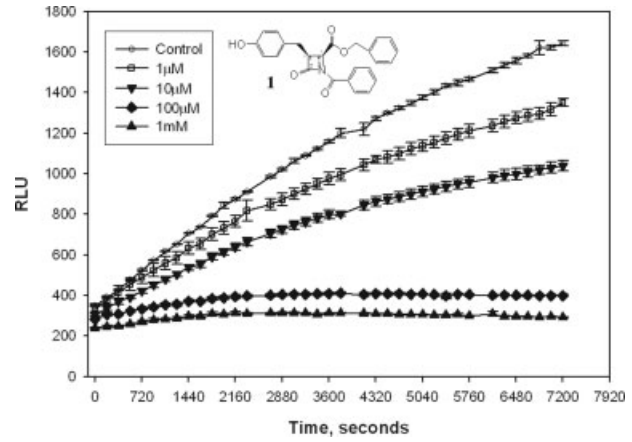

Figure 2

*The kinetic response curve with inhibitor concentrations ranging from 10 nM to 1 mM. On the basis of the average reaction rates within the first hour, the IC_{50} value of compound **1** (chemical structure shown inset) was estimated to be ~10 μM.*

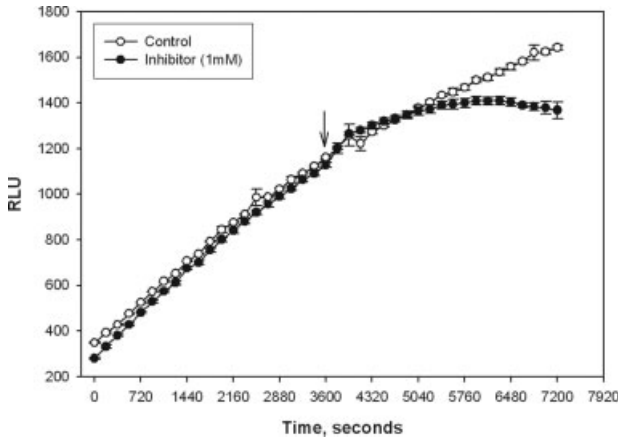

Figure 3

*Measurement of time-dependence of β-lactam inhibition of PSA. The arrow represents the time point (3600 s) at which inhibitor **1** was added at 1 mM concentration to the reaction mixture.*

bition of PSA activity) was evaluated to be ~10 μ*M*. This level of inhibition is similar to the value (9.14 μ*M*) reported previously by Adlington *et al.* and establishes the β-lactam class of compounds as modest inhibitors of PSA, irrespective of the type of substrate used to assess their inhibitory potency.

It has been suggested that β-lactams inhibit PSA in a time dependent fashion with 1:1 stoichiometry,[10] though no specific data has been reported so far. For the β-lactams to be good inhibitors, the rate of their inhibition reaction with PSA has to be competitive with respect to the turnover of natural substrate. To probe the time dependent nature of β-lactam inhibition further, we conducted time course assays in which the enzymatic reaction was allowed to run for 1 h, after which compound **1** was added at a 1 m*M* final concentration and the rate of reaction was recorded for another hour (Fig. 3). The inhibitory effect was not observed instantaneously and the reaction rate was similar to the control until after 5040 s (24 min after the addition of inhibitor) after which a complete inhibition of enzymatic activity was observed. On the basis of these results, it can be concluded that β-lactam compounds react with PSA in a time-dependent fashion since they have to compete with the substrate until the majority of PSA is converted into an irreversible inactive state, leading to the permanent inhibition of PSA.

β-lactams bind covalently with PSA

The β-lactam compounds have previously been shown to inhibit serine proteases such as elastases[14,25] through the formation of a covalent acyl-enzyme complex at the catalytic SER-189 residue. As part of our effort to characterize β-lactam binding to PSA, we conducted an inhibitor wash-out study based on the rationale that if the β-lactam compound binds to PSA in a noncovalent fashion, the wash-out steps will lead to a complete removal of inhibitor from the PSA binding site, resulting in the reactivation of PSA. On the other hand, if compound **1** binds PSA covalently then the protease will remain inhibited even upon overnight incubation and wash-out steps. The results of this study are presented in Figure 4. This study results confirm that PSA remains inhibited even after several wash-out steps. This strongly suggests the

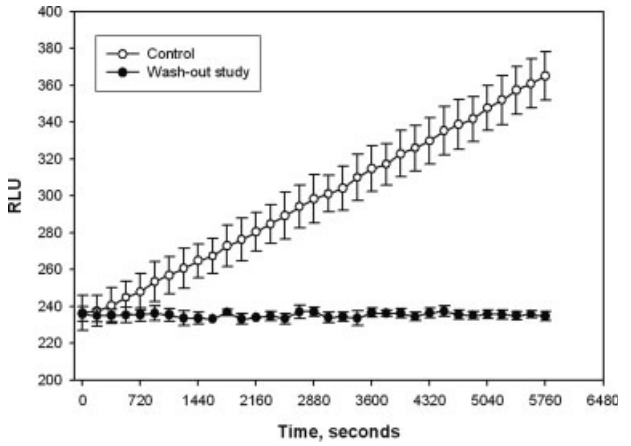

Figure 4

*The inhibitor wash-out study establishing the covalent nature of PSA inhibition by compound **1**. PSA at 5 μg/μL concentration was incubated overnight with (solid circle) and without (open circle) 100 μM of compound **1**.*

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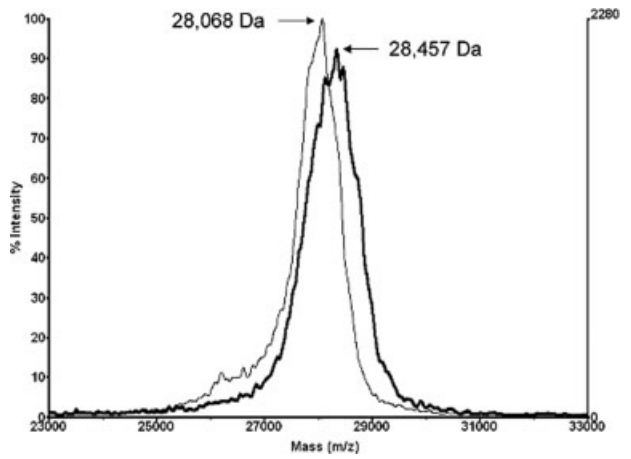

Figure 5

Observation of covalent complex between PSA and β-lactam compound 17. MALDI analysis of PSA alone (peak at 28,068 Da, shown with thin line) and the PSA incubated with compound 17 (peak at 28,457 Da, shown with thick line) shows a mass shift corresponding to the molecular weight of the compound.

covalent mode of binding for the β-lactam binding to PSA.

For further confirmation, we performed MALDI-TOF mass spectrometry experiments on active PSA incubated with the representative β-lactam compound **1** (MW 415.14). The mass spectrometry PSA alone vs. PSA incubated with compound **1** demonstrates a clear mass shift upon the binding of compound **1** with PSA (Fig. 5). This strongly indicates the formation of a covalent complex between the β-lactam compound and PSA. This mass shift will not be observed if the compound was interacting noncovalently with PSA as noncovalent complexes are unstable and are likely to dissociate during the MALDI-TOF ionization process. The magnitude of the mass shift upon the β-lactam binding was ~389 Da, which is only an approximate estimate due to the presence of multiple species because of the fact that PSA sample used in this study exists in multiple processed forms.

Molecular details of β-lactam binding through GOLD docking

While the experimental studies described in the previous sections explored the time dependent and covalent nature of β-lactam binding, the exact molecular details of key conformational states along the reaction path of β-lactam binding to PSA (Fig. 1) cannot be obtained by such studies. To elucidate β-lactam binding at the molecular level, we performed molecular docking of a β-lactam family of compounds using the GOLD docking program. In the absence of crystal structure information, we developed a homology model of PSA based on the hybrid templates of porcine and human tissue kallikreins. The docking was performed in both noncovalent and covalent modes in order to illustrate the initial noncovalent interactions and the molecular details of the final acyl-enzyme product, respectively.

Molecular details of early noncovalent Michaelis complex

β-lactam binding to PSA is initiated by a series of noncovalent interactions at the catalytic site of PSA. To gain insights on the nature of these interactions, compound **1** (3S, 4S enantiomer) was docked to the catalytic binding site of the PSA in a noncovalent mode and the top scoring pose was chosen for subsequent analysis. Figure 6 depicts the overall orientation of the inhibitor and the key protease residues interacting with the inhibitor sidechains. The C-3 sidechain of the inhibitor is docked in the S-1 specificity pocket where the *para*-hydroxyl group is forming a hydrogen bond with the SER183 hydroxyl and backbone of the THR184 residue. The C-2 carbonyl of the lactam ring is stabilized by the oxyanion hole via hydrogen bonds with the backbone amine-hydrogen of GLY187. The C-2 carbon is oriented such that the catalytic SER189 sidechain oxygen is in perfect position to attack the electrophilic C-2 carbon. This orientation is supported by favorable interactions of the N-1 sidechain with VAL25 in the valley region and the C-4 sidechain with the hydrophobic residues TRP205, LEU81, and TYR77 in the upper groove region.

The predicted binding mode of the C-3 sidechain in the S-1 pocket is consistent with a previous report on the crystal structure of a β-lactam compound bound at the catalytic site of trypsin.[26] Our docking predictions are also in close agreement with previously suggested binding modes of lactam compounds.[10] This close agreement validates the molecular insights revealed by our docking methodology. The binding of the C-3 sidechain in the S-1 pocket emphasizes the role of this sidechain in determining the specificity of β-lactams since the residues lining the S-1 pocket are the main determinant of PSA substrate specificity. Thus, the docking results suggest that the specificity of a lactam inhibitor toward PSA can be strengthened by optimizing its C-3 side chain to maximize the hydrophobic and the polar interactions in the S-1 pocket.

Molecular details of covalent acyl-enzyme complex

Upon formation of the initial Michaelis complex, the catalytic serine residue of PSA attacks the C-2 carbon of the lactam ring resulting in the opening of the lactam ring. Further conformational reorganization of the lactam side chains produces a stable acyl-enzyme leading to the long term inhibition of PSA. To predict the molecular details of this stable covalent complex, the (3S, 4S) enantiomer of compound **1** was docked in a covalent mode.

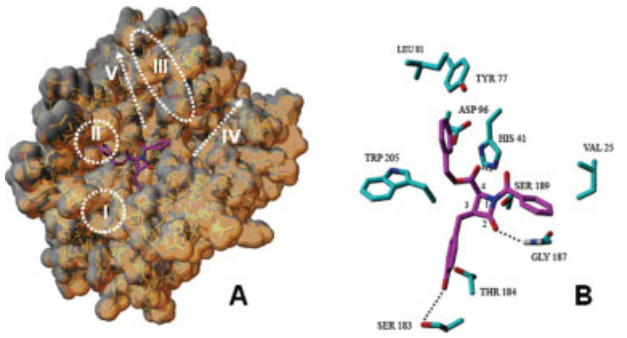

Figure 6

*Initial noncovalent interactions of compound **1** in the catalytic pocket of PSA. (**A**) Surface representation of PSA with the (3S, 4S) enantiomer of compound **1** (shown in magenta). The major structural features of PSA molecule include lower groove (I), upper groove (II), arginine patch (III), valley (IV), and cleft (V). The N-1 sidechain of compound **1** is oriented towards the valley region while C-4 sidechain is docked in the upper groove. The C-3 sidechain is docked in the S-1 specificity pocket. (**B**) The key PSA residues (shown in cyan) interacting directly with the side chains of compound **1**. The dotted black lines denote hydrogen bonds.*

Figure 7(A) presents the binding mode of compound **1** with its C-2 carbon covalently attached to the hydroxyl sidechain of the SER189 residue. The C-3 sidechain remains bound in the S-1 pocket making a hydrogen bond with the SER183 residue. The N-1 nitrogen forms a hydrogen bond with residue HIS41 while its sidechain is bound in the upper groove region, through hydrophobic interactions with LEU81, TYR77, and TRP205. The C-4 sidechain is oriented towards the cleft region on the left side of VAL25 while its carbonyl is making hydrogen bonds with residues (i.e., GLY187) in the oxyanion hole. The C-2 carbonyl is pointing away from the oxyanion hole and oriented towards the HIS41 residue.

The orientation of the N-1 nitrogen towards the HIS41 residue is consistent with our understanding of the role of the catalytic triad residues during substrate hydrolysis.[27,28] It is generally accepted that a proton from the catalytic serine residue is transferred to the nitrogen group via the histidine residue in the catalytic triad. In the case of β-lactam binding, this is only possible if the N-1 nitrogen is oriented towards the HIS41 residue in order to accept the proton upon the opening of the lactam ring. The same rationale is also valid in the case of substrate cleavage but the reason why β-lactams are inhibitors rather than substrates lies in the stability of the final acyl-enzyme complex. In the case of substrate cleavage, the C-terminal part of the cleaved bond dissociates and the remaining acyl-enzyme complex is primed for hydrolytic water attack. In the case of β-lactams, the molecules remain tightly bound even upon lactam ring opening as the N-1 nitrogen is still covalently attached with the C-4 carbon of the lactam ring. The strain energy released by the opening of the lactam ring drives conformational reorganization of the lactam sidechains resulting in a stable covalent complex. The nature of this conformational reorganization can be easily inferred from a comparison between the noncovalent and covalent docking modes as shown in Figure 7(B). The C-2 sidechain remains docked in the S-1 pocket upon the lactam ring opening but there is a drastic difference in the location of the N-1 and the C-4 sidechains. The N-1 sidechain is bound in the valley region in the noncovalent mode while it docks in the upper groove in the covalent mode. In this reorientation, the nitrogen in the N-1 sidechain is making the crucial hydrogen bond with the HIS41 residue. The C-2 carbonyl oxygen moves away from the oxyanion hole and now points towards HIS41. In its place the C-4 sidechain carbonyl is now moved closer to the oxyanion hole and makes a hydrogen bond with the backbone amide nitrogen of GLY187. This particular positioning of the C-4 sidechain carbonyl is likely to stabilize the nascent acyl-enzyme complex. This is consistent with a previous experimental observation[10] that the presence of a reverse ester moiety at the C-4 sidechain of the nontosyl β-lactams eliminates their inhibitory potency. It is likely that the presence of a reverse ester moiety affects the ability of the C-4 carbonyl to make the hydrogen bond crucial for the stability of the acyl-enzyme complex.

Effect of stereochemistry on the inhibition potencies of β-lactams

The presence of two chiral centers on the C-3 and C-4 position of the lactam ring means that four different

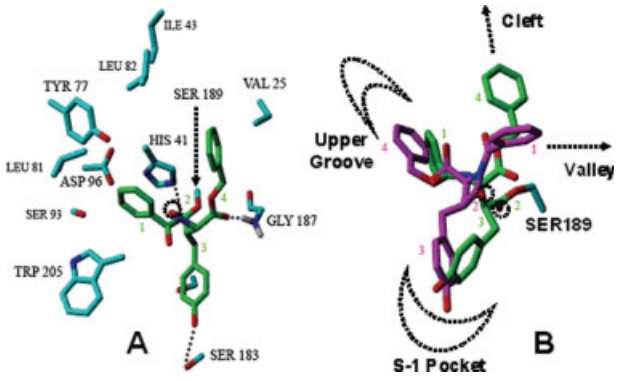

Figure 7

*Molecular details of the covalent complex and conformational reorganization upon lactam ring opening. (**A**) The (3S, 4S) enantiomer of compound **1** (green) covalently bound with the catalytic SER189 residue. The key protein residues interacting with the compound are depicted in cyan. (**B**) Initial noncovalent complex of compound **1** (shown in magenta) superimposed with the final acyl-enzyme complex (shown in green). Small dotted circles denote the position of carbonyl oxygen at the C-2 position of compound **1**. The straight dotted lines denote the presence of hydrogen bonds.*

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enantiomers are possible with the same chemical structure of a β-lactam compound. While a number of studies on serine proteases such as elastase and chymase indicate that the chirality of the β-lactam ring drastically affects the inhibitory potency of the compounds, the molecular basis for the stereoselectivity remains unknown. To understand the molecular rationale behind the stereoselectivity of PSA inhibition, we docked (3S, 4S) and (3R, 4R) enantiomers of compound **1** as well as compound **2** (Table I). The docking was performed in noncovalent as well as covalent mode in order to assess whether the chirality of the lactam ring affects the nature of molecular interactions during the initial Michaelis complex or the final covalent complex. Because of a lack of experimental data, we did not study the (3S, 4R) and the (3R, 4S) enantiomers as it would not be possible to validate the docking results on these particular enantiomers.

Higher potency of (3S, 4S) enantiomers of non-tosyl compounds

The study[10] by Adlington *et al.* documented that the (3S, 4S) enantiomer of the non-tosyl lactam compounds (such as compound **1**) inhibit PSA with greater potency than their (3R, 4R) counterparts. To understand the structural basis of this observation, we docked these two enantiomers of compound **1** in noncovalent and covalent docking modes. Table I presents the GOLD scores and binding modes of these enantiomers. The overall binding conformation and the GOLD scores were similar for the (3S, 4S) and (3R, 4R) enantiomers of compound **1** in noncovalent binding modes, that is, 51.39 and 52.29 respectively, but differed significantly in the covalent mode, that is, 40.88 and 21.23 respectively. This difference in the GOLD scores is due to the difference in the strengths of energetic interactions during formation of the final acyl-enzyme complex. While the initial noncovalent interactions are of the same strength for both of the enantiomers of compound **1**, production of the final acyl-enzyme complex is more favorable in the case of the (3S, 4S) enantiomer due to hydrogen bonding of the C-4 sidechain with the oxyanion hole.

Higher potency of (3R, 4R) enantiomers of tosyl compounds with a reverse ester group

In contrast to the non-tosyl β-lactam compounds, for tosyl compounds with a reverse ester group at the C-4 position (i.e., compound **2**, Table I), the (3R, 4R) enantiomers are reported to be better than the (3S, 4S) in terms of their inhibitory potencies. To determine if docking can provide the molecular rationale behind this observation, we docked both enantiomers of compound **2** (Table I) in the noncovalent and covalent docking mode. As presented in Table I, GOLD scores for the enantiomers of compound **2** were similar in both covalent and noncovalent modes of docking indicating the similar strengths of interactions. The reason why the (3R, 4R) enantiomer of compound **2** is a better inhibitor of PSA than the (3S, 4S) enantiomer may lie in the crucial differences in binding conformation of the two enantiomers. The C-3 sidechain of the compound **2** (3S, 4S) is docked in the cleft region rather than in the S-1 pocket during the initial noncovalent complex formation. In its place, the N-1 sidechain docks at the S-1 pocket, leading to a conformation in which the N-1 nitrogen is pointing away from the HIS41 Nϵ2. In contrast, the C-3 sidechain of the (3R, 4R) enantiomer docks at the S-1 pocket both during the initial noncovalent and final covalent complex. In this orientation, the N-1 nitrogen is in close contacts with the HIS41 sidechain.

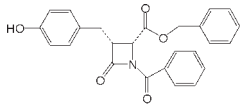
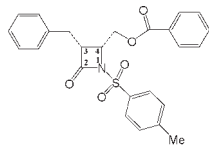
An earlier study reported that when reverse ester moiety at the C-4 position was replaced by the normal ester group, the (3R, 4R) enantiomers of the tosyl compounds were ineffective at inhibiting PSA.[10] To determine if the nature of the ester group has any effect on the ability of the N-1 nitrogen to adopt a favorable orientation towards HIS41, we docked the (3R, 4R) enantiomer of compound **2** in two forms, one containing a normal ester moiety at the C-4 position, the other having a reverse ester group at the same position. The binding modes of the two compounds are similar during the initial Michaelis complex [Fig. 8(A,B)] but differ drastically upon the formation of a covalent bond [Fig. 8(C,D)]. In the final acyl-enzyme complex, the normal ester of the **2**(3R, 4R) enantiomer is oriented in such a way that the tosyl moiety is unable to make a hydrogen bond with the oxyanion hole, thus keeping the nitrogen group at the N-1 position away from catalytic HIS41 Nϵ2. This indicates an unfavorable acyl-enzyme complex in the case of **2** (3R, 4R) enantiomer containing a normal ester group. The presence of reverse ester allows the sulphonyl group of the tosyl moiety to orient itself to make a stabilizing hydrogen bond with the oxyanion hole permitting the nitrogen group to interact with the HIS 41 residue [Fig. 8(D)].

This docking rationale is consistent with the known binding modes of tosyl compounds as revealed by the crystal structure of an acyl-enzyme of the porcine elastase in complex with a β-lactam compound.[25] The tosyl group in this structure was oriented towards the oxyanion hole whereas the N-1 nitrogen was perfectly positioned to make a hydrogen bond with the catalytic histidine residue.

Docking of cis enantiomers of eight additional β-lactam compounds

To confirm that the molecular insights revealed by our docking methodology are not just valid for compounds **1** and **2** but rather applicable to whole β-lactam family, we performed another docking study in which (3S, 4S) and (3R, 4R) enantiomers of eight additional compounds

Table I
The GOLD Scores and the Binding Modes of the (3S, 4S) and the (3R, 4R) Enantiomers of Compound 1 and Compound 2

		Gold score		Sidechain orientation (noncovalent/covalent)							
				N-1 		C-2 		C-3 		C-4 	
Compound structure	Stereochemical isomer	Noncovalent	Covalent								
 **1**	(3S, 4S) 	51.39	40.88	Valley	Upper groove	Oxyanion home	Towards His-41	S-1 pocket	S-1 pocket	Upper groove	Cleft
	(3R, 4R) 	52.29	21.23	Valley	Cleft	Oxyanion home	Away from His-41	S-1 pocket	S-1 pocket	Upper groove	Cleft
 **2**	(3S, 4S) 	66.42	51.75	S-1 pocket	Upper groove	Oxyanion hole	Towards His-41	Cleft	S-1 pocket	Upper groove	Cleft
	(3R, 4R) 	67.02	44.85	Valley	Cleft	Oxyanion hole	Towards His-41	S-1 pocket	S-1 pocket	Upper groove	Upper groove

Compound **2** contains a tosyl moiety at the N-1 position and a reverse ester group at the C-4 position. The docking was performed in noncovalent as well as covalent binding mode.

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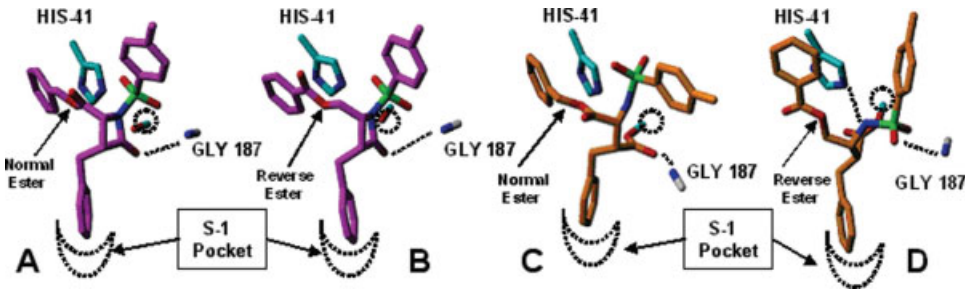


Figure 8

*Influence of the nature of the ester moiety at the C-4 position on the binding modes of the (3R, 4R) enantiomer of compound **2**. Compound **2** contains a tosyl moiety at the N-1 position. The dotted circle denotes the position of catalytic SER189 residue. (**A**) Initial noncovalent complex when normal ester group is present. (**B**) Initial noncovalent complex when reverse ester group is present. (**C**) The covalent acyl-enzyme complex when normal ester is present. (**D**) The covalent acyl-enzyme product when reverse ester group is present.*

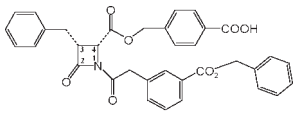
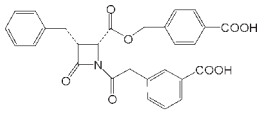
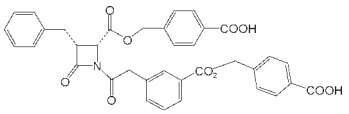
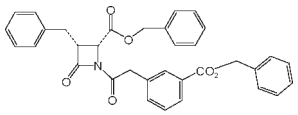
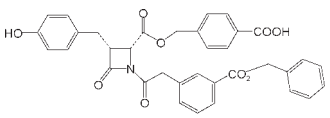
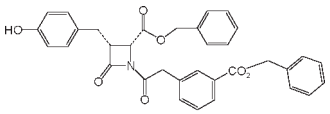
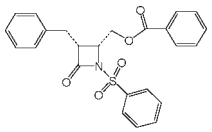
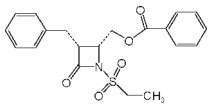
were docked in both covalent and noncovalent modes. These compounds have been tested for their PSA inhibitory potency in a previous study,[10] which reported the IC_{50} values of the racemic mixture of these compounds. Table II presents the GOLD scores and the binding orientation of these compounds. In the absence of IC_{50} data on the individual enantiomers, these compounds cannot be compared with each other to establish any correlation between GOLD scores and reported inhibitory potencies. Rather, we have made the comparison between different enantiomer of the same compound. As seen previously in the case of compound **1**, the (3S, 4S) enantiomer of nontosyl compounds, **3**–**8**, have much higher GOLD scores than their (3R, 4R) counterparts in the covalent binding modes. This correlates with the experimental observation that (3S, 4S) enantiomers of non-tosyl compounds exhibit better inhibition potency than (3R, 4R) enantiomers. In the case of the tosyl compounds, **9** and **10**, the difference in the GOLD scores was less pronounced in the covalent binding mode. This is exactly what was observed previously in the case of tosyl compound **2**. For all the compounds, the C-3 sidechain was bound in the S-1 pocket in the covalent binding mode. The main difference in the binding was in the relative positioning of the N-1 and the C-4 sidechains. This is consistent with our docking observations on compounds **1** and **2**.

Apart from the GOLD scores, the overall binding modes of non-tosyl and tosyl containing compounds were also similar to the binding modes of compounds **1** and **2**, respectively (Table II). This suggests that the β-lactam compounds can be separated into two broad categories, the tosyl containing and non-tosyl compounds. Within a particular category, all the compounds behave similarly in terms of their binding modes and their stereoselectivity. On the basis of the consistency between the docking results of compounds **1** and **2**, and the larger set of β-lactam compounds, we conclude that our docking results are applicable to the whole β-lactam family.

Experimental validation of docking methodology

Docking studies reveal that the orientation of the N-1 nitrogen is strongly dependent on the chemical nature of the N-1 and the C-4 sidechains, and in particular, on the presence of groups such as tosyl and reverse ester moieties. Further, the ability of the N-1 nitrogen group to make a crucial hydrogen bond with the HIS41 Nϵ2 strongly correlates with the inhibitory potency of a particular β-lactam enantiomer. To experimentally validate this docking rationale, we designed two enantiomers of a novel β-lactam compound, that is, **11** (3S, 4S) and **11** (3R, 4R), with an aliphatic sidechain and a normal ester group at the C-4 position (Table III). We conducted docking of these two enantiomers and calculated the GOLD scores in the covalent docking mode. As presented in Table III, compound **11** (3S, 4S) possessed a significantly higher GOLD score than **11** (3R, 4R). More importantly, this difference in the GOLD score was consistent with the fact that the N-1 nitrogen of the **11** (3R, 4R) enantiomer was pointing away from the catalytic triad and was unable to make the crucial hydrogen bond with the HIS41 sidechain. On the basis of this, we predicted that the (3S, 4S) enantiomer would be a better inhibitor of PSA than the (3R, 4R) enantiomer. Subsequently, these novel compounds were synthesized and the activity assays were performed in order to evaluate the inhibitory potencies of these two compounds. In agreement with the docking prediction, the (3S, 4S) enantiomer of compound **11**, a (3S, 4S) with a tosyl moiety and a normal ester group, was now a 10-fold better inhibitor than the (3R, 4R) counterpart in terms of IC_{50} value.

Table II
Gold Scores and Binding Modes of the (3S, 4S) and the (3R, 4R) Enantiomers of 7 β-Lactam Compounds Previously Reported[10]

Compound structure	Stereochemical isomer	Gold score		Sidechain orientation (covalent)		
		Noncovalent	Covalent	C-3	N-1	C-4
 **3:** 8.98μM	(3S, 4S)	62.39	72.49	S-1	Upper groove	Upper groove
	(3R, 4R)	69.17	49.56	S-1	Lower groove	Upper groove
 **4:** > 30μM	(3S, 4S)	59.57	60.24	S-1	Upper groove	Cleft
	(3R, 4R)	61.61	50.71	S-1	Valley	Upper groove
 **5**: 5.84 μM	(3S, 4S)	61.37	72.20	S-1	Upper groove	Upper groove
	(3R, 4R)	67.90	49.03	S-1	Valley	Upper groove
 **6**: 1.43μM	(3S, 4S)	56.55	68.05	S-1	Upper groove	Upper groove
	(3R, 4R)	61.73	56.99	S-1	Valley	Upper groove
 **7**: 3.5μM	(3S, 4S)	57.0	72.20	S-1	Upper groove	Cleft
	(3R, 4R)	62.14	57.17	S-1	Valley	Cleft
 **8**: 0.34μM	(3S, 4S)	54.13	70.92	S-1	Valley	Cleft
	(3R, 4R)	56.13	47.24	S-1	Valley	Upper groove
 **9**: 3.08μM	(3S, 4S)	63.40	57.30	S-1	Upper groove	Cleft
	(3R, 4R)	65.58	44.85	S-1	Cleft	Upper groove
 **10**: 2.19 μM	(3S, 4S)	59.77	57.58	S-1	Upper groove	Upper groove
	(3R, 4R)	71.89	49.72	S-1	Cleft	Upper groove

The IC_{50} values correspond to the racemic mixture of both of the enantiomers.

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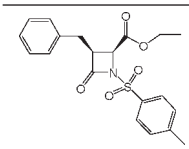
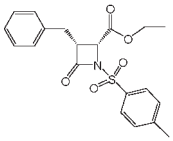
Table III
The IC_{50} Values and GOLD Scores of Two Novel β-Lactam Enantiomers

Compounds	Gold score (covalent)	IC_{50} (μM)
 **11** (3S, 4S)	47.54	11
 **11** (3R, 4R)	26.21	100

These compounds were synthesized using a high-yield chemical synthesis scheme described in the methods section.

CONCLUSION

Our study provides important mechanistic insights into β-lactam inhibition of the serine protease PSA. Through enzymatic assays and mass spectrometry experiments, we have confirmed earlier findings on the β-lactam inhibition of PSA via formation of a stable covalent acyl-enzyme complex. Also, we confirmed that the covalent binding of β-lactam compounds to the catalytic site of PSA is time-dependent and competitive with respect to substrate binding. Additionally, our docking studies elucidated the molecular details of key conformational states along the reaction path of β-lactam binding and explained how the stereoselectivity of the β-lactam compounds modulate their inhibitory potency.

To the best of our knowledge, docking studies on the serine proteases reported thus far have only studied β-lactam binding in the noncovalent mode and our study is the first to describe the formation of a stable acyl-enzyme complex through covalent docking methodology. Our docking results suggest that it is the docking in the covalent mode which provided the important insight into the mechanism and the structure–activity relationships of PSA. The molecular insights revealed by our docking studies were in agreement with the experimental data and correctly predicted the inhibitory potencies of two novel compounds synthesized as a validation of our docking methodology. Since the accuracy of the docking results can be compromised by limited sampling and approximations in energy function, we put a particular emphasis on the consistency between the docking predictions and the experimental information available in literature. In our ongoing studies, we are currently in the process of utilizing these docking predictions for designing more potent and specific β-lactam inhibitors of PSA for their use in probing the physiological role of this protease in prostate carcinogenesis, tumor invasion, and metastasis.

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