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PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205-2196

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6. AUTHOR(S)
Samuel R. Denmeade, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205-2196

E-Mail: denmesa@jhmi.edu

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13. ABSTRACT (Maximum 200 Words)

The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. The hypothesis is that a potent TG analog can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells thus minimizing toxicity to normal tissue. Two enzymatic activities for PSMA have been described: an N-acetyl-α linked acid dipeptidase (NAALADase) activity and a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity. On the basis of preliminary data, the ideal TG prodrug should consist of either an aspartate or glutamate containing TG analog coupled via to a peptide containing a series of α- and γ-linked glutamates and ending in an α-linked aspartyl-glutamate "cap". This substrate would be readily cleaved by PSMA but would be stable to hydrolysis by proteases such as gamma-glutamyl hydrolase present in serum and extracellular fluid of some normal tissue types.

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prostate cancer, prodrug, prostate specific membrane antigen, thapsigargin, drug delivery

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INTRODUCTION:

Currently there is no treatment that significantly prolongs survival in men with metastatic prostate cancer (5). Androgen ablation therapy, while of substantial palliative benefit, has had little impact on overall survival and the mortality rate from prostate cancer has steadily increased since the introduction of this therapy in the 1940's (1). Androgen ablation therapy eventually fails because the metastatic prostate cancer within an individual patient is heterogeneously composed of clones of both androgen dependent and independent cancer cells (6). Due to these androgen independent prostatic cancer cells, the patient is no longer curable using the numerous chemotherapeutic agents that have been tested over the past 30 years (5).

Standard antiproliferative chemotherapeutic agents may be ineffective against androgen independent prostatic cancers because these cancers have a low proliferative rate. Previously we have demonstrated that the median daily proliferative rate of prostate cancer cells within lymph node or bone metastases is <5.0% per day (7). Newer agents are needed that target the >95% of prostate cancer cells within a given metastatic site that are not immediately proliferating. The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. Previously we have identified a natural plant product, thapsigargin, that is able to induce programmed cell death in proliferatively quiescent (i.e. G_0) human androgen independent prostate cancer cells without recruitment into the proliferatively cell cycle (8-9). Because most cells within the human body are also in a quiescent G_0 state, thapsigargin would be difficult to administer systemically without significant toxicity. In this proposal we outline a strategy to develop an inactive prodrug form of thapsigargin that can be activated by the enzymatic activity of a unique protein, prostate specific membrane antigen (10), which is a membrane bound enzyme produced in highest levels by androgen independent metastatic prostate cancer cells (reviewed in 11). The relevance of this innovative approach is that it combines a new type of proliferation independent therapy with a new method of prostate-specific drug delivery and should yield new effective therapy for metastatic prostate cancer.

We have chemically modified thapsigargin to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. Like thapsigargin, these TG-analogs are not prostate cancer-specific cytotoxins. Therefore, the hypothesis of this proposal is that these potent TG analogs can be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). In this way the inactive prodrugs can be efficiently converted back to active killing drugs only by the enzymatic activity of PSMA. Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells.

Two enzymatic activities for PSMA have been described: an N-acetyl-a linked acid dipeptidase (NAALADase) activity and a pteroyl poly-g-glutamyl carboxypeptidase (folate hydrolase) activity. On the basis of preliminary data, the ideal TG prodrug should consist of either an aspartate or glutamate containing TG analog coupled via to a peptide containing a series of a- and g-linked glutamates and ending in an a-linked aspartyl-glutamate "cap". This substrate would be readily cleaved by PSMA but would be stable to hydrolysis by gamma-glutamyl hydrolase (GGH) present in serum and extracellular fluid of some normal tissue types.

Therefore, the specific aims of the study are: (1) to synthesize a series of aspartate or glutamate containing TG analog and characterize their cytotoxicity against human prostate cancer cell lines (2) to synthesize prodrugs by chemically linking the aspartyl and glutamyl containing TG analogs to peptide substrates that require hydrolysis by both the NAALADase activity and folate hydrolase of PSMA. These prodrugs will be assayed for in vitro cytotoxicity against PSMA-producing and non-producing cell lines to determine which of these prodrugs is the best lead compound for future in vivo animal studies; (3) to determine the in vivo efficacy and toxicity of the best (i.e. lead) PSMA prodrug (based upon comparison of the in vitro potency and specificity) against PSMA-producing human prostate cancer xenografts; (4) to develop a molecular model of the PSMA catalytic site and compare binding of peptide substrates tested to date in order to predict optimal PSMA peptide substrate.

BODY:

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 (Horoszewicz). Using a series of monoclonal antibodies, several groups have characterized PSMA expression in various normal and tumor tissues. These studies demonstrated that PSMA is expressed in high levels by both normal and malignant prostate tissue. Low-level expression was only seen in the lumen of the small intestine and the proximal tubules of the kidney. In addition, while normal vascular endothelial cells are PSMA negative, endothelial cells of the tumor vasculature stain positive for PSMA in a large number of tumor types, while the tumor cells themselves do not express PSMA.

In summary, the aforementioned studies highlight the characteristics of PSMA that make it a suitable target for prostate specific therapy. PSMA expression is highly restricted to prostate tissue with strongest expression in both primary and metastatic prostate cancers. 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. In LNCaP cells androgen has been found to downregulate PSMA expression and in patient specimens an increase in immunohistochemically detectable PSMA expression has been observed following androgen ablative therapy. In contrast, PSA expression is downregulated by androgen deprivation. Therefore, PSMA should be readily targetable in the majority of hormone refractory patients because PSMA levels are expected to remain high following androgen ablation.

Two discrete enzymatic functions for PSMA have been described. Initially, Carter et al 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. In addition to the NAALADase activity, PSMA also functions as a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase). 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. In our proposal, we outline an approach that would take advantage of both the prostate specific expression of the PSMA protein in men and its unique NAALADase and folate hydrolase activities.

A successful PSMA activated prodrug must pass three critical tests. The prodrug must be cleaved by PSMA, it must not be toxic to PSMA-negative cells and its toxicity to PSMA positive cells must be secondary to activation specifically by PSMA. In order to rapidly screen a larger number of prodrugs for PSMA activity and specificity, we previously synthesized a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides. This strategy was utilized for several reasons. The coupling of APA to the N-terminal amine of gamma-linked polyglutamates does not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described and the large quantities of the inexpensive APA precursor are available. The APA molecule has an absorbance at 340 nm and hydrolysis of prodrugs can be readily followed by HPLC analysis. Finally, APA-Glu (i.e. methotrexate) is cytotoxic at nanomolar concentrations and therefore activation of prodrugs in vitro can be easily assessed using growth inhibition and clonogenic survival assays. This strategy, therefore, was used to identify peptides that were selectively hydrolyzed by PSMA while at the same time remaining stable to hydrolysis in human plasma..

These studies with methotrexate-based peptides have delineated the limited range of substrates that can be hydrolyzed by PSMA. The only alpha-linked methotrexate substrate (i.e. substrate for PSMA's NAALADase activity) that was hydrolyzed by PSMA was APA-Asp-Glu. This substrate was also stable to hydrolysis by gamma-glutamyl hydrolase (GGH) and other protease activity present in the serum, table 1. These previous studies also demonstrated the enhanced ability of PSMA to hydrolyze a variety of γ-linked substrates. These γ-linked substrates, however, were not specific for PSMA and were readily hydrolyzed in human plasma. On this basis, we reasoned that the ideal substrate would take advantage of the dual ability of PSMA to hydrolyze certain

alpha and gamma linkages between aspartyl and glutamyl residues. Thus, the ideal substrate should incorporate the specificity of the α-linkage with the enhanced efficiency of the γ-linkage. The longer length, negatively charged, substrates would serve two additional purposes: first, they help to make the highly lipophilic more toxins like TG analogs more water soluble; second, the highly charged prodrug will be less likely to cross the plasma membrane, further limiting non-specific cytotoxicity, figure 1.

Table 1. Hydrolysis of PSMA-substrates by purified PSMA and stability in plasma

Substrate	% PSMA[2] Hydrolysis 4 h	24h	18 h Incubation Human Plasma % APA-Glu or Asp	% Prodrug Remaining[1]	18 h Incubation Mouse Plasma % APA-Glu or Asp
α-linked					
APA-Asp-Glu	0	62	1	99	ND
APA-Glu-Glu	0	0	ND	ND	ND
γ-linked					
APA-Glu-Glu-Glu-Glu-Asp	18	86	9	20	72
APA-Glu-Glu-Glu-Glu-Gln	48	92	6	26	62
APA-Glu-Glu-Glu-Glu-Glu	50	96	11	11	78

[1]% Prodrug Remaining equals peak area of starting material/total peak area (sum of prodrug and intermediate peaks). [2] % complete hydrolysis to APA-Asp or APA-Glu by purified PSMA. ND= not done

Using this rationale, additional substrates were synthesized in which a PSMA-hydrolyzable a-linked dipeptide "cap" is introduced that is not a substrate for GGH in order to produce more specific PSMA substrates. One of these substrates APA-Glu*Glu*Glu*Asp-Glu (Note: throughout this report alpha linkage of acidic amino acid will be denoted by hyphen and gamma or beta linkage denoted by the * symbol) was a less efficient PSMA substrate but showed enhanced stability in human serum. A second substrate APA-Glu*Glu*Glu*Asp-Gln was a poor substrate for PSMA, although it had even more enhanced stability in serum. Finally, a substrate containing two α-linkages and two γ-linkages, APA-Asp-Glu*Glu*Asp-Glu was a better PSMA substrate and was completely stable to hydrolysis in human and mouse plasma, table 1. These combination alpha- and gamma-linked PSMA substrates possess the best combination of efficiency and specificity and these substrates will therefore be used to create prodrugs described below.

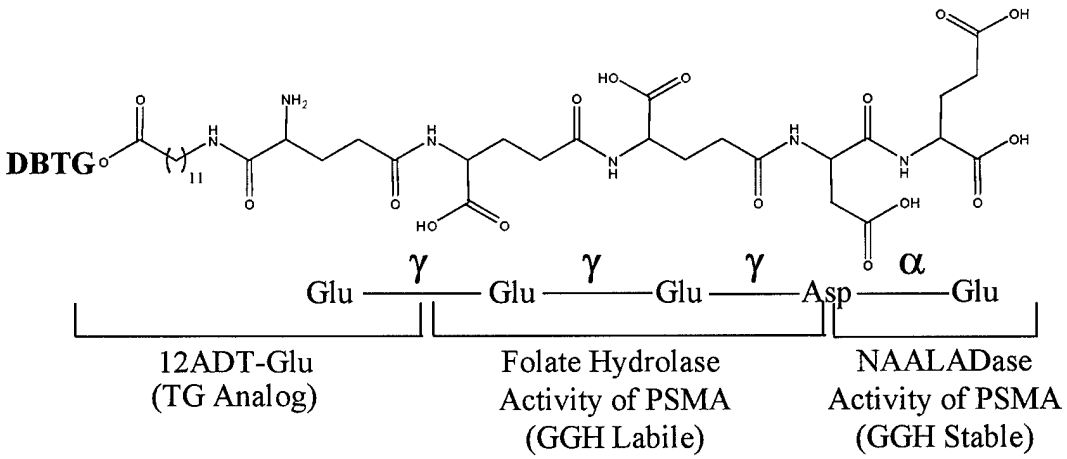


Figure 1. Example of PSMA prodrug containing α-and γ-linked amino acids and TG analog 12ADT-Glu.

Rationale for use of Thapsigargin in the treatment of Prostate Cancer

Thapsigargin (TG) is a sesquiterpene γ-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*, figure 2. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. TG induces programmed cell death of all rapidly proliferating prostate cancer cell lines. Unlike standard antiproliferative agents such as 5-FU or doxorubicin, TG can also induce apoptosis in non-proliferating, G_0 arrested prostate cancer cells. TG represents an excellent choice for treatment of prostate cancer because of its ability to kill these cells in a proliferation-independent manner. This ability to induce apoptosis in a proliferation-independent manner is particularly advantageous for the treatment of prostate cancer since these tumors have been demonstrated to have a remarkably low rate of proliferation (i.e. < 5% cells proliferating/day). Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG has been chemically modified to produce a primary amine containing analog that can be coupled to a water-soluble peptide carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of amino acid. Such a TG analog can be coupled to the alpha (Asp or Glu), beta (Asp) or gamma(Glu) carboxyl of Asp or Glu residues to produce prodrugs that can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSMA, figure 3.

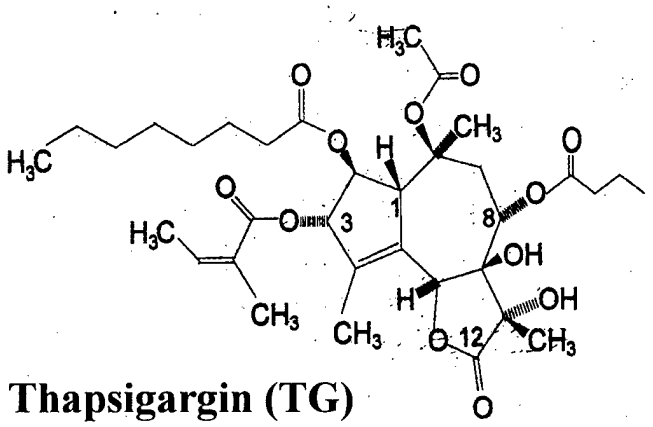


Figure 2. Chemical structure of thapsigargin

Progress over the 2002-2003 funding period.

In **task 1** we proposed the synthesis of a series of TG analogs that could be chemically linked to PSMA specific peptides. Because PSMA is an exopeptidase, hydrolytic processing of any prodrug will result in an end product consisting of a cytotoxin coupled to an acidic amino acid, most likely glutamate or aspartate. Therefore, while a TG analog can be potentially targeted using this prodrug approach, preferred analogs would be those that incorporate glutamic, aspartic or some other dicarboxylic acid into their structure and still maintain their cytotoxicity. Previously we synthesized and characterized a series of primary amine containing TG analogs coupled to amino acids that were potent cytotoxins with IC_{50} values in the low nanomolar range against human prostate cancer cell lines.

Over the past funding year we have synthesized a series of TG analogs that were modified in position 8 of the thapsigargin that incorporated a long hydrocarbon chain terminating in an acidic amino acid or dicarboxylic acid, figure 3. These analogs were tested in cytotoxicity assays against human prostate cancer cell lines and concentration required to reduce clonal survival by 50% (i.e. IC_{50}) was determined for each analog, table 2.

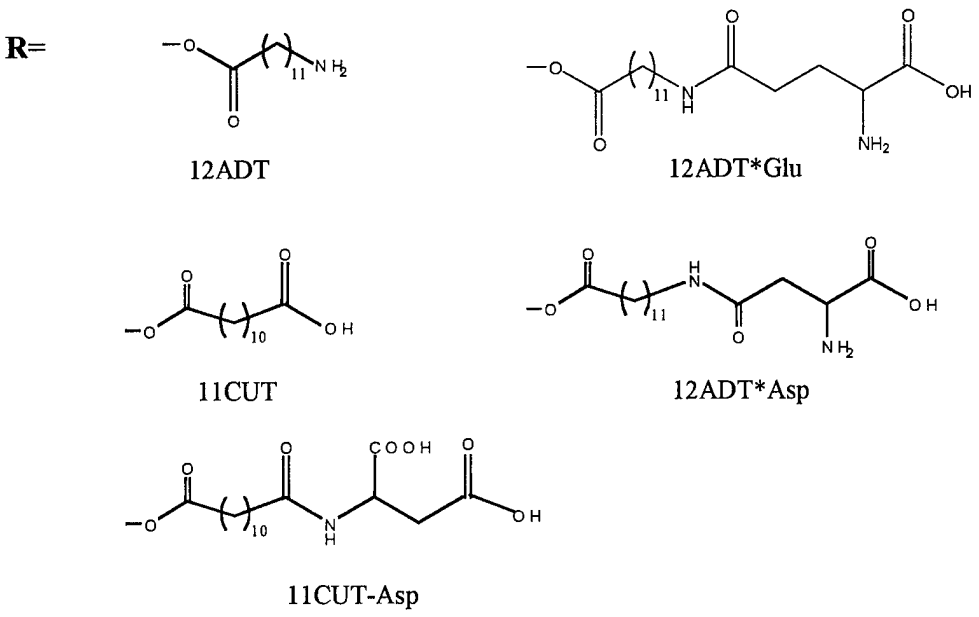


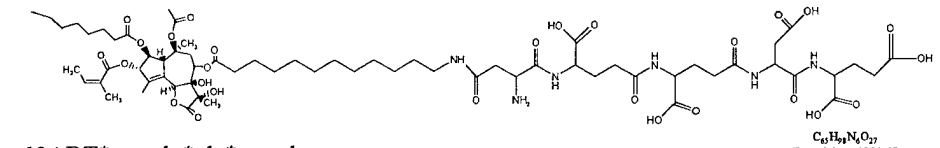
Figure 3. Chemical structure of 8-position side chains in a series of TG analogs. 12ADT= 12-aminododecanoyl; 11CUT= 11-carboxyundecanoyl; 11CUT-Asp=11-(aspartoylcarboxy)undecanoyl;12ADT*Glu= 12-(γ-glutamoylamino)dodecanoyl; 12ADT*Asp= 12-(β-aspartoylamino)dodecanoyl

TG analog	LNCaP IC_{50} (nM)
TG	30 ± 5
12ADT	1100 ± 160
11CUT	>5000
11CUT-Asp	>5000
12ADT*Glu	60 ± 12
12ADT*Asp	25 ± 10

Table 2. IC50 values for TG and TG analogs against human prostate cancer cell line LNCaP

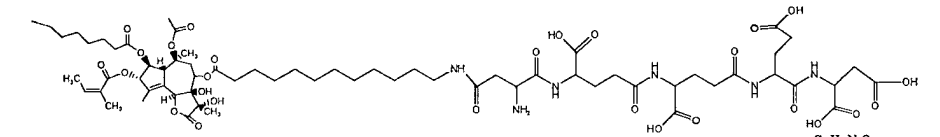
On the basis of these results it appears that the preferred TG analogs are 12ADT*Glu and 12ADT*Asp. These analogs contain no net charge (i.e. zwitterions). In contrast, dicarboxylic acid containing TG analogs are poor cytotoxins possibly due to the net negative charge and poor plasma membrane penetration.

The goal of Task 2 of the proposal was to synthesize prodrugs by chemically linking the TG analogs to peptide substrates that require hydrolysis by either NAALADase activity alone or combined NAALADase and folate hydrolase activity of PSMA. To date, we have synthesized a series of prodrugs, figure 4, and have begun to characterize each on the basis of PSMA hydrolysis, plasma stability and selective cytotoxicity to PSMA producing cells.



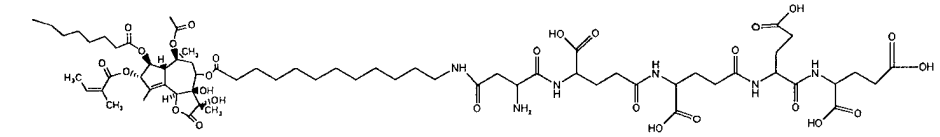
12ADT*asp-glu*glu*asp-glu

$C_{63}H_{94}N_6O_{27}$
Exact Mass: 1394.65
Mol. Wt.: 1395.50
C, 55.94; H, 7.08; N, 6.02; O, 30.96



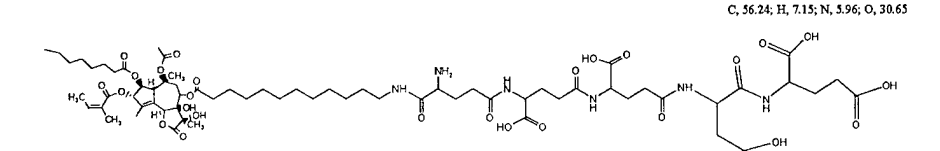
12ADT*asp-glu*glu*glu-asp

$C_{63}H_{94}N_6O_{27}$
Exact Mass: 1394.65
Mol. Wt.: 1395.50
C, 55.94; H, 7.08; N, 6.02; O, 30.96



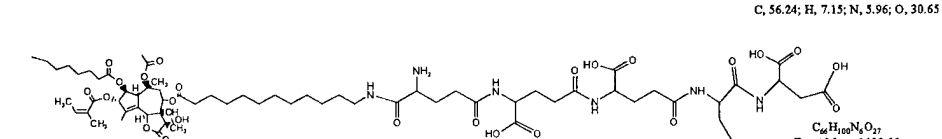
12ADT*asp-glu*glu*glu-glu

$C_{66}H_{102}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



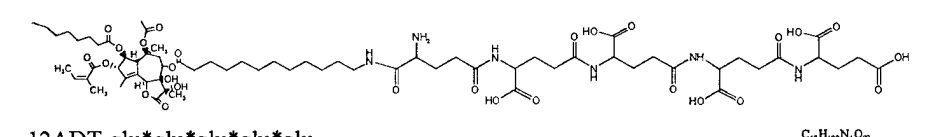
12ADT-glu*glu*glu*asp-glu

$C_{66}H_{102}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



12ADT-glu*glu*glu*glu-asp

$C_{66}H_{102}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



12ADT-glu*glu*glu*glu*glu

$C_{69}H_{102}N_6O_{27}$
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Mol. Wt.: 1423.55
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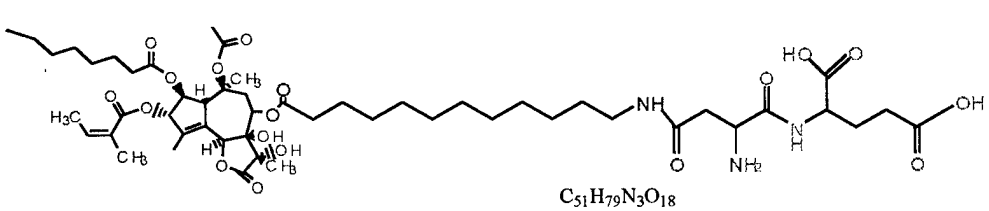

12ADT*ASP-GLU

$C_{51}H_{79}N_3O_{18}$
Exact Mass: 1021.54
Mol. Wt.: 1022.18
C, 59.93; H, 7.79; N, 4.11; O, 28.17

Figure 4. Chemical structures of putative PSMA-activated TG prodrugs

Thus far we have characterized two of these TG prodrugs, 12ADT*Asp-Glu and 12ADT*Asp-Glu*Glu*Asp-Glu for PSMA hydrolysis, figure 5. and for selectivity of cytotoxicity against PSMA-producing (LNCaP) and PSMA non-producing (TSU) cancer cell lines, table 3.

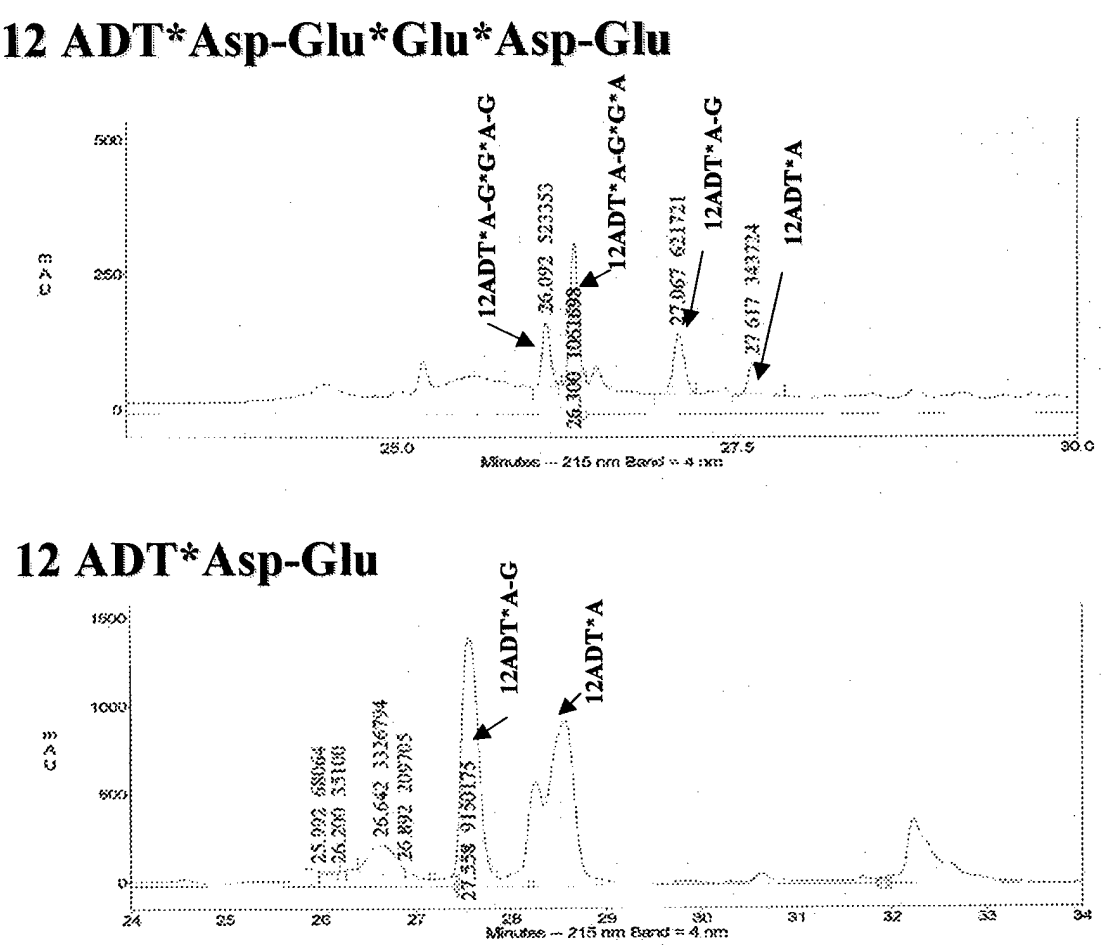

Figure 5. TG prodrugs (50 μM) are incubated with purified, enzymatically active PSMA and hydrolysis determined using HPLC analysis. Figure shows representative trace after 4 hrs incubation. Each peak was isolated and specific products identified by MALDI-TOF mass spectroscopy.

TG analog	PSMA-producing LNCaP IC_{50} (nM)	PSMA non-producing TSU IC_{50} (nM)	Fold Difference
12ADT*Asp-Glu	330	10,800	32
12ADT*Asp-Glu*Glu*Asp-Glu	420	12,600	30

Table 3. IC50 values for inhibition of clonal survival of PSMA-producing and PSMA non-producing human cancer cell lines by PSMA-activated TG prodrugs.

Currently, similar studies are underway to characterize the additional prodrugs pictured in figure 5. In addition, plasma stability of the 12ADT*Asp-Glu and 12ADT*Asp-Glu*Glu*Asp-Glu is being determined. Studies are also underway to determine whether the intact prodrugs can inhibit SERCA pump directly in broken cell microsomal assays. From these ongoing studies we hope to select a lead prodrug that can be tested in vivo

for antitumor efficacy against PSMA-producing human prostate cancer xenografts. These studies are outlined in task 3 of the proposal and will be carried out over the ensuing year of funding.

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized and characterized a series of acidic amino acid containing analogs of the potent natural plant toxin thapsigargin.

2. Determined cytotoxicity of these analogs against a human prostate cancer cell line.

3. Synthesized a series of putative PSMA-activated thapsigargin peptide prodrugs.

4. Demonstrated that two of the TG prodrugs are efficiently hydrolyzed by PSMA.

5. Determined cytotoxicity of two of these prodrugs against PSMA-producing and PSMA non-producing human cancer cell lines and found ~30-fold difference in cytotoxicity in cells that produced PSMA vs. cells that did not.

6. Characterized PSMA production in a series of human prostate cancer cell lines in order to identify optimal line for further prodrug screening.

REPORTABLE OUTCOMES:

Presentations:

"Hydrolysis of alpha and gamma linked polypeptide analogs of methotrexate by prostate-specific membrane antigen (PSMA)". Poster Presentation at American Association of Cancer Research (AACR) meeting, New Orleans, LA, 2001.

"Enzyme activation of Thapsigargin Prodrugs by Prostate-Specific Membrane Antigen as Targeted Therapy for Prostate Cancer". Poster presentation at Fellow's Research Day, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, 2002.
Graduate student Annastasiah Mhaka winner of prize for "Best Translational Research Presentation".

"Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen". Poster presentation at EORTC/American Association of Cancer Research (AACR) meeting, Frankfurt, Germany, 2003

"Targeted Activation of Cytotoxic Prodrugs/Protoxins by Prostate-Specific Proteases" The Brady Urologic Institute Thursday Evening Seminar Series, Johns Hopkins University, Baltimore, MD, 2003.

Manuscripts and Abstracts:

1. Jakobsen, C.M., **Denmeade, S.R.**, Isaacs, J.T., Gady, A.M., Olsen, C.E., Christensen, S.B. Design, Synthesis and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. J. Med. Chem., 44: 4696-4703, 2001.

2. Tombal, B., **Denmeade, S.R.,** Gillis, J-M, Isaacs, J.T. A supramicromolar elevation of intracellular free calcium ([Ca2+]i) is consistently required to induce the execution phase of apoptosis. Cell Death Differentiation 9:561-573, 2002.27.

3. **Mhaka, A., Denmeade, S. R.,** Yao, W., Isaacs, J. T., Khan S. R. A 5-Fluorodeoxyuridine Prodrug as Targeted Therapy for Prostate Cancer. Bioorg. Medicinal Chemistry 12:2459-2461, 2002.

4. **Mhaka, A.,** Janssen, S., Rosen, M., **Denmeade, S.R**. Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen. European J Cancer 38, Suppl 7:S36, 2002.

5. **Denmeade, S.R.,** Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, 54:249-257, 2003.

6. **Denmeade, S.R.,** Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J NCI, in press, 2003.

Employment:

Salary support for Graduate Student Annastasiah Mhaka to carry out experiments outlined in this proposal.

CONCLUSIONS:

At the end of one year of funding we have completed work outlined in task 1 and identified potent, acidic amino acid containing analogs of TG. As outlined in task 2, we have produced a series of putative PSMA-activated prodrugs and are currently screening these for PSMA hydrolysis, plasma stability and selectivity of cytotoxicity against PSMA producing prostate cancer cells. We have two promising lead prodrugs already and have several more to screen. Over the next year of funding we hope to complete work outlined in task 2 and begin in vivo studies outlined in task 3. We have recently acquired the necessary computing tools to begin PSMA modeling studies outlined in task 4 and hope to generate additional putative PSMA substrates based on data generated from these modeling studies.

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