Irreversible Inhibition of Prostate-Specific Membrane Antigen by Phosphoramidate Peptidomimetics

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Supplemental Data

Materials and Methods

LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). The mouse monoclonal antibody 7E11 was gifted from Cytogen Corporation (Princeton, NJ). The goat anti-mouse secondary antibody-TRIC was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). PSMA (GCP II, E.C. 3.4.17.21) was extracted and purified from LNCaP cells according to our previously described protocol *(1)*. The phosphoramidate peptidomimetic inhibitors and enzyme substrate (N-[4-(phenylazo)benzoyl]-glutamyl-γ-glutamic acid, PAB-Glu-γ-Glu) were available from prior studies *(2-4)*. All other chemicals and cell-culture reagents were purchased from Fisher Scientific (Sommerville, NJ), Pierce (Rockford, IL), or Sigma-Aldrich (St. Louis, MO).

Reversibility of enzyme inhibition

A solution of purified PSMA *(1)*, at a concentration 100-fold greater than that required to achieve approximately 15% conversion of 10 μM substrate to product in 15 min, was incubated with inhibitors at a concentration 10-fold greater than their respective IC_{50} values at 37°C for 10 minutes. This mixture was then diluted 100-fold into a reaction buffer (50 mM Tris buffer, pH 7.4) containing a saturating concentration of substrate (10 μM PAB-Glu-γ-Glu) and kept at 37°C. At increasing time points (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) a 250 μL aliquot was removed and quenched with a 25 μL methanolic solution of TFA (2% by volume) followed by vortexing and centrifugation (10 min at 7000 g). An 85 μL aliquot of the resulting supernatant was subsequently analyzed by HPLC and enzyme substrate and product were quantified as described previously *(1)*. Progress curves of product formation were generated to monitor the recovery of enzymatic activity for inhibited PSMA and compared to a control sample in which no inhibitor was added.

Inhibitor-mediated PSMA Internalization Studies

Cell culture: PSMA-positive cells (LNCaP) were grown in T-75 flasks with complete growth medium [RPMI 1640 containing 10% heat-inactivated fetal calf serum (FBS), 100 units of penicillin and 100 μg/mL streptomycin] in a humidified incubator at 37°C and 5% CO_2. Confluent cells were detached with 0.25% trypsin-0.53 mM EDTA solution, harvested, and plated in 2-well slide chambers at a density of 4 x 10^4 cells/well. Cells were grown for 3-4 days before conducting the following experiments.

Cell incubation with inhibitors: Cells grown on the slides were first washed twice with warm medium A (phosphate-free RPMI 1640 containing 10% FBS), then incubated with 1 mL of each inhibitor (100 μM) except from fluorescent inhibitor (2 μM) in warm medium A for 150 min in a humidified incubator at 37°C and 5% CO_2.

Detection of internalized PSMA: All the above treated Cells were first washed once with cold medium B (phosphate-free RPMI 1640 containing 1% FBS), then twice with cold-KRB bufferpH7.4 (mmol/L: NaCl 154.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, HEPES 5.0, D-glucose 5.0), fixed with 4% paraformaldehyde in KRB for 15 min at room temperature, permeabilized with cold-methanol for 5 min at -20°C. The Fixed cells were blocked with 1% BSA, KRB for 30 min and incubated with primary antibody 7E11 (6 mg/mL, 300X diluted in 1%BSA, KRB) for 60 min at room temperature. After washing, the cells continued to incubated with second antibody (goat anti-mouse IgG-TRITC, 50X dilution in 1%BSA, KRB) for 60 min, counterstained with DAPI (according to manufacturer's instructions; Invitrogen), and mounted for microscopy. Cells were visualized under 40X oil objective using a LSM 510 META Laser Scanning Microscope with Diode Laser (405 nm) for DAPI, Ar Laser (488 nm) for Fluorescein, and HeNe Laser (543 nm) for Rhodamine. The merged pictures were edited by National Institutes of Health (NIH) Image J software (**http://rsb.info.nih.gov/ij/**) and Adobe Photoshop CS2.

References:

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