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Phosphoramidate derivatives of hydroxysteroids as inhibitors of prostate-specific membrane antigen

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Abstract—Prostate-specific membrane antigen (PSMA) is a membrane-bound cell surface peptidase which is over-expressed in prostate cancer cells. The enzymatic activities of PSMA are understood but the role of the enzyme in prostate cancer remains conjectural. We previously confirmed the existence of a hydrophobic binding site remote from the enzyme's catalytic center. To explore the specificity and accommodation of this binding site, we prepared a series of six glutamate-containing phosphoramidate derivatives of various hydroxysteroids (**1a**–**1f**). The inhibitory potencies of the individual compounds of the series were comparable to a simple phenylalkyl analog (**8**), and in all cases IC_{50} values were sub-micromolar. Molecular docking was used to develop a binding model for these inhibitors and to understand their relative inhibitory potencies against PSMA.

A notable discovery in prostate cancer research has been the identification of an over-expressed membrane-bound cell surface protein on prostate cancer cells, namely, prostate-specific membrane antigen (PSMA). PSMA, also known as folate hydrolase I (FOLH1) and glutamate carboxypeptidase II (GCPII),[1,2] is a 750-amino acid type II membrane glycoprotein[3] and was discovered during the development of the LNCaP cell line; one which retains most of the known features of prostate cancer.[4]

Although PSMA is primarily expressed in normal human prostate epithelium, the importance of this enzyme lies with the fact that it is upregulated and strongly expressed in prostate cancer cells, including those of the metastatic disease state.[5] It has also been demonstrated that PSMA expression is present in the endothelium of tumor-associated neovasculature of multiple non-prostatic solid malignancies,[6] including metastatic renal carcinoma.[7] As such, it is not surprising that PSMA has attracted a great deal of attention as a target for immunotherapy.[8–11] In addition to its immunological importance, PSMA is also reported to possess two predominant, yet poorly understood enzymatic activities: the hydrolytic cleavage and liberation of glutamate from γ-glutamyl derivatives of folic acid[12,13] and the proteolysis of the neuropeptide *N*-acetylaspartylglutamate (NAAG).[1] With respect to its function, recent studies suggest that PSMA plays a regulatory role in angiogenesis.[14]

Recently, we identified the presence of a hydrophobic binding site remote from the catalytic center of PSMA using a series of phenylalkylphosphonamidate derivatives of glutamic acid.[15] Based upon ongoing substrate and inhibitor-based studies in our laboratory, we have determined that PSMA can accommodate a variety of structural motifs remote from the catalytic center. The focus of this work was to determine if PSMA could specifically accommodate steroidal motifs in auxiliary binding sites remote from the catalytic center. To this end, we prepared a series of putative hydroxysteroid-containing phosphoramidate inhibitors of PSMA (Fig. 1) and determined their inhibitory potency against purified PSMA.

A general method (Scheme 1) was employed for the preparation of hydroxysteroid phosphoramidate inhibitors (Fig. 1). Briefly, bis(diisopropylamino)-chlorophosphine (**2**) was conjugated with benzyl alcohol to generate

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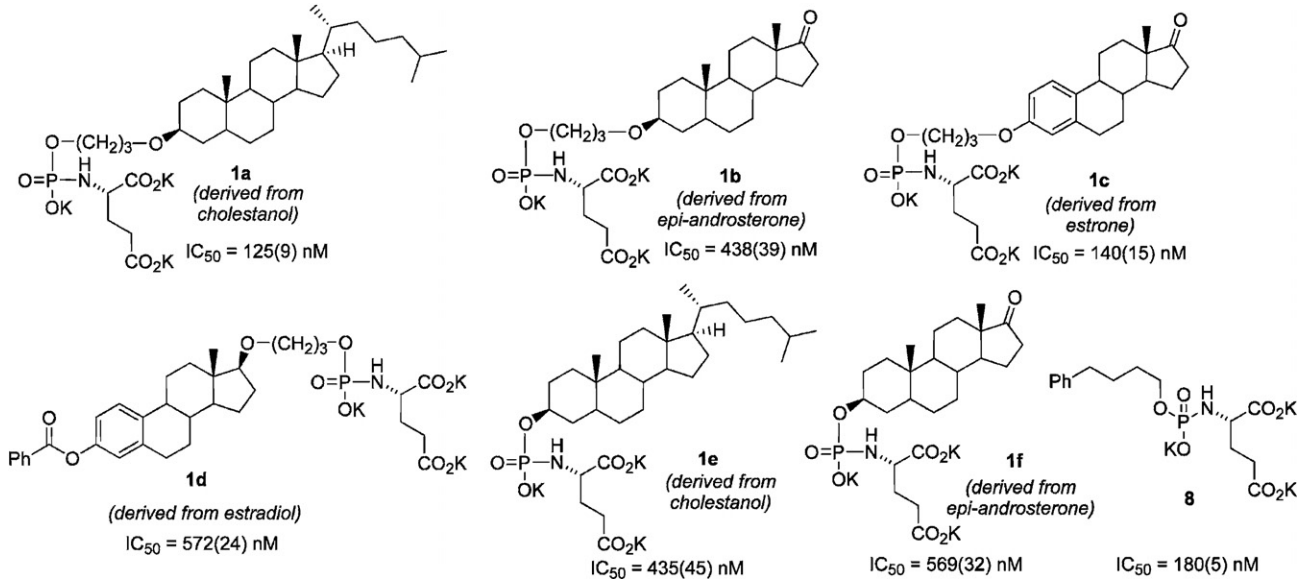
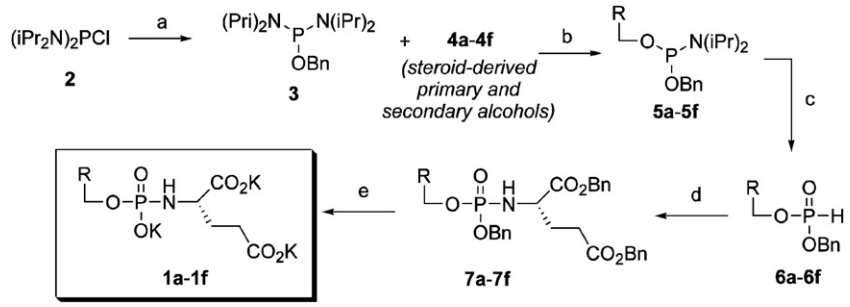


Figure 1. Structures and inhibitory potencies of steroid-derived phosphoramidate inhibitors (**1a**–**1f**) of PSMA and non-steroidal reference compound (**8**). Standard deviation for IC_{50} values in parentheses.



Scheme 1. Synthesis of hydroxysteroid-derived phosphoramidate inhibitors of PSMA. Reagents and conditions: (a) benzyl alcohol (3.0 equiv), Et_3N (3.0 equiv), CH_2Cl_2, 0 °C, 1 h; (b) diisopropylammonium tetrazolide (1.05 equiv), steroid-derived alcohols **4a**–**4f**, CH_2Cl_2; (c) 5-(ethylthio)-*1H*-tetrazole (1.1 equiv), CH_3CN, H_2O; (d) CH_3CN, Et_3N (2.0 equiv), *p*-TsOH·H-Glu(OBn)-OBn (1.3 equiv); then CCl_4 (10 equiv); (e) H_2, cat. Pd (10% on C), K_2CO_3 (1.5 equiv), THF–H_2O, 3 h, rt.

benzyloxyphosphine **3**, and then coupled with steroid-derived alcohols **4a**–**4f** to generate phosphoramidites **5a**–**5f** (see Supporting Information for the structures, synthesis, and characterization of **4a**–**4f**), using conventional methodology.[16,17] These molecules were hydrolyzed to the corresponding steroid-conjugated phosphites **6a**–**6f**. Oxidative coupling with benzyl protected glutamate was accomplished with CH_3CN–CCl_4 to generate **7a**–**7f**. Lastly, removal of benzyl protecting groups was performed under hydrogenolysis conditions to yield the phosphoramidate inhibitors **1a**–**1f** as the tripotassium salts.

Once prepared, the steroid-containing inhibitors **1a**–**1f** were assayed for inhibitory potency against purified PSMA using methods described previously (Fig. 1).[15,18,19] For comparison, the IC_{50} value for a simple phenylalkyl phosphoramidate **8** identified in a previous study is also presented.[20] All of the inhibitors maintained sub-micromolar inhibitory potency against PSMA, while two inhibitors (**1a** and **1c**) achieved a slight improvement over **8**. These results suggest that the enzyme can accommodate organic structures of considerable size and lipophilicity in proximity to the catalytic center. In at least one case, the propyl ether linker had an interesting effect on the potency of the inhibitors toward PSMA. For example, propyl ether linked inhibitor **1a** was significantly more potent than the unlinked analog of the same steroid **1e**. The effect was much less pronounced in linked inhibitor **1b** versus its unlinked counterpart **1f**. Unfortunately, the unlinked form of the relatively potent inhibitor **1c** could not be readily prepared with our synthetic scheme.

Overall, the inhibitors appear to be broadly differentiated into two classifications based on their inhibitory potency against PSMA. Inhibitors **1a** and **1c** exhibit greater potency than **1b**, **1d**, **1e**, and **1f**, and within these groups, the compounds attain fairly narrow ranges of IC_{50} values. The activity of the more active compounds (**1a** and **1c**) is comparable to that of the simple phenylalkyl phosphoramidate inhibitor **8**. That all compounds achieved sub-micromolar potency, including the less active inhibitors in the series, sug-

gests that the general glutamyl phosphoramidate scaffold dominates the interactions with PSMA for this class of compounds. The significance of these results is that this scaffold appears to be capable of accommodating a wide range of linked hydrophobic molecular fragments while still retaining notable potency.

To establish a tentative mode of binding and to rationalize the observed differentiation of the inhibitors by their potencies against PSMA, phosphoramidates **1a**–**1f** were computationally docked into the active site of PSMA (Fig. 2 and Supporting Information). The results were obtained employing a recently determined high-resolution X-ray crystal structure[21] in which the enzyme was co-crystallized with a phosphonate inhibitor (PDB = 2C6C). Docking of each inhibitor was performed with FRED2 (OpenEyes) employing a library of ligand conformations generated by OMEGA (OpenEyes). To filter docking poses non-productive for enzymatic inhibition, a SMARTS pharmacophore constraint was utilized requiring the phosphoramidate oxygen atoms to be within 3 Å of the two catalytic zinc atoms of PSMA. Subsequently, the top consensus scoring pose for each inhibitor was then minimized, without constraint, in the MMFF94 force field as implemented in SZYBKI (OpenEyes).

Upon docking this series of inhibitors, the most notable observation was the effect of the propyl ether linker on the steroid conformation in compounds **1a** (vs **1e**) and **1b** (vs **1f**). Interestingly, the presence of the linker caused the steroid nucleus to flip its docked orientation relative to the inhibitor without a linker. A result of this phenomenon is that it dictated the positions of the steroidal angular methyl groups, which have a propensity to sterically clash with residues in the PSMA active site. Thus, the docked conformations of **1a** (linked) and **1e** (unlinked) show opposite orientations of the steroid systems, and in the case of **1e**, the A-B ring angular methyl is oriented in a manner which potentially clashes with the Tyr^{700} aromatic ring. We speculate that such a steric effect could explain the significant potency difference between **1a** and **1e**. The propyl linker also extends **1a** through the PSMA binding cavity in a manner that could allow the steroid alkyl chain to be involved in favorable hydrophobic interactions with distant residues Pro^{502} and Tyr^{460}, further explaining the improved potency of this compound. A similar conformational flip was observed with **1b** (linked) and **1f** (unlinked), but in this case **1b** exhibited potential steric clashing with Tyr^{700}, while **1f** was observed in a relatively unhindered conformation. This model could explain the relatively poor potency of **1b**, but the lack of inhibitory

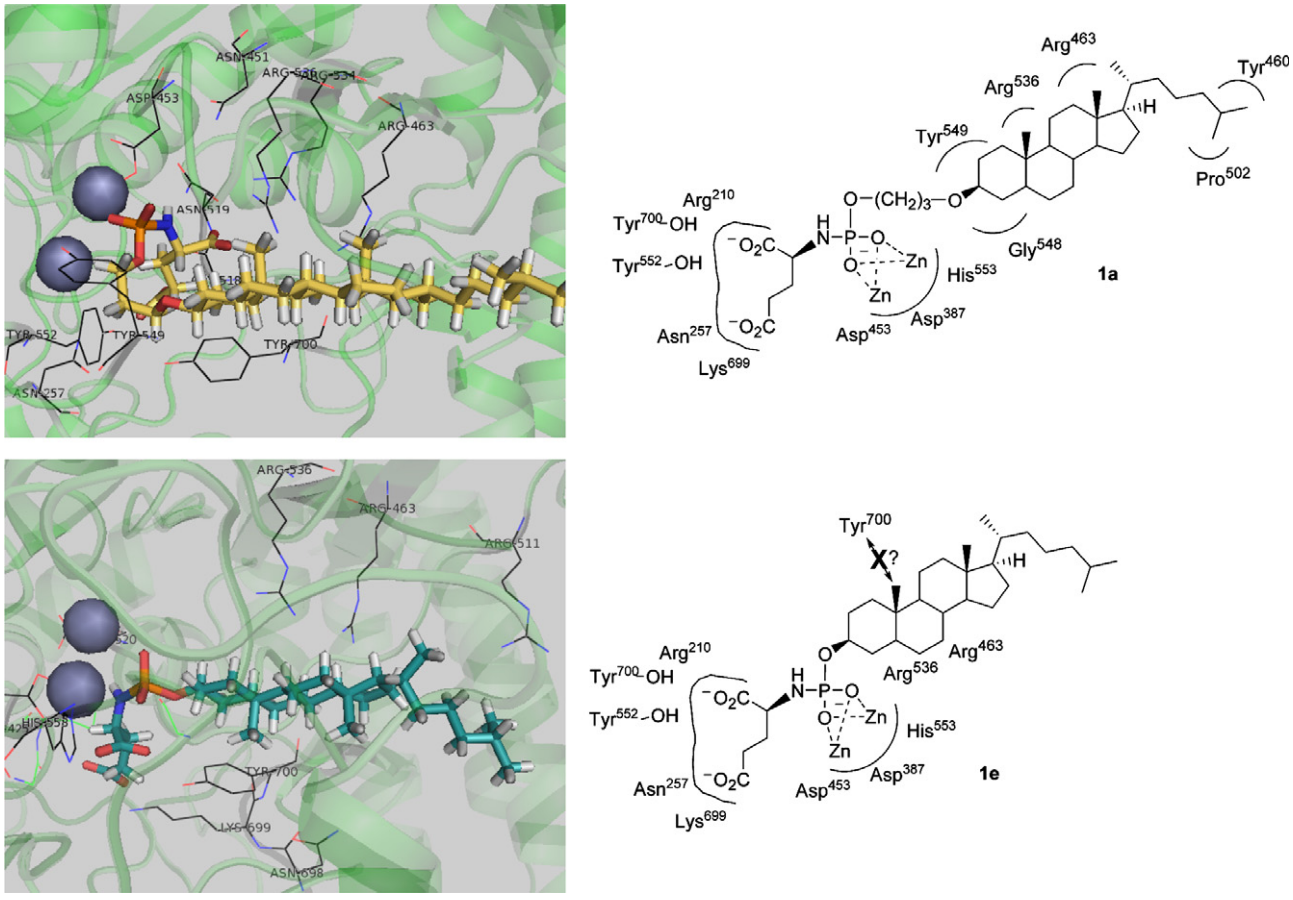


Figure 2. Results from docking inhibitors **1a** and **1e** into the active site of PSMA (PDB = 2C6C). Dark gray spheres represent the zinc atoms in the PSMA active site. Docking results of other inhibitors are in Supporting Information. Structures were visualized with PYMOL.

potency in **1f** is not readily explained with this model. However, the docked pose of the relatively potent inhibitor **1c** (linked) displayed an orientation of the steroid framework in which little steric hindrance was observed overall. Lastly, compound **1d** (linked) is unique relative to the other inhibitors due to the linker attachment at the steroid D ring. The docked conformation of this inhibitor presented the angular methyl in a manner which could potentially clash with Tyr^{700}, which may explain its relative low potency.

In summary, a small library of hydroxysteroid-containing phosphoramidate PSMA inhibitors was prepared and evaluated for inhibitory potency against PSMA. The relative potencies were explained by a model generated through computational docking studies. Namely, the least potent compounds in the series docked with their steroidal angular methyl groups projected in an opposite orientation from Tyr^{700} avoiding a potential steric clash. In contrast, the remaining compounds presenting docked poses in which the angular methyls projected away from Tyr^{700} all exhibited improved potency (with the exception of **1f**). The activity of the most potent inhibitor (**1a**) may be explained by additional hydrophobic contacts with Pro^{502} and Tyr^{460}. Notably, the entire series of inhibitors exhibited sub-micromolar potency against PSMA suggesting that the binding of these analogs may be driven by the *N*-phosphoglutamate core. Results from the designs of subsequent generations of PSMA inhibitors will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.096.

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