

## 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<sub>50</sub> 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.

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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),<sup>1,2</sup> is a 750-amino acid type II membrane glycoprotein<sup>3</sup> and was discovered during the development of the LNCaP cell line; one which retains most of the known features of prostate cancer.<sup>4</sup>

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.<sup>5</sup> It has also been demonstrated that PSMA expression is present in the endothelium of tumor-associated neovasculature of multiple non-prostatic solid malignancies,<sup>6</sup> including metastatic renal carcinoma.<sup>7</sup> As such, it is not surprising that PSMA has attracted a great deal of attention as a target for immunotherapy.<sup>8–11</sup> In addition to its immunological impor-

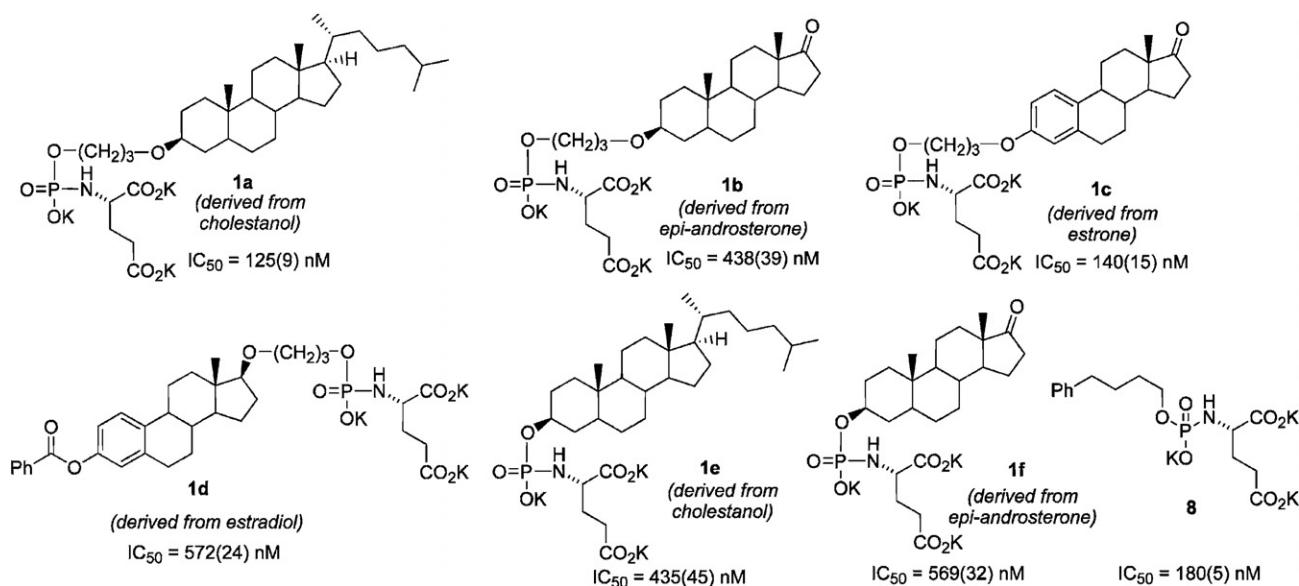
tance, PSMA is also reported to possess two predominant, yet poorly understood enzymatic activities: the hydrolytic cleavage and liberation of glutamate from  $\gamma$ -glutamyl derivatives of folic acid<sup>12,13</sup> and the proteolysis of the neuropeptide *N*-acetylasparylglutamate (NAAG).<sup>1</sup> With respect to its function, recent studies suggest that PSMA plays a regulatory role in angiogenesis.<sup>14</sup>

Recently, we identified the presence of a hydrophobic binding site remote from the catalytic center of PSMA using a series of phenylalkylphosphoramidate derivatives of glutamic acid.<sup>15</sup> 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.

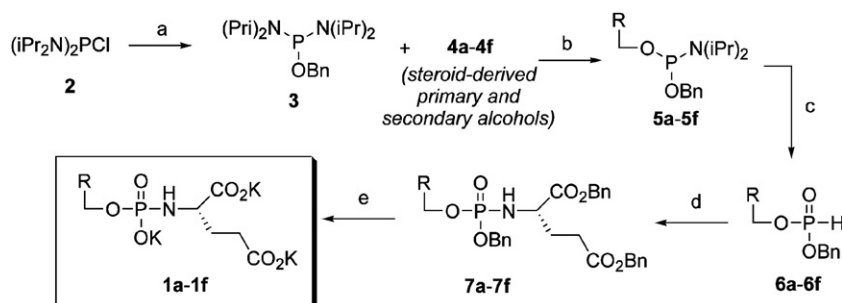
A general method (Scheme 1) was employed for the preparation of hydroxysteroid phosphoramidate inhibitors (Fig. 1). Briefly, bis(diisopropylamino)-chlorophosphine (**2**) was conjugated with benzyl alcohol to generate

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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<sub>50</sub> values in parentheses.



**Scheme 1.** Synthesis of hydroxysteroid-derived phosphoramidate inhibitors of PSMA. Reagents and conditions: (a) benzyl alcohol (3.0 equiv), Et<sub>3</sub>N (3.0 equiv), CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 1 h; (b) diisopropylammonium tetrazolide (1.05 equiv), steroid-derived alcohols **4a–4f**, CH<sub>2</sub>Cl<sub>2</sub>; (c) 5-(ethylthio)-1*H*-tetrazole (1.1 equiv), CH<sub>3</sub>CN, H<sub>2</sub>O; (d) CH<sub>3</sub>CN, Et<sub>3</sub>N (2.0 equiv), *p*-TsOH·H-Glu(OBn)-OBn (1.3 equiv); then CCl<sub>4</sub> (10 equiv); (e) H<sub>2</sub>, cat. Pd (10% on C), K<sub>2</sub>CO<sub>3</sub> (1.5 equiv), THF–H<sub>2</sub>O, 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.<sup>16,17</sup> These molecules were hydrolyzed to the corresponding steroid-conjugated phosphites **6a–6f**. Oxidative coupling with benzyl protected glutamate was accomplished with CH<sub>3</sub>CN–CCl<sub>4</sub> to generate **7a–7f**. Lastly, removal of benzyl protecting groups was performed under hydrogenolysis conditions to yield the phosphoramidate inhibitors **1a–1f** as the tri-potassium salts.

Once prepared, the steroid-containing inhibitors **1a–1f** were assayed for inhibitory potency against purified PSMA using methods described previously (Fig. 1).<sup>15,18,19</sup> For comparison, the IC<sub>50</sub> value for a simple phenylalkyl phosphoramidate **8** identified in a previous study is also presented.<sup>20</sup> 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<sub>50</sub> 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-

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