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Prostate targeting ligands based on *N*-acetylated α-linked acidic dipeptidase

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Abstract

To identify inhibitors of the intrinsic *N*-acetylated α-linked acidic dipeptidase (NAALADase) activity of prostate specific membrane antigen (PSMA) that may be useful for targeting imaging agents or chemotherapeutic drugs to disseminated prostate cancer, analogs of the tetrahedral transition state for hydrolysis of the natural substrate, *N*-acetylaspartylglutamate (NAAG), were synthesized. These compounds were assayed for their ability to inhibit the membrane-associated enzyme isolated from LNCaP prostate cancer cells. Active inhibitors were further assayed for their cytotoxicity and membrane binding. We have identified nine compounds, including fluorescent and iodine-labeled conjugates, which inhibit NAALADase enzyme activity with IC_{50}s at, or below, 120 nM. The binding of these compounds to the cell surface of viable LNCaP prostate tumor cells appears to be specific and saturable, and none of the compounds alter the cell cycle kinetics or induce apoptosis in LNCaP cells, suggesting that they are relatively innocuous and are suitable for targeting imaging agents or cytotoxic drugs to disseminated prostate cancer.

Keywords: Glutamate carboxypeptidase; PSMA; NAALADase; Prostate cancer; Metastasis

In 1999, an estimated 180,000 men were diagnosed with prostate cancer in the US and 37,000 succumbed to the disease [1]. The underlying cause of death lies in the progression and metastasis of the primary cancer. More extensive PSA screening has led to the identification of many more early stage (A1 and A2) tumors. While serum PSA is widely used to monitor tumor progression, it cannot be used to localize metastatic foci, and since it is a secreted protein that is largely inactive in the circulation, it is not readily amenable to targeting of localized tumors or metastatic foci. The ability to identify and treat of localized aggressive, clinically significant tumors early in their progression is hampered by the lack of highly specific markers that can identify and target metastatic cells in the circulation or lymphatics [2]. Several approaches have already been taken to develop new diagnostic and prognostic markers, using prostate specific membrane antigen (PSMA), including RT-PCR and immunological methods [3–5]. We have chosen to develop methods for in situ identification of tumors expressing PSMA, a 110 kDa, type II, integral transmembrane glycoprotein [6] which is found associated with the secretory epithelium of the prostate, prostatic tumor cells, and the tumor associated neovasculature of several other tumor types [7–9]. Since extra-prostatic expression of PSMA is highly restricted, it may be a very useful target for both diagnostic technologies and therapeutic intervention, as is evidenced by the use of monoclonal antibodies specific for PSMA for the detection of occult prostate cancer [CYT-356 (ProstaScint)] and for immunotherapy for prostate cancer [10–13].

Prostate specific membrane antigen is highly homologous to the neuropeptidase *N*-acetylated α-linked acidic dipeptidase (NAALADase) that releases the

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neurotransmitter, glutamate, from the neuronal peptide *N*-acetylaspartylglutamate (NAAG) or folate polyglutamate [14]. Unlike the brain isoform of NAALADase, which is found in the cytoplasm, there are two isoforms of PSMA found in prostate cancer: the predominant membrane associated protein, designated PSM, and a cytosolic form of the protein, PSM′ [15,16]. These two isoforms appear to be generated by differential splicing of the PSM transcript [17,18] that appears to be transcribed from a single gene, designated FOLH1, localized to 11p11.2 [19,20]. We have chosen to identify highly selective inhibitors of the NAALADase activity of membrane associated PSMA that can subsequently be coupled to imaging agents for magnetic resonance imaging (MRI) or to cytotoxic drugs for systemic delivery to localized, primary and disseminated, metastatic tumors. In this report, we describe the synthesis and initial characterization of a panel of compounds that inhibit the NAALADase activity of PSMA isolated from the LNCaP prostate cancer cell line.

Materials and methods

Materials. *N*-Acetyl-L-aspartyl-glutamate (NAAG) was purchased from Sigma Chemical (St. Louis, MO). *N*-Acetyl-L-aspartyl-L-[3,4-^{3}H]glutamate was purchased from Dupont NEN (Boston, MA). AG50 W-X4 resin was purchased from Bio-Rad. All other chemicals were from Sigma Chemical (St. Louis, MO), Aldrich Chemical (Milwaukee, WI), or Acros Organics and were of reagent grade or higher.

Synthesis of NAALADase inhibitors. A total of 70 different compounds were synthesized and assayed to determine whether they inhibit the membrane associated NAALADase activity isolated from LNCaP cells. The rationale for the synthesis of these compounds is based on the design of compounds that mimic the transition state of NAAG, containing a phosphate isoteric mimic of the transition state tetrahedral intermediate generated during cleavage. Several phosphonic ester derivatives (VA-033C, VA-035C, VA-040H, and VA-048H) of the known NAALADase inhibitor, 2-(phosphonomethyl)pentanedioic acid (2-PMPA), were prepared as reference compounds for structure–activity relationship analysis, according to general methods commonly used for substituted phosphonic and phosphinic acids [21]. The fluorescent conjugates (VA-041Y, VA-042Y, and VA-043H) and iodine-labeled derivative (VA-064Y) were synthesized from corresponding aminoalkyl precursors by condensation with fluorescent reagents or 5-iodophthalic anhydride, respectively, using tertiary butyl as the acid protecting group. All compounds possessing an unsymmetrical center were characterized and tested in the racemic form. A number of phosphonate and phosphinic acid derivatives demonstrate the ability to inhibit the NAALADase activity of the membrane associated isoform of the prostatic enzyme (see Table 1 for individual structures and activities).

Preparation of LNCaP cell lysate. LNCaP and DU-145 cells were purchased from ATCC and cultured at 37 °C in RPMI 1640 medium containing 10 nM testosterone and 10% FBS serum. To harvest cells, one T-150 flask of confluent cells was centrifuged at 1000 rpm for 3 min, washed once with 0.32 M sucrose, and re-centrifuged at 1000 rpm for 3 min. The cell pellets were re-suspended in 1 mL 50 mM Tris–HCl, pH 7.4, and 0.5% Triton X-100 and homogenized. The homogenates were then centrifuged at 900*g* to precipitate the nuclei. The post-nuclear supernatants were divided into 50 μL aliquots and stored at −20 °C. Aliquots of the cell preparations were thawed and sonicated for 5 s to resuspend the particulate components and the protein concentration was determined using the BCA based protein assay (Pierce, Rockford, IL).

Assay of the prostatic membrane associated NAALADase activity. NAALADase activity was assayed as previously described with minor modifications [22]. The assays were performed in 600 μL of buffer (50 mM Tris–HCl, pH 7.4, 20 mM $CoCl_2$, and 32 mM NaCl), containing 5 μg lysate protein and varying concentrations of NAALADase inhibitors. The reaction mixture was pre-incubated at 37 °C for 3 min prior to the addition of 1 μM [^{3}H]NAAG (1×10^5 cpm) and subsequently incubated at 37 °C. At the indicated times, 100 μL of the reaction mixture was removed and mixed with an equal volume of ice-cold 0.25 M KH_2PO_4, pH 4.3, to stop the reaction. Half of the mixture was applied to a pre-washed (0.1% Triton X-100) 1.0 mL AG50WX4 cation exchange column (200–400 mesh, H^+ form). Tritiated glutamate was eluted with 1.5 mL of 3 M KCl. The radioactivity was monitored by scintillation spectrophotometry after addition of 15 mL Ecoscint. Zero time background control values were subtracted from experimental time points and the results were expressed as picomoles [^{3}H]glutamate formed/min/mg protein. To ensure linearity of the reaction, the incubations were performed for a maximum of 10 min, at which point no more than 20% of the substrate was metabolized. Initial rates were calculated based on the first 2 min and used to calculate double reciprocal plots.

Binding of fluorescent derivatives (VA-041Y and VA-043H) to LNCaP and DU-145 cells. LNCaP and DU145 cells were grown and harvested as described above. Cell pellets were re-suspended in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) and resuspended in 200 μL of the same buffer. Two microliters of 10 mM VA-041Y (final concentration, 100 μM) was added to each tube and incubated for 20 min in the dark at room temperature. The cells were centrifuged at 1000 rpm for 3 min, washed twice with GC medium, and suspended in GC medium prior to analysis by flow cytometry. In parallel experiments cells were incubated in culture medium with 100 μM VA-043H for 20 min in the dark at room temperature, rinsed several times with with culture medium and PBS, coverslipped, and viewed by fluorescent microscopy.

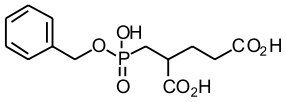
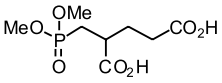
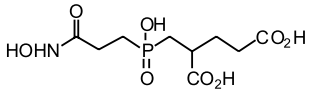
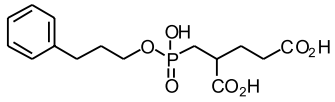
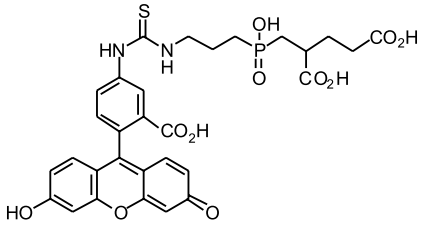
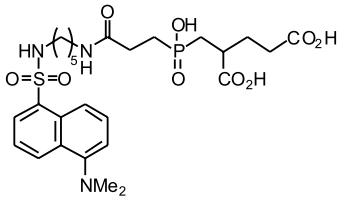
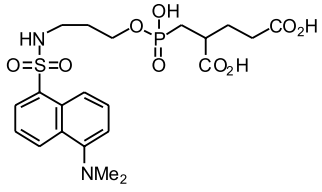
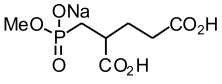
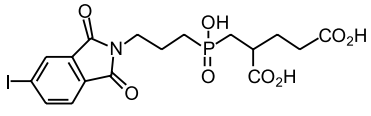
Effect of VA-033C on the viability of LNCaP cells. The viability of LNCaP cells in the presence of VA-033C was tested using flow cytometry. Cells were grown to 35% confluency in 4 mL RPMI medium supplemented with 10 nM testosterone and 10% FBS in T-25 flasks as described above. A 32.5 μL portion of 30 mM VA-033C was added to each flask, to a final concentration of 250 nM, and the cells were incubated for an additional three days prior to harvest. The cells were trypsinized and collected by centrifugation. After centrifugation, the cell pellets were washed twice with PBS and then fixed with 90% ethanol at −20 °C for 30 min. The pellets were resuspended in 130 μL PBS containing 2.5 mM EDTA to a concentration of 10^6 cells/ml. A 100 μL portion of the cell suspension was mixed with 1 mL RNase (15 U/mL) and 10 μL of propidium iodide (50 mg/mL). After incubation for 20 min at room temperature in the dark, the cells were immediately assayed by flow cytometry. A total of 5×10^5 events were analyzed in an Epics-Profile II cytofluorometer (Coulter) and the percentages of cells in the $< G_0$ (apoptotic), G_0/G_1, S, and G_2/M phases of the cell cycle were determined by planimetry using the "Elite" software.

Results

Kinetics of NAALADase

To determine the IC_{50} of each of the compounds, a modification of the assay developed to measure the activity of the brain enzyme was used [22]. The activity of the prostatic isoform of the enzyme was characterized using

Table 1
Compound number, structure, and IC_{50} for active inhibitors of NAALADase

Compound No.	Structure	IC_{50} (nM)
VA-033C		12.5
VA-035C		90
VA-037C		90
VA-040H		20
VA-041Y		25
VA-042Y		25
VA-043H		25
VA-048H		120
VA-064Y		85

membrane extracts from LNCaP cells. The time course of NAALADase activity at 1 μM NAAG is shown in Fig. 1A; the titration of NAAG concentration from 0.1 to 1 nM is shown in Fig. 1B and the double reciprocal plot is shown in Fig. 1C. From these data, we have established that the K_m of the prostatic membrane isoform of NAALADase, measured in lysates from LNCaP cells, is approximately 2.6 nM NAAG. The corresponding enzyme from rat brain appears to have a K_m of approximately 540 nM [23,24], indicating that the membrane associated enzyme from prostate has a higher affinity for the substrate than the enzyme isolated from rat brain.

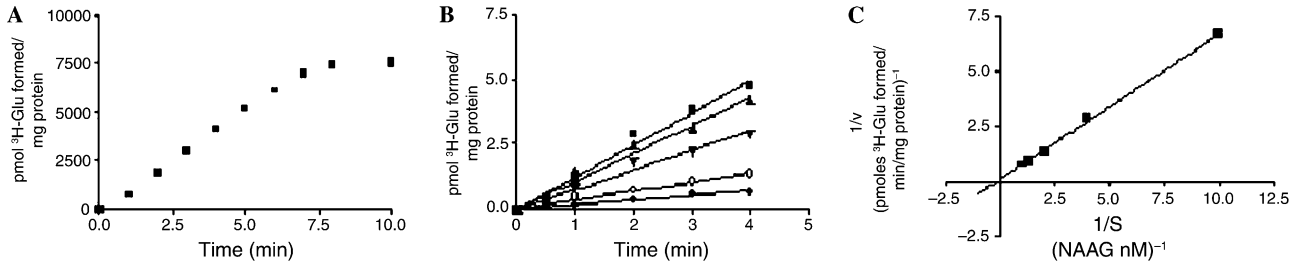


Fig. 1. Kinetics of NAALADase activity in LNCaP cell lysate. (A) Time course of NAALADase activity at 1 μM NAAG. (B) Time course of NAALADase activity titration of NAAG concentration. From the top to the bottom: 1 nM (■), 0.8 nM (▲), 0.5 nM (▼), 0.25 nM (○), and 0.1 nM (●) NAAG. (C) Reciprocal plot of the titration of NAAG concentration in B. K_m was calculated from x intercept ($-1/K_m = 1/X$).

In the course of these studies, 70 potential inhibitor structures were synthesized and assayed, nine of which showed significant inhibition with IC_{50} values at concentrations of 100 nM or below Table 1). The most active inhibitors are hydrolysis transition state analogs of NAAG that contain a phosphate isoteric mimic of the tetrahedral intermediate generated during cleavage. Among these, VA-033C shows strong inhibition of NAALADase activity and serves as the prototype inhibitor. The kinetics of VA-033C inhibition of NAALADase activity isolated from LNCaP cell lysate is shown in Fig. 2. The calculated IC_{50} of VA-033C for NAALADase is 12.5 nM (Table 1), indicating that VA-033C is a potent inhibitor of prostatic NAALADase. Fluorescent modification of VA-033C, by dansylation

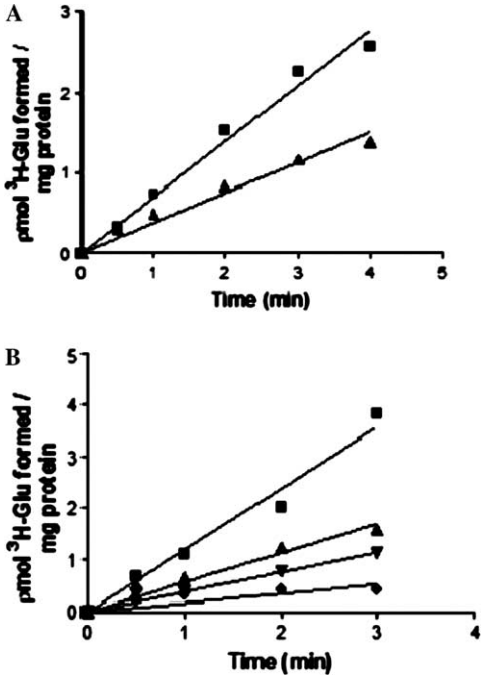


Fig. 2. Kinetics of inhibition of NAALADase activity by VA-033C. (A) Inhibition of NAALADase activity by VA-033C in LNCaP cell lysate at 0.5 nM NAAG: no inhibitor (■), in the presence of 10 nM VA-033C (▲). (B) Titration of the inhibition of VA-033C: no inhibitor (■), 1 nM VA-033C (▲), 5 nM VA-033C (▼), and 25 nM VA-033C (●).

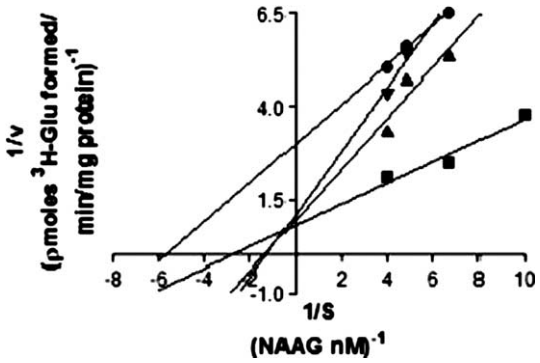


Fig. 3. Double reciprocal plot of VA-064Y inhibition of NAALADase activity in LNCaP cell lysate. The assay of NAALADase activity was performed at the NAAG concentration range from 0.10 to 0.25 nM in the presence of increasing concentrations of VA-064Y: 0.9 nM (■); 1.0 nM (▲); 1.1 nM (▼), and 1.2 nM (●).

(VA-043H) or conjugation to fluorescein (VA-041Y), does not substantially alter the IC_{50} (25 and 28 nM, respectively), demonstrating that modifications to this region of the prototype do not appear to influence the inhibitory activity of the molecule. To establish the nature of the inhibition of NAALADase activity by VA-033C, VA-041Y, and VA-064Y, detailed studies of kinetics of inhibition of NAALADase activity were performed. The reciprocal plots of VA-064Y (Fig. 3) show that both its K_m and V_{max} values are altered at the different concentrations of both substrate and inhibitor, suggesting that VA-064Y and the other phosphonate and phosphinic acid derivatives (see Table 1) are mixed competitive, non-competitive inhibitors of NAALADase activity isolated in the membrane fraction of LNCaP cell lysates.

The effect of VA-033C on cell viability

The effect of VA-033C on the viability of LNCaP cells in culture was analyzed using flow cytometry to monitor changes in cell cycle kinetics and the appearance of apoptotic cells. The histograms of LNCaP cells show no difference in the $<G_0$, G_0/G_1, S, or G_2/M phases of the cell cycle either in the absence (top panel) or the presence

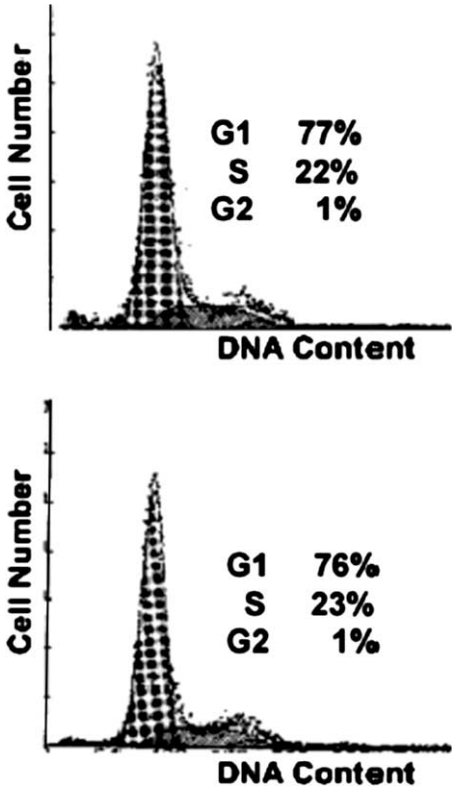


Fig. 4. Flow cytometry analysis of the cytotoxicity of VA-033C on LNCaP cells. (Upper) Control LNCaP cells. (Lower) LNCaP cells grown in the presence of 250 μM VA-033C for 48 h.

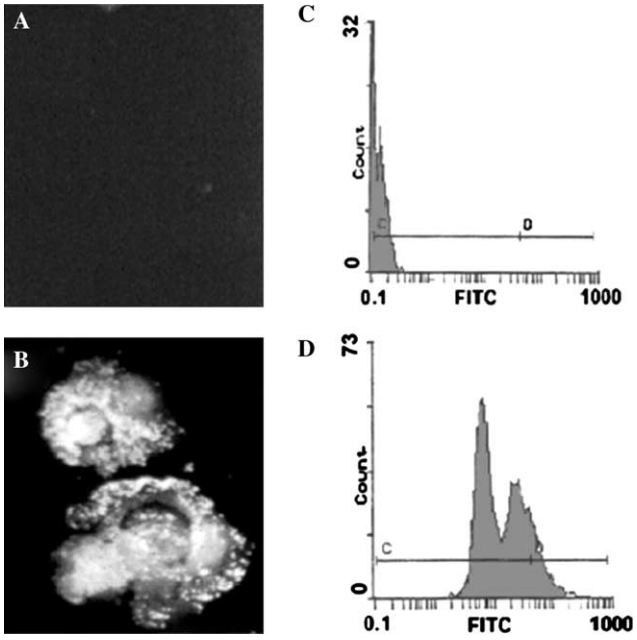


Fig. 5. Analysis of the binding of fluorescent analog to LNCaP cells by fluorescence microscopy (VA-043H, panels A and B) and flow cytometry (VA-041H, panels C and D). (A,C) Control DU-145 cells. (B) LNCaP cells incubated with 100 μM VA-043H for 20 min at 37 °C. (D) LNCaP cells incubated with 100 μM VA-041Y for 20 min at 37 °C.

(bottom panel) of 250 μM VA-033C, indicating that this compound is neither cytostatic nor cytotoxic (Fig. 4). This suggests that VA-033C and the other phosphonate and phosphinic acid derivatives may be excellent reagents for targeting cytotoxic cargoes or imaging reagents to the membrane-bound enzyme in the prostate.

The binding of VA-041Y and VA-043H to LNCaP cell membranes

To determine whether these compounds are bound to and localized on the membrane of LNCaP cells, the fluorescein derivative VA-041Y was incubated with LNCaP and DU-145 cells in vitro, and the binding was analyzed by flow cytometry and by fluorescent microscopy. Fig. 5 shows that incubation of VA-041Y with LNCaP cells for as little as 20 min results in the stable binding of VA-041Y to the membrane of viable LNCaP cells but not to DU-145 cells which do not express the membrane associated form of PSMA, as monitored by fluorescent microscopy (Figs. 5A and B). The stability of the surface binding was further demonstrated by flow cytometry as shown in Figs. 5C and D). The binding to LNCaP cells appears to have two components (Fig. 5D), but without further study it is not possible to determine the biological significance of this observation. However, since this binding is subjected to competition by preincubation with VA-033C (not shown) and is not seen in DU-145 cells (Fig. 5C), it is clear that this fluorescently labeled derivative could be used as a diagnostic marker for prostate derived cells in the circulation.

Discussion

The long term aim of this research is to develop a biologically innocuous targeting compound that can be used to deliver imaging agents for MRI or scintigraphy, and cytotoxic agents, to both localized and metastatic prostate cancers. Since PSMA is expressed on approximately 90% of localized and disseminated prostate cancers, and is expressed on the external membrane of the cell, we have chosen to identify inhibitors of the intrinsic NAALADase activity that can be used as targeting agents.

Prostate specific membrane antigen was first identified as a membrane antigen on LNCaP cells, and the first efforts to utilize PSMA as a target for imaging-using ^{111}In-labeled anti-PSMA monoclonal antibody 7E11.C5 were first reported almost 10 years ago [10]. The utility of ProstaScint for diagnosis of prostate cancer and the identification of occult soft tissue metastases has been demonstrated recently. A number of factors, however, may limit the utility of PSMA as the cellular target for

the identification of micro-metastases. First, it is likely that not all disseminated prostate tumors express the membrane form of PSMA [25,26]. Indeed, several well-established prostate tumor cell lines, including DU-145 and PC-3, do not express PSMA [27]. Second, it is possible that the antibody or other peptide-directed targeting moieties cannot adequately penetrate the microvasculature of the tumor or are rapidly proteolyzed. While any methodology utilizing PSMA as a target will be prone to the same inability to detect tumors that do not express PSMA, it is possible to design innocuous small molecule inhibitors that can carry imaging agents to the tumors which should not be impeded by the microvasculature.

We have identified several inhibitors of the NAALADase activity of the membrane associated PSMA (including VA-033C, VA-41Y, and VA-064Y) that are very potent, having IC_{50} values below 120 nM. These compounds appear to inhibit the enzyme by a mixed competitive non-competitive mechanism, and the most effective of the inhibitors (VA-033C) has a very slow off rate. It is of interest that several of the compounds previously reported to be effective inhibitors against the soluble NAALADase isolated from the brain do not appear to be active inhibitors of the membrane associated isoform of PSMA isolated from the prostate. There are several possible reasons for these observations. First the use of a membrane preparation as the source of the enzyme may alter the kinetics of the reaction used in this assay simply based on the relative hydrophilicity of the inhibitors. While this is an important consideration we have not attempted to compare the relative activities of the inhibitors against those of the membrane and soluble and membrane bound forms of the prostatic enzyme to determine whether the differences in K_m of the soluble and membrane forms of the enzyme and the prostatic protein (PSM′ and PSM, respectively), and the apparent differences in the efficacy of the inhibitors represent a biologically significant difference or represent a difference in the assay conditions used.

As exemplified by VA-041Y, these inhibitors bind to the cell surface of LNCaP in a saturable manner. This binding appears to have two components, but without further study it is not possible to determine the biological significance of this observation. None of the NAALADase inhibitors appear to alter the cell cycle kinetics of LNCaP cells (as exemplified by the lack of effect of VA-033C), suggesting that they are not themselves cytotoxic. In this regard they appear to be ideal targeting agents for siderophores incorporating iron or gadolinium (for imaging purposes) or cytotoxic drugs (for therapeutic purposes), since they do not show any significant association with cells that do not express the membrane associated form of the enzyme. Using combinatorial chemistry it should be possible to modify the active groups of the pharmacophore to alter the solubility and to optimize the localization of these inhibitors to prostatic tumors and to improve the penetration of the imaging agent through the microvasculature.

Acknowledgments

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