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Research Paper

Use of Methotrexate-Based Peptide Substrates to Characterize the Substrate Specificity of Prostate-Specific Membrane Antigen (PSMA)

Annastasiah Mhaka[1]
Alyssa M. Gady[1]
D. Marc Rosen[1]
Kin-Ming Lo[2]
Steven D. Gillies[2]
Samuel R. Denmeade[1]

[1]The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins; The Johns Hopkins School of Medicine; Baltimore, Maryland USA

[2]EMD-Lexigen Research Center; Billerica, Massachusetts USA

*Correspondence to: Samuel R. Denmeade; Bunting Blaustein Cancer Research Building; Rm 1M43; 1650 Orleans; Baltimore, Maryland 21231-1001 USA; Tel.: 410.502.3941; Fax: 410.614.8397; E-mail: denmesa@jhmi.edu

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KEY WORDS

folate hydrolase, methotrexate, NAALADase, peptide, prostate cancer, prostate-specific membrane antigen, substrate

ABBREVIATIONS

PSMA	prostate-specific membrane antigen
NAALADase	N-acetyl α-linked acidic dipeptidase
NAAG	N-acetyl-aspartyl-glutamate
Glu	glutamic acid
Asp	aspartic acid
Asn	asparagine
Gln	glutamine
NAD	b-nicotinamide adenine dinucleotide
APA	4-N[N-2,4diamino-6-pteridinyl-methyl)-N-methylamino-benzoate]
DHFR	dihydrofolate reductase

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NOTE

Throughout this report "-" denotes α-linkage, "*" denotes γ-linkage and "$" denotes β-linkage.

ABSTRACT

Prostate-Specific Membrane Antigen (PSMA) is a glutamate carboxypeptidase II that is highly expressed by both normal and malignant prostate epithelial cells and by the neovasculature of many tumor types but is not expressed by endothelial cells in normal tissue. PSMA possesses the hydrolytic properties of an N-acetylated α-linked acidic dipeptidase (NAALADase) and also functions as a pteroyl poly-γ-glutamyl carboxypeptidase (i.e., folate hydrolase). Therefore, PSMA can be targeted for activation of peptide-based prodrugs within the extracellular fluid of prostate cancers. In this study, methotrexate-based peptide analogs were evaluated to identify PSMA selective substrates that are also stable to nonspecific hydrolysis in human and mouse plasma. These methotrexate analogs were also characterized for in vitro toxicity against PSMA and nonPSMA producing human cancer cell lines. Analogs containing γ-linked glutamate residues were most efficiently hydrolyzed by PSMA, but were unstable in plasma. Analogs containing both α- and γ-linked acidic amino acids were less efficiently hydrolyzed by PSMA but were most stable in plasma. Analogs were 5–10 fold more selectively toxic in vitro in the presence of active PSMA. These studies have identified PSMA selective, plasma stable peptide substrates that can be incorporated into prodrugs targeted for activation by PSMA within prostate cancer sites.

PSMA is a 100 kDa prostate epithelial cell type II transmembrane glycoprotein that was originally isolated from a cDNA library from the androgen responsive LNCaP human prostate cancer cell line.[1] Immunohistochemical studies using monoclonal have demonstrated that PSMA is expressed by normal prostate epithelium and is even more highly expressed by a large proportion of primary and metastatic prostate cancers.[2-4] In these studies, low levels of PSMA protein were detected in the duodenal mucosa and in a subset of proximal renal tubules.[4,5] In all other human tissues, including normal vascular endothelium, PSMA expression was not detectable.[4,5] In these studies, PSMA expression was also undetectable in other nonprostatic primary tumors.[2,4,5] Others studies, however, have demonstrated low level PSMA expression in primary renal and transitional cell carcinomas.[6,7] In addition, PSMA expression, has been demonstrated in the neovasculature of a variety of cancer types including panels of breast, renal, colon and transitional cell carcinomas.[8,9]

The PSMA protein detectable in prostate cancers is an integral membrane protein and therefore has an extracellular domain that is accessible to agents in the extracellular peritumoral fluid making it possible to target this protein with antibodies and prodrugs. A final interesting aspect of PSMA expression is that the PSMA mRNA is upregulated upon androgen withdrawal.[10,11] In LNCaP cells, androgen has been found to downregulate PSMA expression[10] and in patient specimens an increase in immunohistochemically detectable PSMA expression has been observed following androgen ablative therapy.[11] In contrast, PSA expression is downregulated by androgen deprivation.[12,13] Recently, Ross et al. demonstrated that prostate cancer cells expressed relatively increased levels of PSMA compared to benign prostate tissue.[14] In this study, increased PSMA expression correlated with tumor grade, and pathological stage.[14] Most importantly, this study demonstrated for the first time that overexpression of PSMA in primary prostate cancer is a predictive indicator for biochemical recurrence.[14]

The aforementioned studies highlight some of the characteristics of PSMA that make it a suitable target for prostate specific therapy. Functionally, PSMA has been classified as a glutamate carboxypeptidase II.[15] Two discrete enzymatic activities for PSMA have been described. Initially, Carter et al.[16] demonstrated that PSMA possesses the hydrolytic properties of an N-acetylated α-linked acidic dipeptidase (NAALADase). NAALADase is a membrane hydrolase activity that is able to hydrolyze the neuropeptide N-acetyl-*l*-aspartyl-*l*-glutamate (NAAG) to yield the neurotransmitter glutamate and N-acetyl-aspartate.[17,18] NAALADase was originally characterized in the mammalian nervous system.[18,19] Carter et al.[16] performed immunoscreening of a rat brain cDNA expression library using anti-NAALADase antisera

and identified a 1428 base pair partial cDNA that showed 86% sequence homology with a 1428 base pair region of PSMA cDNA.[16] Transfection of a cDNA coding for PSMA isolated from LNCaP into two NAALADase negative cell lines conferred NAAG hydrolyzing activity that was inhibitable by the NAALADase specific inhibitors quisqualic acid, β-NAAG, and (phosphonomethyl)pentanedioic acid (PMPA).[16,20] Tiffany et al. later demonstrated that the kinetics of NAAG hydrolysis are remarkably similar to NAALADase isolated from rat brain.[21]

In addition to the NAALADase activity, PSMA also functions as a pteroyl poly-γ-glutamyl carboxypeptidase (i.e., folate hydrolase).[22] PSMA exhibits exopeptidase activity and is able to progressively hydrolyze γ-glutamyl linkages of both poly-γ-glutamated folates and methotrexate analogs with varying length glutamate chains.[5,22] The role that PSMA's NAALADase or folate hydrolase activity plays in the physiology of normal or malignant prostate cells is presently unknown.

A variety of strategies can be used to target PSMA. Our approach is to take advantage of both the prostate specific expression of the PSMA protein in men and its unique enzymatic activities. Inactive prodrugs consisting of a cytotoxin coupled to a peptide carrier can be designed such that the active cytotoxin is liberated only by the enzymatically active PSMA present on normal and malignant prostate epithelial cells. These prodrugs can be used to target PSMA-positive prostate cancer and could potentially be used to target the vascular endothelial cells of a wide variety of epithelial cancers.

In the present study a number of peptide substrates for PSMA were screened in an attempt to identify a PSMA selective substrate for the NAALADase and/or folate hydrolase activity of PSMA. Putative PSMA substrates were synthesized in which the free carboxyl group of the 4-N[N-2,4diamino-6-pteridinyl-methyl)-N-methylamino-benzoate] (APA) portion of methotrexate was coupled to the N-terminal amine of small peptides composed of predominantly acidic amino acids. These substrates were characterized on the basis of rates of PSMA hydrolysis, stability in human serum and in vitro cytotoxicity against PSMA producing and nonproducing human cancer cell lines.

MATERIALS AND METHODS

Materials. All methotrexate prodrugs, with the exception of poly-γ-glutamated methotrexate were synthesized by California Peptides Inc. (Napa, CA). Synthesis was accomplished by coupling indicated amino acids to 4-N[N-2,4diamino-6-pteridinyl-methyl)-N-methylamino-benzoate] (APA) (Aldrich). Compounds were purified by HPLC (>98% purity). The peptide sequence was confirmed by amino acid analysis and molecular weights were confirmed by mass spectroscopy. Poly-γ-glutamate methotrexate was purchased from Schircks Laboratories (Jona, Switzerland). Di- and tripeptides were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Bachem (King of Prussia, PA).Unless otherwise specified, all other reagents were from Sigma-Aldrich.

Purified PSMA. To perform PSMA enzymatic assays a PSMA fusion protein (Fc-PSMA) was used. The production of the Fc-PSMA vector and characterization of the Fc-PSMA fusion protein have been described previously by Lo et al.[23] Purified Fc-PSMA fusion protein was produced by transfecting cells with a secretion cassette consisting of a leader sequence from a variable region gene of a mouse kappa chain fused to a DNA fragment encoding the hinge, CH2 and CH3 domains of the human immunoglobulin γ-1 gene. The coding sequence for the soluble portion of PSMA (i.e., transmembrane portion deleted) was then ligated in frame. The fusion protein was purified using protein A Sepharose. Fc-PSMA produced and purified in this manner[23] was provided by Lexigen Pharmaceuticals (Billerica, MA).

Cell Lines. The LNCaP androgen responsive human prostate cancer and TSU human bladder cancer cell lines were obtained from ATCC (Rockville, MD) and maintained by serial passage in RPMI 1640 media containing 10% fetal calf serum with 100 units/ml penicillin G, and 100 units/ml streptomycin sulfate (antibiotics from M.A. Bioproducts, Walkerville, MD) as standard media in 5% CO_2/95% air at 37°C. The origins and characteristics of the LNCaP and TSU cell lines have been described previously.[24-26]

PSMA Enzymatic Assay. LNCaP cells (~1–5 x 10^7) were sonicated and membranes isolated after centrifuged at 100,000xg for 10 min at 4°C. Tris buffer was added to bring the concentration of protein to 0.5 mg/ml. The enzymatic activity assay was adapted as previously described by Tiffany et al.[21] Briefly, 5 μg of total protein from LNCaP cells or purified Fc-PSMA were added to PSMA assay buffer (10 mM $CoCl_2$, 50 mM Tris, pH 7.4). Following a 10 min incubation at room temperature, N-acetyl-aspartyl-^{3}H glutamate (^{3}H-NAAG) (NEN, Boston, MA) was added (50 nM final concentration) and reactions incubated for indicated times at room temperature. A column was constructed by placing a 3mm solid glass bead into a 9 inch pasteur pipette and 5 cm of preconditioned AG 1-X8 ion exchange resin (Bio-Rad) added. At the end of the incubation period, an aliquot from each assay was loaded onto the top of the column. The column was washed with 2 ml of 1M formic acid into 10 ml of scintillation cocktail and total counts determined using a Beckman LS 8100 scintillation counter. A standard curve was plotted using increasing concentration of ^{3}H-NAAG in order to convert measured counts to pmoles ^{3}H-glutamate released.

Western Blot. Samples containing 100 ng Fc-PSMA or cellular extracts were separated by denaturing SDS-PAGE using 12% denaturing gel followed by transfer to Hybond-ECL nitrocellulose membranes (Amersham, Arlington, IL) The membranes were processed by standard western blot procedures and incubated with mouse monoclonal antibody J591 hybridoma conditioned media kindly provide by Dr. Neil Bander, Cornell University, NY at a dilution of 1:500. PSMA protein was visualized with the ECL detection system (Amersham, Arlington Heights, IL) according to the manufacturers' specifications.

Determination of Glutamate and Aspartate Production. Hydrolysis of α- and γ-linked di- and tripeptides by PSMA production of free glutamate or aspartate were assayed as follows. Substrates (50 μM) were incubated with 10 μg Fc-PSMA in PSMA assay buffer at room temperature for 3 hrs. For glutamate determination, a commercially available glutamate assay kit was utilized (Sigma) with minor modifications to the manufacturer's instructions. Briefly the reaction mixture (1:1) was added to a buffer consisting of 0.095 M Tris, 1.9mM EDTA, 3.2% Hydrazine, pH 9.0. To this mixture, 50 μl of 30 mM NAD and 5 μl of 100 mM ADP were added and the volume brought to 1 ml with dH2O. After mixing gently, 10 μl of Glutamate Dehydrogenase (GDH) (1200 U/ml) was added and the mixture incubated for an additional 40 minutes at room temperature. In this way free glutamate is converted to α-ketoglutarate with the coupled production of NADH. NADH fluorescence (ex. 340 nm and em. 460 nm) was determined using a fluorometer from Photon Technologies International (PTI) (New Brunswick, NJ) equipped with photomultiplier tube (PTI). A standard curve was plotted using increasing concentrations of glutamate in the GDH/NADH assay in order to convert measured fluorescence to amount of glutamate released.

To measure free aspartate, 70 μl of 1.8 mM α-ketoglutarate and 25 μl of glutamic oxaloacetic transaminase (GOT) were added to the initial reaction mixture (i.e., substrate + Fc-PSMA in PSMA buffer). In this method free aspartate is directly converted to oxaloacetic acid and α-ketoglutarate is converted to glutamic acid. After 3 hr incubation at room temperature, 250 μl of this reaction mixture were assayed for glutamate as described above.

Hydrolysis of Methotrexate Prodrugs. Methotrexate prodrugs at a final concentration of 50 μM were incubated at room temperature in PSMA buffer containing 10 μg total Fc-PSMA. At indicated time, aliquots were analyzed by reverse phase high-pressure liquid chromatography (HPLC). The HPLC system consisted of a dual-pump (Model 126, Beckman Instruments, Columbia, MD) with a manual injection valve (Rheodyne, Cotati, CA) fitted with a 200μl injection loop. A reversed phase C_{18} Ultrasphere analytical column [(Beckman) 15 cm x 4.6 mm (I.D.)] was used together with a 4.5 cm x 4.6 mm (I.D.) Ultrasphere reversed phase guard

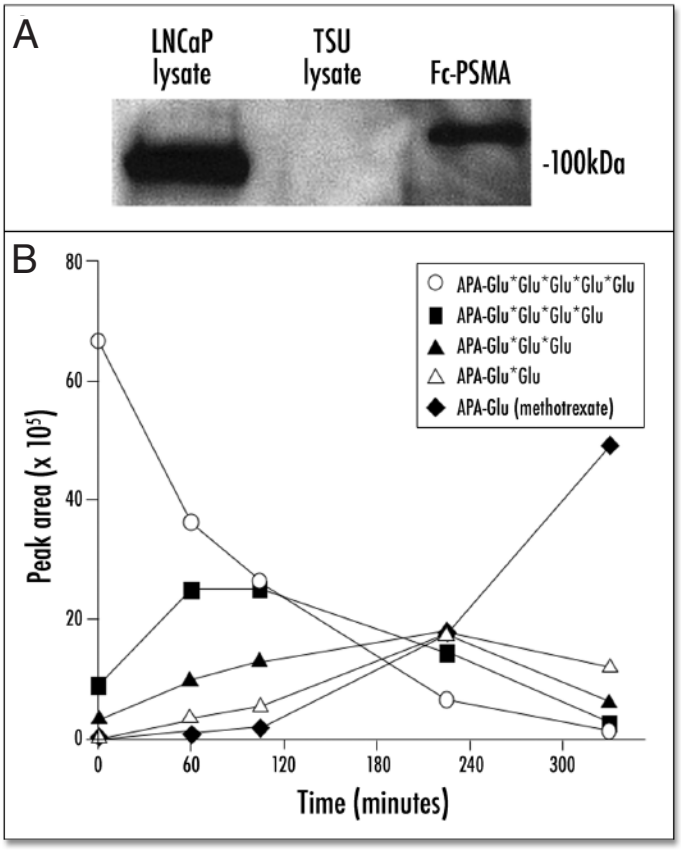

Figure 1. Characterization of Fc-PSMA fusion protein. (A) Western blot analysis of Fc-PSMA compared to cellular extracts from PSMA-positive LNCaP and PSMA-negative TSU human cancer cell lines. PSMA visualized using mouse monoclonal antibody J591 (1:500 dilution). (B) Fc-PSMA is a folate hydrolase that progressively hydrolyzes γ-linked glutamate residues from polyglutamated methotrexate. PSMA (10 μg) incubated with 50 μM polyglutamated methotrexate. Aliquots removed at indicated times and analyzed by HPLC. Identity of peaks confirmed by mass spectroscopy. Peak area represents milliabsorbance units x 10^5. Total peak area remained relatively constant throughout the time course of the experiment.

column (Beckman). A gradient elution was performed consisting of eluent A, 0.1 M sodium acetate, pH 5.1 and eluent B, 50% acetonitrile/0.1 M sodium acetate, pH5.1 with a gradient of 0–50% B over 15 min with a flow rate of 1 ml/min. A diode array detector (Model 168, Beckman) was used to monitor the effluent at 314 nm. All analyses were conducted at ambient temperature. Data processing was performed using Gold Chromatography Data System (Beckman). Purified methotrexate and methotrexate analogs were used as standards and coelution used to confirm production of methotrexate and other hydrolysis products.

Cytotoxicity Assays. Clonal survival of TSU (5 x 10^4 cells) following 48 hr exposure to varying concentrations of methotrexate prodrugs or vehicle control with or without exogenously added PSMA were performed as previously described.[27] Percent inhibition of clonal survival was calculated from the ratio of number of colonies observed in treated group to number of colonies in control group. Inhibition of LNCaP cell growth (1 x 10^5 starting cell number) was determined by counting total cell number after 72 hr exposure to drug or vehicle control. Percent inhibition calculated from ratio of cell number in treated group to cell number in control group. All cytotoxicity assays were performed in triplicate and experiments repeated twice.

RESULTS

Characterization of Enzymatic Activity of Fc-PSMA Fusion Protein. The Fc-PSMA fusion protein was detectable by Western blot analysis using the J591 anti-PSMA mouse monoclonal antibody. As expected, western blot revealed that the Fc-PSMA fusion protein was larger than wild type PSMA isolated from LNCaP cell membranes (Fig. 1A). Purified Fc-PSMA was found to have a specific NAALADase activity of 85.6 ± 5 nmol/min/mg protein. In comparison, PSMA specific NAALADase activity from LNCaP cell membrane preparations was 29.0 ± 2.6 pmol/min/mg protein.[28] Previous studies demonstrated that PSMA can be inhibited by high concentrations of phosphate (10–200 μM).[21] In this study, no significant difference in rates of hydrolysis of ^{3}H-NAAG was observed when Fc-PSMA was incubated in PSMA buffer (i.e., Tris, $CoCl_2$) versus RPMI 1640 (5.63 mM Na_2HPO_4) ± 10% fetal calf serum or 1X phosphate-buffered saline (1.54 mM KH_2PO_4, 2.71 mM Na_2HPO_4). The Fc-PSMA protein was unstable to freezing and thawing and lost >75% activity after one freeze-thaw cycle. Similar to the wild type PSMA, the Fc-PSMA also functioned as an exopeptidase with pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity as demonstrated by its ability to progressively hydrolyze γ-glutamyl linkages of poly-γ-glutamated methotrexate (Fig. 1B). This purified Fc-PSMA was used in all subsequent substrate assays.

Hydrolysis of α-linked Peptides by PSMA. PSMA has been previously demonstrated to function as a NAALADase because of its ability to hydrolyze the neurotransmitter NAAG.[16,21] Previously, Carter et al. described the ability of a series of peptides to competitively inhibit NAAG hydrolysis by purified PSMA. In these studies, hydrolysis of peptides by PSMA was not assayed.[16] In a more recent study, Barinka et al. characterized PSMA hydrolysis of a complete set of N-acetylated dipeptides.[29] As expected, this study identified Ac-Asp-Glu, Ac-Glu-Glu and Ac-Asp-Glu as substrates for PSMA.[29] Additional, albeit less active, substrates included Ac-Asp-Met, Ac-Glu-Met, and Ac-Ala-Met.[29] To more fully delineate the substrate specificity of PSMA, we also analyzed a series of commercially available α-linked acidic dipeptides hydrolysis by Fc-PSMA. Dipeptides consisted of either combinations of the acidic amino acids glutamate and aspartate (i.e., Asp-Asp, Asp-Glu, Glu-Asp, Glu-Glu), amidated derivatives (i.e., Asn-Glu, Gln-Glu, Gln-Gln), or other amino acids combined with glutamate (i.e., Leu-Glu) (Table 1). Hydrolysis of these substrates was determined using coupled enzymatic assays in which free glutamate, released by PSMA hydrolysis, is converted to α-ketoglutarate by glutamate dehydrogenase. Coupled production of NADH can then be measured fluorometrically (ex.340nm and em.460) and correlates directly with glutamate concentration. Amounts of hydrolysis of these dipeptide substrates by Fc-PSMA were compared to hydrolysis of NAAG.

The results of these assays are summarized in Table 1. PSMA was able to hydrolyze most of these dipeptides. Unacetylated Asp-Glu was a better substrate than NAAG. Substitution of the carboxy terminal glutamate with Asp, or Asn did not markedly affect hydrolysis. Asp was the preferred N-terminal amino acid with substitution of Glu, Gln, or Asn resulting in decreased hydrolysis. In contrast, in the study of Barinka et al. N-acetylated Asp-Asp, Asn-Glu, and Gln-Glu were not found to be substrates for PSMA. The difference in hydrolysis between these our study and that of Barinka et al. may be due to difference in N-acetylation state of the peptides in the two studies. In our study, no hydrolysis was detectable when the N-terminus was the hydrophobic amino acid leucine, when both residues (i.e., Gln-Gln) were amidated or when *D*-amino acids where used. Interestingly, minimal hydrolysis of the α-linked tripeptides, Glu-Glu-Glu, Asp-Asp-Asp or Glu-Glu-Asp was observed. In comparison a small amount of hydrolysis of a γ-linked glutamate tripeptide occurred.

These results suggest that PSMA prefers acidic α-linked dipeptides with aspartate preferred at the amino terminus. Acetylation of the amino-terminus was not required and resulted in decreased activity. Substitution of glutamate with aspartate or amidated amino acids Asn and Gln did not markedly affect hydrolysis suggesting that a free gamma carboxyl is not critical for substrate hydrolysis. Longer α-linked tripeptides were not detectably hydrolyzed by PSMA, perhaps due to inability to enter the catalytic site in the correct orientation. γ-linked tripeptides, however, were readily hydrolyzed by PSMA.

Table 1 **PSMA Hydrolysis of Di- and Tripeptides**

Peptide Hydrolysis	Percent
N-Acetyl-Asp-Glu	41
D-Glu-*D*-Glu	0
Gln-Gln	0
Leu-Glu	0
Gln-Glu	53
Glu-Asp	72
Glu-Glu	84
Asn-Glu	57
Asp-Glu	78
Asp-Asp	89
Glu-Glu-Asp	0
Glu-Glu-Glu	5
Asp-Asp-Asp	22
Glu*Glu*Glu	85

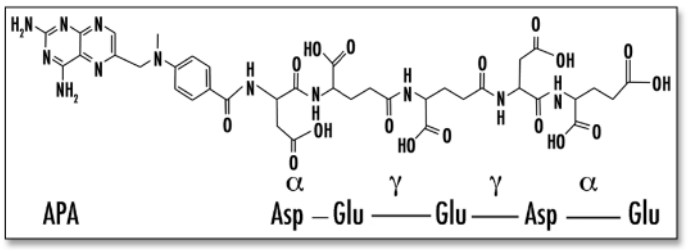

Figure 2. Chemical structure of 4-N[N-2,4diamino-6-pteridinyl-methyl)-N-methylamino-benzoate] (APA) and representative peptide depicting both α- and γ-linked amino acids.

Characterization of PSMA-Hydrolysis of α- and γ-linked Methotrexate-based Substrates. One approach to targeting PSMA is to design prodrugs that can be activated by the NAALADase activity of PSMA. In order to be effective, the prodrug must not possess significant cytotoxicity until activated by PSMA and the prodrug must be readily hydrolyzed by PSMA. Due to its inherent exopeptidase activity, PSMA activated prodrugs must be designed in such a way that the cytotoxin is inactivated by coupling to the N-terminal amino acid of a peptide. This is in contrast to a similar strategy targeting an endopeptidase such as prostate-specific antigen (PSA) in which the cytotoxin is coupled to the C-terminal carboxyl group of the carrier peptide.[27,30]

Therefore, in order to identify a PSMA selective substrate, a series of substrates were synthesized in which small peptides of 2-5 amino acids were coupled to the APA portion of methotrexate (Fig. 2). This strategy was utilized for several reasons. Previously it has been demonstrated that the coupling of APA to the N-terminal amine of γ-linked polyglutamates did not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described[31] and large quantities of the inexpensive APA precursor are commercially available (Sigma Aldrich). The APA molecule has an absorbance maximum at 310 nm and therefore hydrolysis of prodrugs can be readily followed by HPLC analysis. Finally, methotrexate is a cytotoxic agent. Therefore, these methotrexate-based substrates can also be tested as for their potential as prodrugs in vitro cytotoxicity assays.

In order to determine if similar degree of hydrolysis could be observed when the α-linked dipeptide substrates identified above were coupled to APA, a series of α-linked dipeptide methotrexate analogs were synthesized and assayed for hydrolysis by PSMA. On the basis of the results of PSMA hydrolysis of dipeptides from Table 1, analogs consisting of methotrexate coupled to either a second Glu residue, Asp or Gln were synthesized (i.e., APA-Glu-Glu, APA-Glu-Asp, APA-Glu-Gln). Other analogs were also synthesized in which Asp, Asn or Leu was substituted for the glutamate moiety of methotrexate (i.e., APA-Asp-Glu, APA-Asn-Glu, APA-Leu-Glu). These analogs were then assayed for Fc-PSMA hydrolysis. From this series, the only α-linked dipeptide methotrexate analog that was significantly hydrolyzed by PSMA was APA-Asp-Glu. After 24 hrs incubation with Fc-PSMA ~70% of this substrate was hydrolyzed to APA-Asp and 99% hydrolysis is observed after 48 hrs (Table 2). APA-Glu-Glu was minimally hydrolyzed after 48 hrs incubation (Table 2). No appreciable hydrolysis of the other α-linked dipeptide substrates was observed following incubation with PSMA (Table 2).

A second approach to targeting PSMA is to design prodrugs that can be activated by the pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity PSMA. Previously it has been demonstrated that PSMA is able to progressively hydrolyze γ-glutamyl linkages of both poly-γ glutamated folates and poly-γ glutamated methotrexate analogs with varying length glutamate chains.[22] These polyglutamated analogs, however, can also be readily hydrolyzed by gamma glutamyl hydrolase, a lysosomal enzyme.[32,33] Gamma glutamyl hydrolase is also secreted by hepatocytes and by a variety of tumor cell types.[32] Therefore, an ideal PSMA-prodrug would be specifically hydrolyzed by PSMA with minimal to no hydrolysis by the ubiquitous gamma glutamyl hydrolase.

The first step toward accomplishing this goal is to more clearly define substrate specific for the pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity of PSMA. Therefore, a series of γ-linked methotrexate analogs were synthesized and assayed for hydrolysis by PSMA (Table 3). Initially, γ-linked dipeptide analogs were screened (Table 3). Jodrell et al. had previously characterized the serum stability of a series of dipeptide methotrexate analogs to determine hydrolysis by gamma glutamyl hydrolase.[35] This group demonstrated that the analog APA-Glu*Asp was partially hydrolyzed in mouse plasma and in mouse liver after in vivo administration. In contrast, APA-Glu**D*-Glu and APA-Glu*GABA (GABA; gamma amino butyric acid) were stable to hydrolysis in mouse serum and by mouse kidney and liver. These analogs, therefore, do not appear to be substrates for gamma glutamyl hydrolase. These APA-Glu**D*-Glu and APA-Glu*GABA analogs were synthesized and tested for hydrolysis by PSMA. After 48 hr incubation no detectable hydrolysis of these analogs was observed (Table 2). In contrast, two other γ-linked dipeptide analogs, APA-Glu*Asp and APA-Glu*Gln were readily hydrolyzed by PSMA (Table 2).

The PSMA peptide substrates are intended to function as prodrug carriers that can both better solubilize hydrophobic cytotoxic agents as well as prevent nonspecific uptake of drug into cells due to presence of multiple charged amino acids. On this basis, additional analogs were synthesized consisting of APA coupled to a series of pentapeptides. The analogs APA-Glu*Glu*Glu*Glu*Asp and APA-Glu*Glu*Glu*Glu*Gln, in which the terminal glutamate is replaced by γ-linked Asp or Gln were both sequentially hydrolyzed by PSMA (Table 2). These analogs were as efficiently hydrolyzed as APA-Glu*Glu*Glu*Glu*Glu, the polyglutamated methotrexate analog with similar γ-glutamyl chain length (Table 2).

These results with both the di- and pentapeptide substrates demonstrate that PSMA does not require a C-terminal γ-linked glutamate for hydrolysis as evidenced by its ability to hydrolyze C-terminal, γ-linked aspartate nor does it require a C-terminal dicarboxylic acid as evidenced by its ability to hydrolyze C-terminal γ-linked glutamine. In addition, although β-NAAG in which the glutamate is coupled to aspartate via a β-linkage has been reported to inhibit PSMA, these results suggest that PSMA can also hydrolyze peptides containing β-linked Asp$Glu.

Plasma Stability of α- and γ-Linked Methotrexate Analogs. It has been previously demonstrated that poly-γ-glutamate peptides as well as poly-γ-glutamated folate and methotrexate (APA- Glu*Glu*Glu*Glu*Glu) can be readily hydrolyzed by purified γ-glutamyl hydrolase.[32,34] γ-Glutamyl hydrolase is a cysteine protease found predominantly in the lysosomes of most cell types.[32] However, γ-glutamyl hydrolase is also secreted by a variety of transformed cell lines as well as some normal cell types, particularly hepatocytes.[32] γ-Glutamyl hydrolase activity is also present within human and

Table 2 **Hydrolysis of Substrates by Purified PSMA and Stability in Plasma**

	% PSMA[2] Hydrolysis		18 h Incubation Human Plasma		18 h Incubation Mouse Plasma
Substrate (50 μM)[1]	24 h	48 h	% APA Glu or Asp[3]	%Prodrug Remaining[4]	% APA Glu or Asp[5]
α-linked					
APA-Asp-Glu	70	99	1	99	1
APA-Glu-Glu	0	20	6	-	-
γ-linked					
APA-Glu*Asp	38	85	-	-	-
APA-Glu*Gln	48	95	-	-	-
APA-Glu**D*-Glu	0	0	-	-	-
APA-Glu*GABA	0	0	-	-	-
APA-Glu*Asp$Glu	38	86	-	-	-
APA-Glu*Glu*Glu*Glu*Asp	86	100	9	20	72
APA-Glu*Glu*Glu*Glu*Gln	92	100	6	26	62
APA-Glu*Glu*Glu*Glu*Glu	96	100	11	11	78

[1]α-linkage denoted by hyphen (-), γ-linkage denoted by star (*), β-linkage by S; [2]% complete hydrolysis to APA Asp or APA Glu by purified PSMA (10 μg total/assay); [3]Denotes % of starting material completely hydrolyzed to -APA Asp or APA Glu; [4]Peak area of starting material/total peak area (total peak area= starting material + intermediate peaks + product); [5]No intermediate peaks seen in mouse plasma, only starting material and APA Asp or APA Glu; [6]Dash denotes hydrolysis not assayed

rodent plasma.[32,33] In addition, Gingras et al. characterized a human blood plasma glutamate carboxypeptidase (PGCP) that has significant sequence homology to PSMA and glutamate carboxypeptidase activity.[36] Therefore, because PSMA-activated peptide prodrugs will be administered systemically via the blood, it is important to determine their stability to hydrolysis by other carboxypeptidases present in plasma. To assay for hydrolysis by γ-glutamyl hydrolase and other serum carboxypeptidases like PGCP, the γ-linked pentapeptide APA analogs were incubated in human and mouse plasma and hydrolysis determined by HPLC analysis after 18 h incubation (Table 2). For each of the γ-linked analogs tested, >75% of the starting material was hydrolyzed to methotrexate or to other intermediate length species, consistent with sequential hydrolysis by the exopeptidase activity of γ-glutamyl hydrolase present in human serum (Table 2). In mouse plasma, ~ 60–75% conversion of each analog directly to APA-Glu (i.e., methotrexate) was observed after 18 h (Table 2). This finding is consistent with previous reports that γ-glutamyl hydrolase found in rodent sera possesses both exo- and endopeptidase activity.[32] To confirm that hydrolysis in plasma was due to γ-glutamyl hydrolase, p-hydroxymercuribenzoate, a nonspecific inhibitor of γ-glutamyl hydrolase and other cysteine proteases,[34] completely inhibited hydrolysis when added to either human or mouse plasma (data not shown).

To date, no studies have documented whether γ-glutamyl hydrolase, like PSMA, is able to hydrolyze α-linked acidic peptides.[34] To characterize the stability of α-linked analogs, the only α-linked analog that was hydrolyzed by PSMA, APA-Asp-Glu, was tested for stability in human serum (Table 2). After 18 hr incubation in human serum, HPLC analysis demonstrated no significant hydrolysis, confirming the inability of serum γ-glutamyl hydrolase and other serum carboxypeptidases like PGCP to cleave α-linked acidic peptides (Table 2).

Characterization of Analogs Containing Both α- and γ-Linked Amino Acids. The above results suggest that, while γ-linked acidic amino acid-based peptides are readily hydrolyzed by PSMA, these peptides are not stable to hydrolysis in human or mouse plasma. In contrast, although PSMA has limited ability to cleave α-linked peptides coupled to APA, this α-linkage is stable to hydrolysis in plasma. On the basis of these results, several APA-analogs were designed and synthesized that incorporated both α- and γ-linked peptides to take advantage of PSMA's ability to hydrolyze both types of linkages (Table 3). These analogs as a group were not hydrolyzed as efficiently as the analogs containing only γ-linked amino acids. However, these analogs were much more stable to hydrolysis in human and mouse plasma (Table 3). The best of this group in terms of both PSMA hydrolysis and plasma stability was the analog APA-Asp-Glu*Glu*Asp-Glu containing two central γ-linked amino acids flanked by α-linked amino acids at the N- and C-terminus. This analog was completely stable in plasma as evidenced by no detectable hydrolysis to product or any intermediate after 18 hr incubation in human plasma.

Cytotoxicity of α- and γ-Linked Methotrexate Analogs. While the goal of this study was to use these peptide-based methotrexate analogs to characterize the substrate specificity of PSMA, these analogs, due to the cytotoxicity of methotrexate, can also be used to determine if a therapeutic index between PSMA-producing and nonproducing cells can be achieved. Therefore, each of the α- and γ-linked methotrexate analogs were tested for cytotoxicity against PSMA non producing TSU cells using clonal survival assays. All of the analogs tested, with the exception of APA-Glu-Asp and APA-Asn-Glu and APA-Asp-Glu*Glu*Asp-Glu inhibited clonal survival by >95% at a concentration of 10 μM (data not shown). Although IC_{50} values were not determined, most of the analogs inhibited clonal survival by ≥ 50% of control clonal survival at a concentration of 1 μM (Table 4). The most potent cytotoxic analogs were APA-Glu* Gln, APA-Glu*Glu*Glu*Glu (polyglutamated

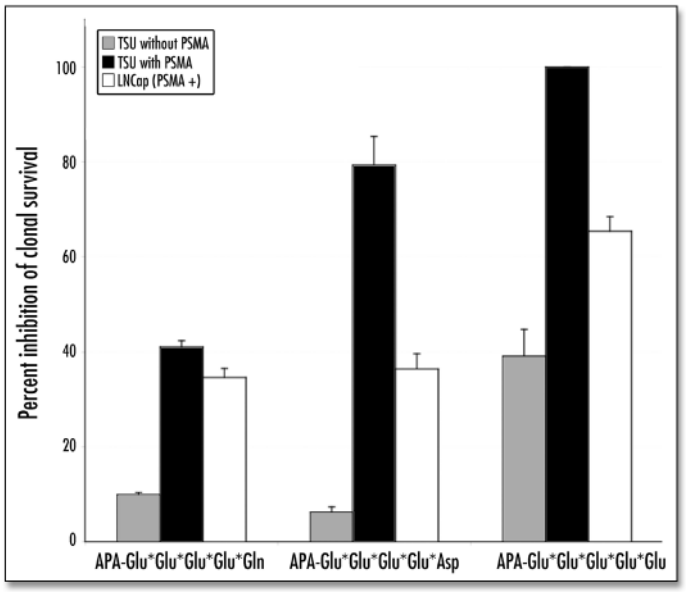

Figure 3. Inhibition of clonal survival of human cancer cell lines by γ-linked methotrexate analogs. Cells were incubated with 100 nM concentration of analogs for 72 hours. TSU cells were incubated in standard media ± Fc-PSMA (8 μg total/well). LNCaP (PSMA positive) incubated in standard media only. Data presented represent average (n = 5) percent inhibition compared to control cells treated with vehicle only (0.3% DMSO) ± standard error. Each experiment performed in duplicate.

Table 3 **HYDROLYSIS OF α- AND γ-LINKED PSMA-SUBSTRATES BY PURIFIED PSMA AND STABILITY IN PLASMA**

Substrate (50 μM)[1]	% PSMA[2] Hydrolysis 24h	% PSMA[2] Hydrolysis 48h	18 h Incubation Human Plasma % APA-Glu or Asp[3]	18 h Incubation Human Plasma % Prodrug Remaining[4]	18 h Incubation Mouse Plasma % APA-Glu or Asp[5]
α- and γ-linked					
APA-Glu*Glu*Glu-Glu*Glu	1	5	-	-	-
APA-Glu*Glu*Glu*Asp-Glu	30	65	23	57	5
APA-Glu*Glu*Glu*Asp-Gln	5	10	13	76	-
APA-Asp-Glu*Glu*Asp-Glu	36	77	0	100	2

[1]α-linkage denoted by hyphen (-) and γ-linkage denoted by star(*); [2]% complete hydrolysis to APA-Asp or APA-Glu by purified PSMA (10 μg/assay); [3]Denotes % of starting material completely hydrolyzed to APA-Asp or APA-Glu; [4]Peak area of starting material/total peak area (total peak area = starting material + intermediate peaks + product); [5]No intermediate peaks seen in mouse plasma, only starting material and APA-Asp or APA-Glu

Table 4 **CYTOTOXICITY OF ANALOGS AGAINST NON-PSMA PRODUCING TSU CELLS**

Analog	% Inhibition of Clonal Survival[1,2] 1 μM	0.1 μM
APA-Asp	58 ± 3	0[3]
APA-Glu	97 ± 1	88 ± 2
α-linked		
APA-Asp-Glu	65 ± 2	5 ± 4
γ-linked		
APA-Glu*Asp	37 ± 5	22 ± 8
APA-Glu*Gln	93 ± 1	21 ± 5
APA-Glu*Asp$Glu	47 ± 2	41 ± 2
APA-Glu*Glu*Glu*Glu*Glu	88 ± 6	43 ± 4
APA-Glu*Glu*Glu*Glu*Gln	50 ± 6	0
APA-Glu*Glu*Glu*Glu*Asp	56 ± 5	0
α and γ-linked		
APA-Glu*Glu*Glu-Glu*Glu	50 ± 20	21 ± 14
APA-Glu*Glu*Glu*Asp-Glu	15 ± 5	0
APA-Glu*Glu*Glu*Asp-Gln	65 ± 2	10 ± 10
APA-Asp-Glu*Glu*Asp-Glu	6 ± 8	0

[1]TSU cells (5×10^4) exposed to analog for 48 hrs; [2]% Inhibition of Clonal Survival= (1-(# Colonies in treated group/# control)) * 100; [3]Zero percent inhibition indicates average number colonies at this concentration is ≥ to control colony number; Results presented as average % inhibition ± standard error (n = 6)

methotrexate) (Table 4), and APA-Glu*GABA (data not shown). In contrast, the least potent analogs were those containing both α- and γ- linked amino acids, APA-Glu*Glu*Glu*Asp-Glu and APA-Asp-Glu*Glu*Asp-Glu (Table 4).

The ultimate hydrolysis product of each of these analogs is either APA-Glu (i.e., methotrexate) or APA-Asp. APA-Glu (methotrexate) is a potent cytotoxin that inhibited clonal survival of TSU cells by ~90% at a concentration of 0.1 μM. In contrast, the APA-Asp analog was far less cytotoxic and showed no inhibition of survival at this concentration (Table 4). Having defined the cytotoxicity of these analogs against a PSMA negative cell line, the next step was to determine if the cytotoxicity of these analogs could be potentiated in the presence of enzymatically active PSMA. To accomplish this, PSMA nonproducing TSU cells were exposed to 100 nM of three γ-linked analogs in media containing either vehicle or purified Fc-PSMA. The cytotoxicity of these analogs ± PSMA was then compared to PSMA-producing LNCaP cells (Fig. 3). In this assay the cytotoxicity of each analog against TSU cells was greatly enhanced in the presence of enzymatically active PSMA (Fig. 3). A more marked potentiation of cytotoxicity was observed in TSU cells grown in PSMA containing media compared to PSMA producing cells LNCaP cells (Fig. 3). This enhanced effect most likely was due to the fact that higher concentration of exogenous PSMA was added to TSU media then are present in membranes of LNCaP cells (Fig. 3).

At a concentration of 0.1 μM APA-Glu*Glu*Glu*Glu*Glu completely inhibited clonogenic growth in the presence of PSMA while <50% inhibition of clonogenic survival was seen in the absence of PSMA (Fig. 3). APA-Glu*Glu*Glu*Glu*Gln was less active at 0.1 μM then APA-Glu*Glu*Glu*Glu*Glu in the absence of PSMA (Fig. 3). The calculated dose of APA-Glu*Glu*Glu*Glu*Gln that produced 50% inhibition of growth (i.e., LD_{50}) in the absence of PSMA was 1.0 μM while the LD_{50} in the presence of PSMA was 0.12 μM. Thus, in the presence of PSMA the cytotoxicity of APA-Glu*Glu*Glu*Glu*Gln increases ~8 fold. APA-Glu*Glu*Glu*Glu*Asp was the least active of the three analogs at 0.1 μM in the absence of PSMA. However the calculated LD_{50} of APA-Glu* Glu*Glu*Glu*Asp in the absence of PSMA was 1.1 μM while the LD_{50} in the presence of PSMA was 0.10 μM. Thus, in the presence of PSMA the cytotoxicity of APAGlu*Glu*Glu*Glu*Asp increases ~12 fold.

To determine the extent of nonspecific hydrolysis, each γ-linked analogs was incubated for 48 hrs in conditioned media from TSU cells (i.e., 5 days conditioning) and then analyzed by HPLC. No significant hydrolysis of APA-Glu*Glu*Glu*Glu*Glu, APA-Glu*Glu*Glu*Glu*Gln, or APA- Glu* Glu*Glu*Glu*Asp was observed (data not shown). These results suggest that cytotoxicity of the analogs in the absence of PSMA is not due to nonspecific hydrolysis of analogs in serum containing media, but rather, may be due to nonspecific uptake of intact (i.e., unhydrolyzed) analogs possibly via folate receptor mediated mechanisms.

As stated, the analogs containing both α- and γ-linked analogs were the least effective against nonPSMA producing TSU cells. When PSMA was added to the media of TSU cells, some potentiation of effect was observed at 10 μM concentration for APA-Asp-Glu*Glu*Asp-Glu and 1 μM concentration for APA-Glu*Glu*Glu*Asp-Glu (Fig.4). Unlike the γ-linked analogs described above, no potentiation of cytotoxicity was observed at 0.1 μM concentrations of either drug in the presence of PSMA. This decreased potentiation in the presence of PSMA may reflect both decreased rate of PSMA hydrolysis of α-linked amino acids in both analogs in addition to decreased cytotoxicity of the APA-Asp cleavage product compared to APA-Glu for the APA-Asp-Glu*Glu* Asp-Glu analog.

DISCUSSION

A variety of treatment approaches are under development that take advantage of the relatively prostate-restricted expression of PSMA. These strategies include monoclonal antibody-cytotoxin conjugates, PSMA vaccines, and gene therapies. In the present study, peptide substrates have been characterized in terms of PSMA hydrolysis, plasma stability, and nonspecific cytotoxicity that could form the basis for prodrugs targeted to the enzymatic activity of PSMA. These studies demonstrated that γ-linked amino acids substrates are more readily hydrolyzed by PSMA as compared to α-linked substrates. These γ-linked substrates are less stable to nonspecific hydrolysis in

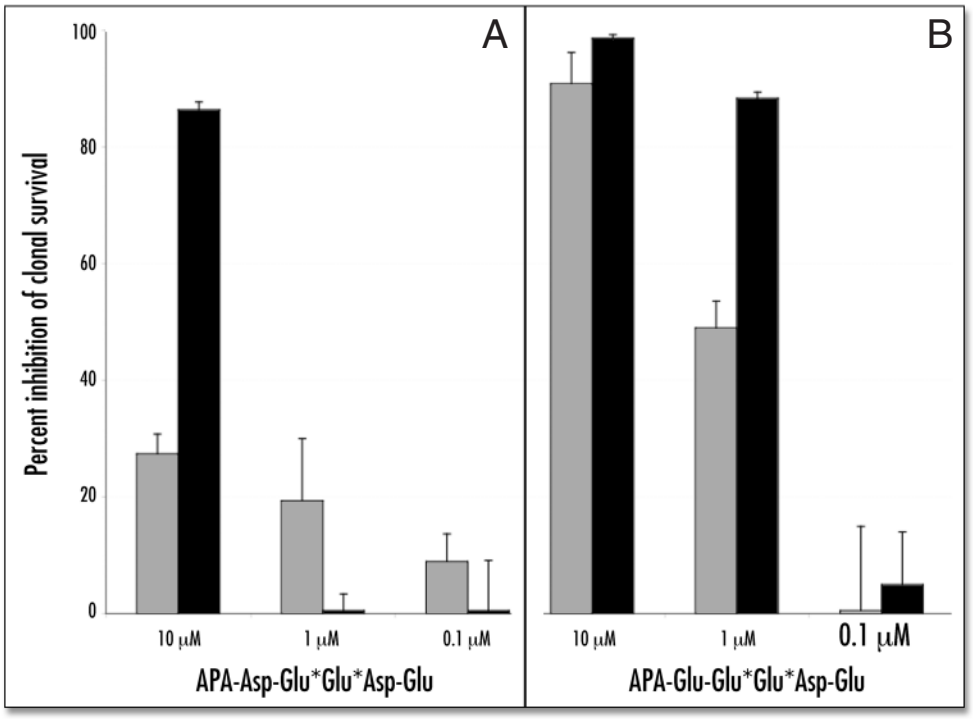

Figure 4. Inhibition of clonal survival of human TSU cancer cell lines by α- and γ-linked methotrexate analogs. Cells were incubated with indicated concentration of analogs for 72 hours. Shaded bars represent TSU cells in treated in standard media. Black bars indicate TSU cells treated in standard media + Fc-PSMA (8 μg/well). Data presented represent average (n = 5) percent inhibition compared to control cells treated with vehicle only (0.3% DMSO) ± standard error. Each experiment performed in duplicate.

human plasma. Substrates containing both α- and γ-linked amino acids, while less efficiently hydrolyzed completely by PSMA, are more stable to hydrolysis in plasma and may therefore represent best carriers for PSMA-based prodrug strategies.

Methotrexate has been evaluated as treatment for metastatic prostate cancer either as a single agent or in combination with other agents.[37] The results of the studies using methotrexate as a single agent have demonstrated response rate of <10%. Resistance to methotrexate occurs frequently and can be secondary to cellular alterations in uptake, polyglutamation, or amplification of DHFR gene expression.[38] Methotrexate, therefore, does not represent the ideal agent for prodrug development as treatment of prostate cancer. In this study, methotrexate was chosen as the cytotoxic agent because poly-γ-glutamated methotrexate was already known to be a substrate for PSMA. Using methotrexate made it possible to study the effect of peptide sequence changes on the hydrolytic activity of PSMA and compare it to a known PSMA substrate.

Folates and antifolates enter cells through two major mechanisms; the reduced folate carrier (RFC), and via membrane folate binding proteins (mFBP) also called folate receptors (i.e., FR-α, FR-β).[39,40] The folate-dependent enzyme inhibitors have differing affinities for each of these transport mechanisms. Different cell types can also utilize one transport mechanism more than another but often use both. Little data exist as to specific folate transport mechanisms in prostate cancer cells, although Holm et al.[41] have demonstrated a high affinity binding protein in normal prostate tissue and Horn et al.[42] demonstrated carrier mediated uptake of methotrexate and folate in human PC-3 prostate cancer cells. In addition the affinities of the RFC and mFBP for longer chain poly-γ-glutamated species like polyglutamated methotrexate has not been well-studied. However, folates have been coupled to proteins, DNA, imaging agents and incorporated into liposomes and dendrimers to facilitate entry into cells via the folate transporters.[43-47]

In this study, each of the longer chain γ-linked analogs (i.e., APA-Glu* Glu*Glu*Glu*Glu, APA-Glu*Glu*Glu* Glu*Gln, or APA-Glu*Glu*Glu* Glu*Asp) are potent cytotoxins at doses of 1–10 μM. These analogs were not significantly hydrolyzed to APA-Glu (methotrexate) in conditioned media from TSU cells. This suggests that these analogs are transported into the TSU cells via one of the folate transport mechanisms. The folate transporters appear to specifically recognize the pteridine moiety of folate and antifolates. Therefore, the longer chain analogs can enter the TSU cells via a folate transporter. Once in the cell, the polyglutamated forms of methotrexate and other antifolates have been demonstrated to be more potent at inhibiting folate-dependent enzymes like DHFR then the monoglutamated species.[38]

Due to this folate-specific uptake that is independent of PSMA hydrolysis, prodrugs containing methotrexate or other antifolates might not be the preferred agents for targeting PSMA activation. Coupling acidic amino acid containing peptides to other cytotoxic agents that do not contain the pteridine ring present in antifolates might represent a better approach to PSMA-based prodrug therapies. The addition of these acidic amino acids would produce a charged prodrug that should not easily cross the plasma membrane without PSMA hydrolysis of the amino acids. These prodrugs would also not be recognized by the folate transporters. Because PSMA is an exopeptidase, hydrolytic processing of any prodrug will result in an end product consisting of a cytotoxin coupled to the acidic amino acids glutamate or aspartate. Therefore, while a variety of agents could be potentially targeted using this prodrug approach, preferred agents would be those that incorporate glutamic or aspartic acid into their structure and still maintain their cytotoxicity.

Current studies are underway in our laboratory to identify analogs of cytotoxic agents that maintain potent cytotoxicity after incorporation of aspartate or glutamate into their chemical structures. These analogs can then be coupled to the α- and γ-linked PSMA peptide substrates characterized in this present study to generate prodrugs that are stable to hydrolysis when administered systemically via the blood and which only become converted to active cytotoxic agents in the presence of PSMA.

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