Pseudoirreversible Inhibition of Prostate-Specific Membrane Antigen by Phosphoramidate Peptidomimetics†

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ABSTRACT: The mode of inhibition for phosphoramidate peptidomimetic inhibitors of prostate-specific membrane antigen was determined by inhibition reversibility experiments. The results revealed that these inhibitors can be classified into three types: pseudoirreversible (compounds **1**−**3**), moderately reversible (compounds **4**−**9**), and rapidly reversible inhibitors (compounds **10** and **11**). Representative compounds from each class were further evaluated for their ability to induce cellular internalization of PSMA. Results from these experiments revealed that the pseudoirreversible inhibitor **1** induced the greatest PSMA internalization. The discovery of pseudoirreversible PSMA inhibitors is expected to provide a new avenue of investigation and therapeutic applications for prostate cancer and neurological disorders.

The cell-surface enzyme prostate-specific membrane antigen (PSMA) continues to serve as an important biomarker and target in prostate cancer research. Also known as folate hydrolase I (FOLH1) and glutamate carboxypeptidase II (GCPII) (*1, 2*), PSMA 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*). PSMA is upregulated and strongly expressed on prostate cancer cells, including those that are metastatic (*5*). Endothelial expression of PSMA in the neovasculature of a variety of nonprostatic solid malignancies has also been detected (*6, 7*). As a consequence, PSMA has attracted significant attention as a target for the delivery of imaging (*8*−*11*) and therapeutic agents (*12*−*15*).

PSMA is reported to possess two predominant yet poorly understood enzymatic activities: the hydrolytic cleavage and liberation of glutamate from γ-glutamyl derivatives of folic acid (*16*) and the proteolysis of the neuropeptide *N*-acetylaspartylglutamate (NAAG) (*1*). There is emerging evidence that with respect to its function, PSMA plays a regulatory role in angiogenesis (*17*). Various chemical scaffolds have been developed as inhibitors of this enzyme (*18*−*20*). Recently, we synthesized a series of analogues to identify the pharmacophore for phosphoramidate peptidomimetic inhibitors of PMSA (*20*). The design of the lead inhibitor (**1**) was based upon *N*-acyl derivatives of the endogenous substrate folyl-γ-Glu and incorporated a phosphoramidate group to interact with catalytic zinc atoms within the active site of PSMA. The scope of the analogue library was designed to test the importance of various functional groups to the inhibitory potency of the lead phosphoramidate (Figure 2). The focus of the work described herein was to examine the postinhibitory profiles of the inhibitors from our recent analogue library. We have now identified three classes of PSMA inhibitors (reversible, moderately reversible, and pseudoirreversible) and further correlated their modes of inhibition to PSMA internalization in LNCaP cells.

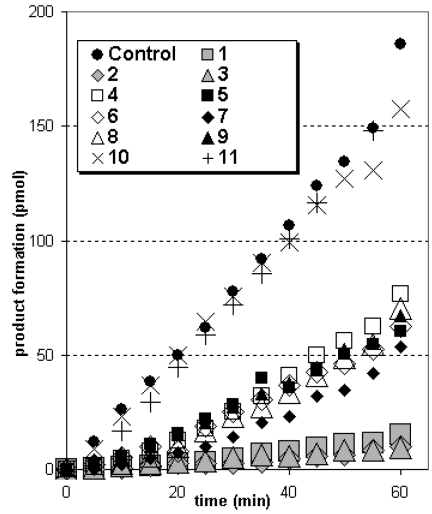

FIGURE 1: Activity recovery profiles for PSMA inhibitors. Enzymatic activity after 100-fold dilution of PSMA incubated with inhibitors at 10-fold IC_{50}. On the basis of recovery profiles, inhibitors are rapidly pseudoirreversible (**1**−**3**), moderately reversible (**4**−**9**), and rapidly reversible (**10** and **11**). Uninhibited PSMA served as a control (●).

In our previous report, we determined the IC_{50} values for analogues in a library of phosphoramidate inhibitors of PSMA (Figure 2) (*20*). The data from that study allowed a relative determination of the individual affinities of the library entries for PSMA. To definitively determine enzyme affinity for these compounds, the mechanism of inhibition must now be ascertained. A first step toward this end is to identify whether the inhibition of enzymatic activity is rapidly reversible, moderately reversible, or pseudoirreversible. To determine the reversibility of PSMA inhibition by our phosphoramidate analogues, we monitored the recovery of enzyme activity following rapid dilution of the enzyme−inhibitor complex (*21*).

In these experiments, the concentration of enzyme (2.5 μg/mL) is 100-fold greater than that used under typical assay conditions. The enzyme is then incubated with inhibitor at a

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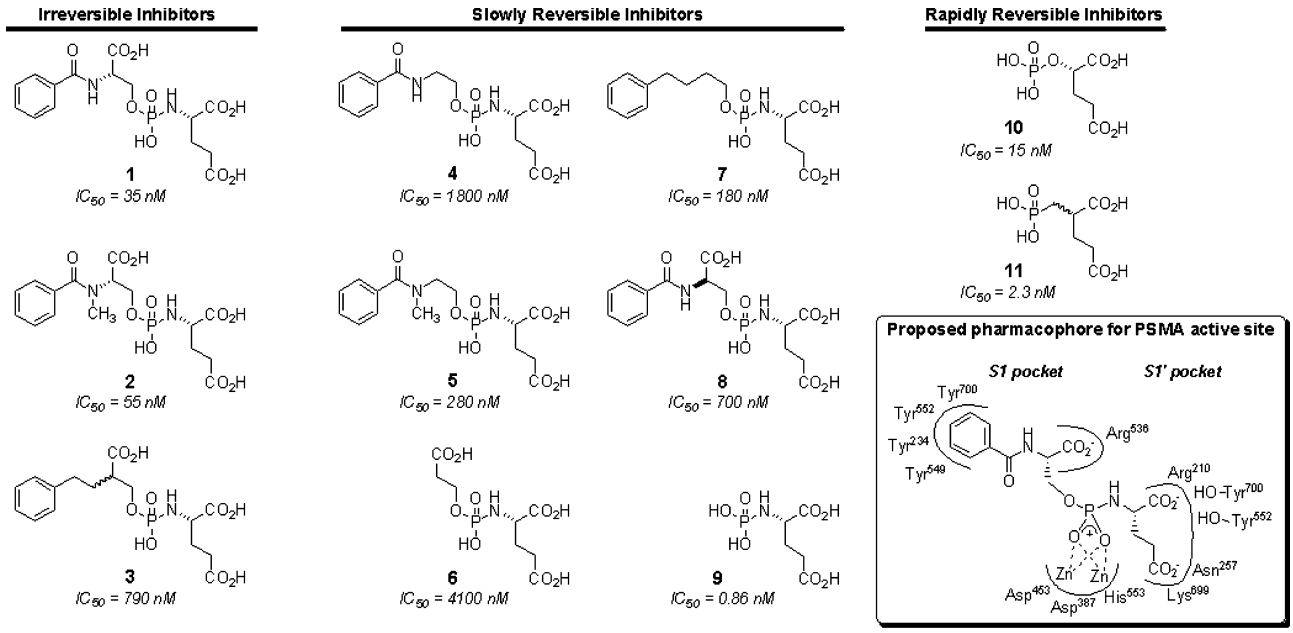

FIGURE 2: Peptidomimetic inhibitors and proposed pharmacophore for PSMA.

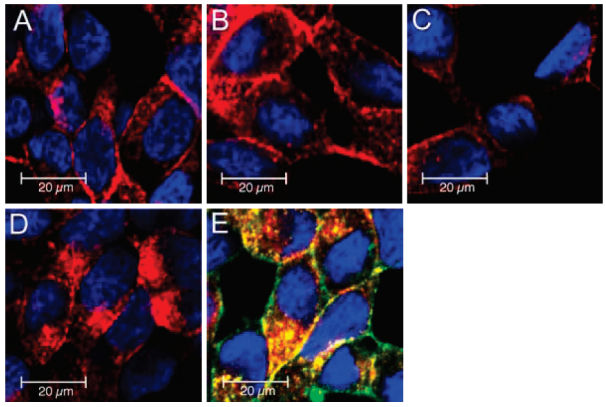

FIGURE 3: Inhibitor-mediated PSMA internalization in LNCaP cells. Live LNCaP cells incubated with (A) no inhibitor with 10% FBS and phosphate-free RPMI 1640, (B) 100 μM compound **10**, (C) 100 μM compound **9**, (D) 100 μM compound **1**, or (E) 2 μM fluorescent inhibitor (*24*). All cells were fixed; PSMA was detected with the antibody-based immunofluorescence method, and nuclei were stained with DAPI. The distance scale is 20 μm.

concentration 10-fold greater than their IC_{50}. Upon rapid dilution (100-fold) with a saturating concentration of substrate (10 μM), the final enzyme concentration is approximately equal to that used in a typical activity assay while the inhibitor concentration will have been diluted to 1/10th of the IC_{50}. If the inhibitor is rapidly reversible, the progress curve should be linear with a slope nearly equal the slope of an uninhibited control sample. If the inhibitor is pseudoirreversible, then only ~9% residual activity can be measured after dilution. If the inhibitor is moderately reversible on the time scale of the activity assay, the progress curves will be curvilinear and increase with time. The results from the inhibition reversibility experiments confirm the presence of all three classes of inhibitors of PSMA from our library of analogues (Figure 1).

The phosphate (**10**) and phosphonate (**11**) entries exhibited rapidly reversible profiles as the recovery of PSMA inhibited by these compounds tracked close to the uninhibited control sample. The main structural difference between these compounds and the remaining inhibitors examined is the lack of a phosphoramidate P−N linkage in the P1′ residue. These results suggest that although bioisosteric replacement of the phosphoramidate nitrogen is sufficient to confer considerable inhibitory potency toward PSMA, the phosphoramide motif is necessary for moderately reversible or pseudoirreversible inhibition of the target enzyme. As observed for phosphonamidate inhibitors of thermolysin (*21*), the phosphoramide nitrogen likely participates in a significant hydrogen bonding interaction with active site residues which could be revealed in future cocrystallization studies.

Phosphoramidates **4**−**9** were moderately reversible inhibitors as demonstrated by their curvilinear recovery of PSMA activity. More interestingly, it was found that compounds **1**−**3** were pseudoirreversible inhibitors of PSMA. We recently proposed a pharmacophore model for phosphoramidate inhibitors of PSMA based upon experimental and docking results with available crystal structure data (*22*) (Figure 2) (*20*). In addition to an essential P1′ glutamate residue and a zinc-binding group (ZBG), a P1 group was found to be optional for activity but, if present, should contain hydrophobic functionality to interact in π-stacking or hydrophobic interactions with nearby aromatic residues Tyr^{234}, Tyr^{549}, Tyr^{552}, and Tyr^{700}. On the basis of the results from this study, only those inhibitors that possess both a P1 hydrophobic and carboxylate group exhibit pseudoirreversible inhibition of PSMA. Compound **8** represents an exception to this generalization, although it possesses inverted stereochemistry at the P1 α center, presumably altering its mode of binding from that of **1**. Phosphoramidate peptidomimetics lacking either a P1 carboxylate or hydrophobic group (**4**−**9**) exhibit moderately reversible inhibition of PSMA but not pseudoirreversible inhibition. These results suggest that interactions in the putative S1 site may be additive, leading to a pseudoirreversible enzyme−inhibitor complex. Formation of an initial enzyme−inhibitor complex may be rapid and reversible but may not initially involve all structural elements. Insertion of both P1 carboxylate and hydrophobic groups into complementary binding sites in S1 may represent a poorly reversible second kinetic step in

formation of a tight-binding and pseudoirreversible complex. Consequently, such inhibitors would exhibit a slow, tight-binding mode of action displaying time-dependent inhibition. Although structurally unrelated to the compounds examined here, a similar phenomenon has been observed for indomethacin in which the insertion of a methyl group into a hydrophobic pocket is responsible for the time-dependent, pseudoirreversible inhibition of cyclooxygenases (*23*). In contrast to pseudoirreversible inhibitors **1**−**3**, phosphoramidates not capable of both types of interactions in S1 are hypothesized to lead to a moderately reversible enzyme−inhibitor complex. To elucidate the mechanism of the interaction between PSMA and its pseudoirreversible inhibitors described here, time-dependent inhibition and covalent modification analysis are required and are currently underway.

To explore the impact on cellular events, specifically on the internalization of PSMA, representatives of the three types of inhibitors were incubated with PSMA-positive LNCaP cells in vitro. The pseudoirreversible inhibitor (**1**) induced the internalization of PSMA, which was largely focused in the perinuclear region (Figure 3D). This result was consistent with the endosomal localization of internalized PSMA induced by a fluorescent PSMA inhibitor (Figure 3E), which was studied and described in detail in our previous report (*24*). In contrast, reversible inhibitor **10** and moderately reversible inhibitor **9** displayed weaker effects on PSMA internalization (Figure 3B,C) as compared to the no-inhibitor control (Figure 3A). Despite PSMA's propensity for internalization, antibody binding is known to induce this process (*25*), as well as small-molecule inhibitors albeit more moderately (*24*). On the basis of our understanding, the efficiency of PSMA internalization is possibly dependent on the extent of PSMA conformational changes, which can contribute to affecting interactions of PSMA's cytoplamic domain with clathrin and the clathrin adaptor protein-2 (AP-2) complex, leading to the internalization of the PSMA complex via clathrin-coated pits (*26*). Therefore, the extent of the conformation changes resulting from different modes of inhibition for PSMA inhibitors may be correlated with the different effects on PSMA internalization. We anticipate that pseudoirreversible inhibition and an increased level of PSMA internalization can be exploited both in the treatment of neurological disorders and as a mechanism for transporting drugs into PSMA-positive prostate tumors. When harnessed to therapeutic agents, such compounds are expected to serve as selective homing elements to achieve greater uptake of drug conjugates in target cells.

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SUPPORTING INFORMATION AVAILABLE

Detailed materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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