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PSMA-Activated Imaging Agents for Prostate Cancer

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14. ABSTRACT In preliminary studies, a potent TG analog (12ADT) was coupled to a series of pentapeptides composed of varying combinations of Asp and Glu to create PSMA-activated prodrugs. One of these prodrugs with the sequence 12ADT-Asp-Glu*Glu*Glu*Glu was efficiently hydrolyzed by PSMA and resulted in accumulation of high levels of the cleaved product in tumor tissue compared to normal tissue. The goal of this study is to take advantage of this selective accumulation of the TG analog to make a prostate cancer specific PSMA targeted imaging agent. Specific Aims: The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells. (3) To determine the <i>in vivo</i> efficacy toxicity, pharmacokinetics and biodistribution of ¹²⁵ I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts; (4) To evaluate added therapeutic efficacy produced by ¹³¹ I labeling of the PSMA-activated prodrug in vivo against PSMA producing xenografts. Progress: Over the past year we have developed a 14-step synthesis to generate precursor phenolic TG analog. We documented the analogs ability to bind to the SERCA pump target. We then developed methods to couple the analog to the carrier peptide and confirmed cleavage by PSMA. Finally we developed methods to synthesize and purify the iodinated PSMA-activated agent. This compound is now under evaluation in vivo in biodistribution and imaging studies.				
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INTRODUCTION:

Our laboratory has been actively engaged in the preclinical development of methods to selectively target a highly potent cytotoxin, thapsigargin (TG), to prostate cancer cells. TG is an abundant natural product that makes up 1% of the weight of the seeds of the umbelliferous plant, *Thapsia garganica*, which grows as a weed throughout the Mediterranean basin. We have been interested in developing TG as a drug on the basis of its ability to kill both proliferating and non-proliferating prostate cancer cells at low nanomolar concentration. Previously our laboratory has demonstrated that prostate cancer cells have a remarkably low rate of proliferation (<5%/day), a finding that, in part, may explain their relatively poor response to standard antiproliferative chemotherapies. In addition, prostate cancers, like most malignancies, are a heterogeneous collection of cells that often express variable amounts of certain target proteins that are not required for cell survival. TG therapy, therefore, could overcome problems of therapeutic resistance due to low proliferative rate and heterogeneity of target expression within prostate tumors because it activates proliferation independent cell death due to its ability to inhibit the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump, a critical intracellular protein whose normal function is required by all cell types for survival. TG inhibition of the SERCA pump results in sustained elevation of intracellular calcium that leads to activation of endonucleases within the nucleus, ER stress responses and release of apoptotic factors by the mitochondria (11). TG's potent cytotoxicity, however, is not prostate cancer specific. Therefore, a strategy must be developed to target TG selectively to prostate cancer cell types while avoiding toxicity to normal, non-proliferating normal host tissues.

Our strategy for targeted TG therapy for prostate cancer has been to inactivate TG's cytotoxicity by coupling it to peptide carriers that are recognized as substrates by prostate tissue specific proteases. Since TG is a highly lipophilic compound that readily partitions into lipid membranes, coupling to a water soluble peptide carrier helps to solubilize TG while at the same time preventing it from passively entering the cell. These TG "prodrugs" can only become activated by release of the peptide by proteases present within sites of prostate cancer. Using this approach we have developed TG prodrugs that are selectively activated by prostate-specific antigen (PSA) (1) and human glandular kallikrein 2 (hK2).

In the course of developing a PSMA-activated TG therapy we identified a prodrug that was readily hydrolyzed by PSMA and selectively toxic to PSMA-producing human prostate cancers in vitro and in vivo. In the course of this work, we began to analyze tissue levels of the TG analog (12ADT-Asp) that is released from the peptide carrier by PSMA, figure 1.

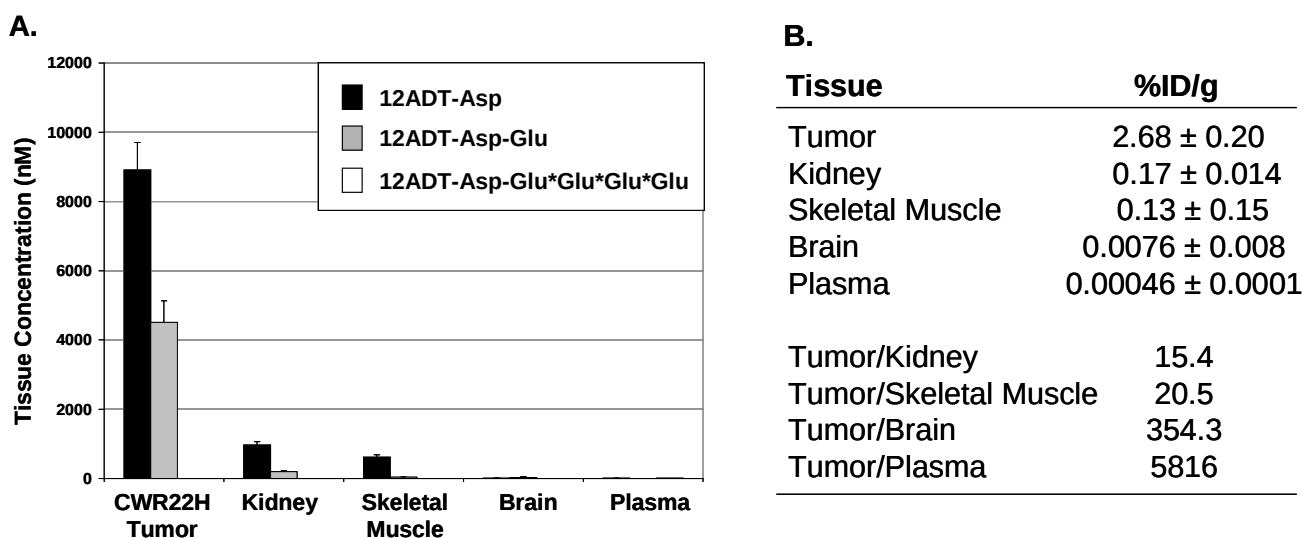


Figure 1. (A) Selective accumulation of 12ADT-Asp and 12ADT-Asp-Glu in tumor tissue compared to indicated normal tissue five days after single intravenous dose of 2 μ mole (120 mg/kg); (B) Biodistribution of TG species (sum of 12ADT-Asp, 12ADT-Asp-Glu and 12ADT-Asp-Glu*Glu*Glu*Glu) in CWR22H tumor bearing mice. Data presented as % Initial dose (ID)/gram and tumor/tissue ratios (n=4 mice).

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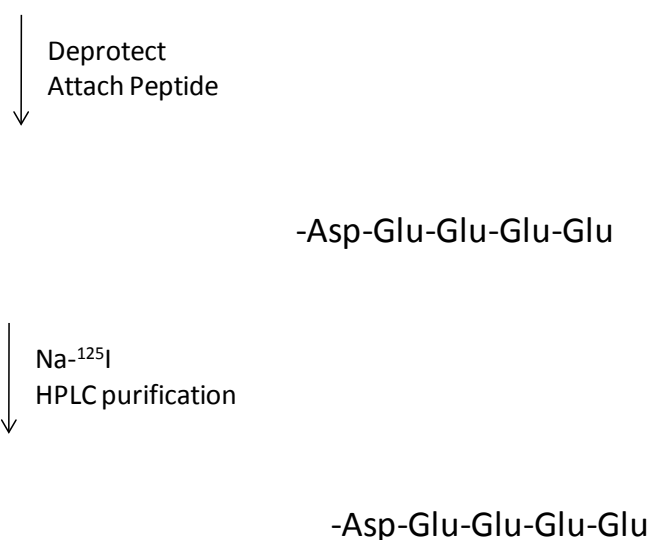
Hypothesis: The hypothesis is that a PSMA-Activated Imaging Agent can be achieved by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times. This would then allow PSMA positive prostate cancers to be imaged. As a secondary objective, the cytotoxicity and antitumor efficacy of these PSMA-activated prodrugs will also be evaluated.

Specific Aims: The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity and stability of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro; (3) To determine the in vivo toxicity, pharmacokinetics and biodistribution of ^{125}I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts.

Progress for Reporting Period 2009-2010.

In the previous progress report for this grant we described the 14 step synthesis we developed to a radiolabeled PSMA-activated prodrug. Over the past year we developed methods to optimize the final step in the process which involved coupling the thapsigargin analog to the carrier peptide followed by addition of $\text{Na-}^{125}\text{I}$. The difficulty for this procedure was in the separation of the ^{125}I labeled peptide analog from non-labeled analog. After extensive HPLC-based studies, an efficient method of separation was developed that allowed for purification of the compound in high yield (i.e. >75%). The final step in the synthesis of the radiolabeled thapsigargin analog peptide is shown in figure 1.

Figure 1.



Previously we synthesized iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro. In initial studies we evaluated the hydrolysis of the non-iodinated PSMA prodrug (PSMA-14) by PSMA producing LNCaP human prostate cancer cells in vitro. In this assay we saw cleavage of the phenolic TG analog-Asp-Glu-Glu-Glu-Glu prodrug to two forms, phenolic TG (Ph12ADT) and the Asp-Ph12ADT (D-Ph12ADT) consistent with PSMA hydrolysis of the gamma linked glutamate residues, figure 2. Both analogs were observed in the conditioned media and in the cells, consistent with ability of the analogs to penetrate cell membrane once liberated from the peptide carrier. In contrast, no intact PSMA-14 was observed in the cell extract, consistent with the ability of the highly charged carrier peptide to keep the agent out of cells, figure 2A.

Figure 2.

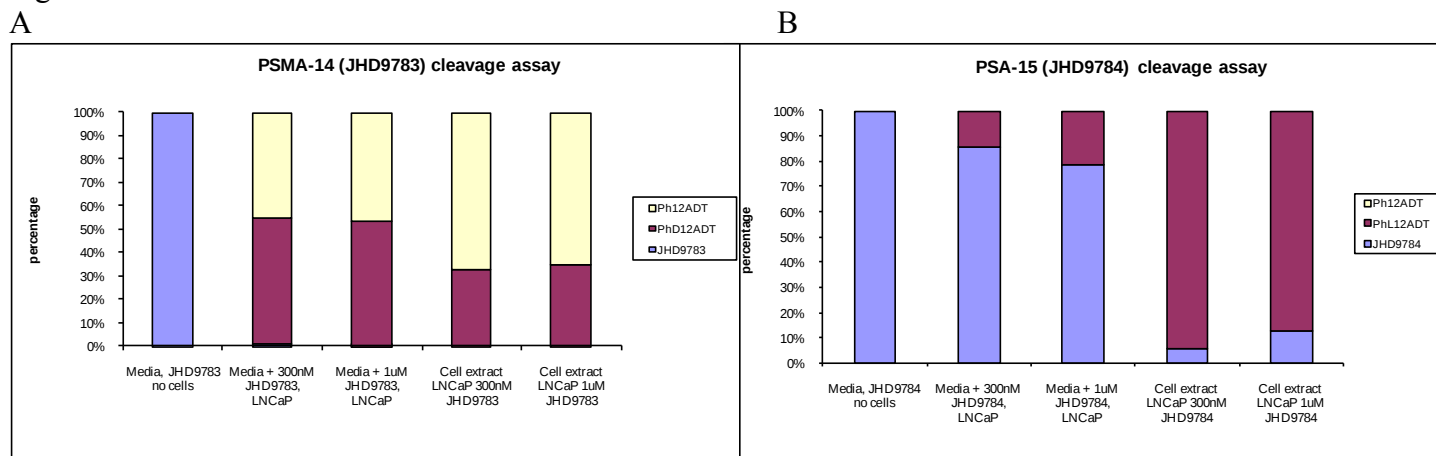
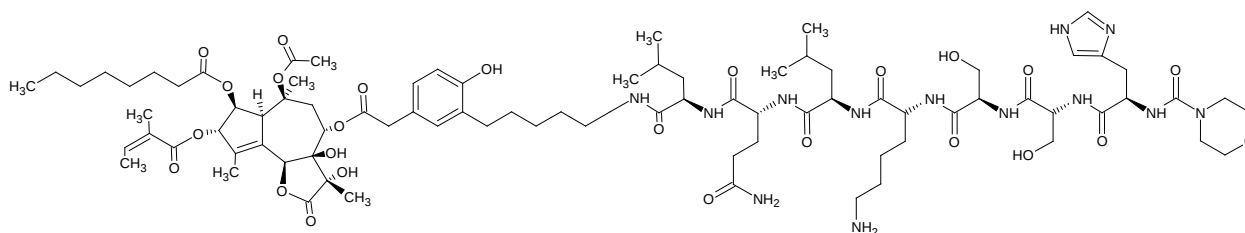


Figure 2. Relative Hydrolysis of (A) PSMA-14 by LNCaP cells. LNCaP cells (confluent) were treated with media and PD-14 for 3 days. After 3 days the media was collected and the cells were scraped and extracted with acetonitrile. The media and the cell extract were run on the LCMS. (B) Similar results for PSA-activated agent PSA-15 using LNCaP cells.

Over the course of the funding year, we also compared cleavage and selectivity of an agent containing a different peptide that targeted the prostate cancer specific protease PSA. The selection of PSA was based on previous studies with a PSA-prodrug demonstrating the enhanced specificity of this approach compared to a PSMA prodrug. In addition, it was based on data from the previous funding year with the PSMA-14 agent demonstrating non-specific uptake in the GI tract and kidneys. On this basis, we used similar methods to generate a PSA-activated imaging agent (PSA-15 or JHD9784) that contained the targeting peptide Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-Ph12ADT, figure 3.

Figure 3. Structure of PSA-15 (JHD9784)



The PSA-activated PSA-15 was efficiently cleaved by purified PSA (data not shown) and was also hydrolyzed by LNCaP cells to the single amino acid analog PhL12ADT. Unlike the PSMA-14, further hydrolysis to Ph12ADT was not observed. In addition, the highly charged PSMA-14 peptide was better at keeping the intact agent out of cells compared to the PSA-15 peptide, figure 2.

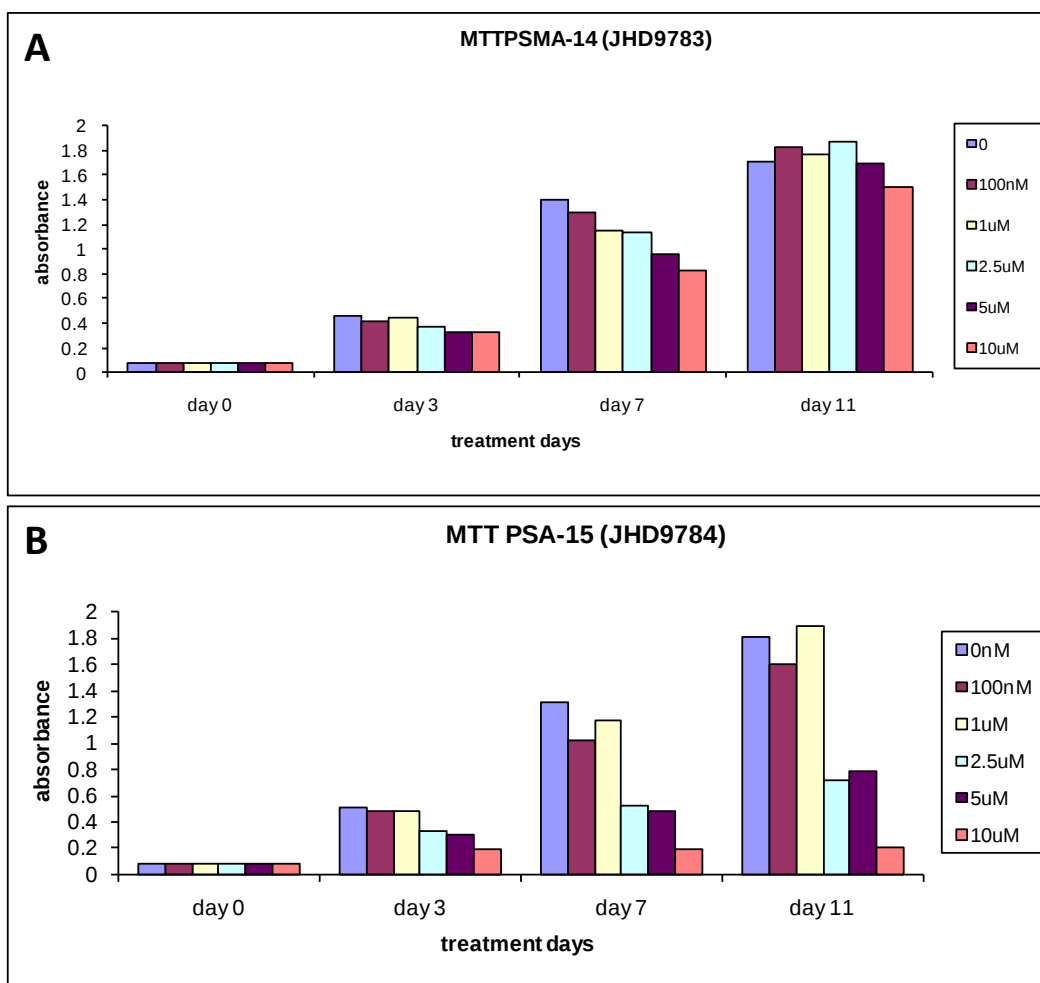


Figure 4. MTT assay evaluating varying concentrations of I-PSMA-14 and I-PSA-15 against PSMA and PSA producing LNCaP cells.

Subsequent we developed methods to iodinate PSMA-14 and PSA-15 and to purify to homogeneity. Evaluation of these compounds for cytotoxicity against LNCaP cells, which produce both PSA and PSMA, in tissue culture revealed some toxicity of the I-PSMA-14 compound at the relatively high concentration of 10 μ M with an IC₅₀ that was even higher, figure 3. This result suggests that, while PSMA-14 is cleaved as evidenced by cleavage data in figure 2, insufficient TG analog is released at concentrations < 10 μ M to produce a cytotoxic effect. In contrast, the I-PSA-15 compound demonstrated an IC₅₀ of ~ 2.5 μ M after 7 and 11 days of exposure. These results suggest that more of the I-PSA-15 compound is cleaved to the toxic TG analog in vitro.

The goal of Specific Aim 3 was to determine the in vivo toxicity, pharmacokinetics and biodistribution of 125-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts. To accomplish this goal we synthesized prodrug and established CWR22R xenografts in nude mice. In previous studies we demonstrated that CWR22R produce reasonably high levels of PSMA, although at lower levels that observed in primary human prostate cancer. However, sufficient PSMA is

present in these tumors to make them suitable for these imaging studies. In contrast, these tumors make very low levels of PSA compared to human primary cancers.

In subsequent, we injected 500 μ Ci of PSMA-14 intravenously and then sacrificed the animals 96 hrs post injection and measured total counts within the tissues, figure 4A. In comparison we also injected 500 μ Ci of a PSA-15, figure 5B. The biodistribution results demonstrate that both prodrugs are predominantly cleared by the liver through the intestine. High levels of PSMA-14 compound were observed in the PSMA-positive CWR22R xenografts, figure 5a. Lower levels were observed in the kidney, which also expresses a low level of PSMA. In contrast, for PSA-15 compound, minimal activity was observed in tumor tissue compared to most other organs, figure 5b. These results may be due to the relatively low expression of PSA by this xenograft compared to PSMA. These results also suggest that following exposure to PSMA-14, tumor uptake is due to PSMA cleavage of the compound to release 125 I-PhD12ADT and 125 I-Ph12ADT, which then accumulates in tumor tissue.

A.

B.

Figure 5. Uptake of (A) 125 I-PSMA-14 or (B) 125 I-PSA-15. Counts were determined on whole organs using gamma-counter (Beckman-Coulter) at 96 hours post injection of compounds. Counts/gram are shown for representative animals.

Based on these biodistribution data we proceeded to compare the PSMA-14 vs PSA-15 as an imaging agent. Previously, we utilized the human prostate cancer cell line PC-3 transfected with PSMA to document that PSMA-14 could generate a signal in the PSMA-producing PC-3 line compared to a non-PSMA producing control, (figure 5 from prior progress report 2009). To compare uptake of PSMA-14 vs. PSA-15 we needed to use a line that produced both enzymes. For this experiment we selected the human prostate cancer xenograft LNCaP. We compared ability of PSMA-14 and PSA-15 to image tumors in this model. As shown in figure 6 tumor uptake can be easily visualized at 20 hrs post injection of PSMA-14. In contrast, no uptake was observed at this time point following injection of PSA-15.

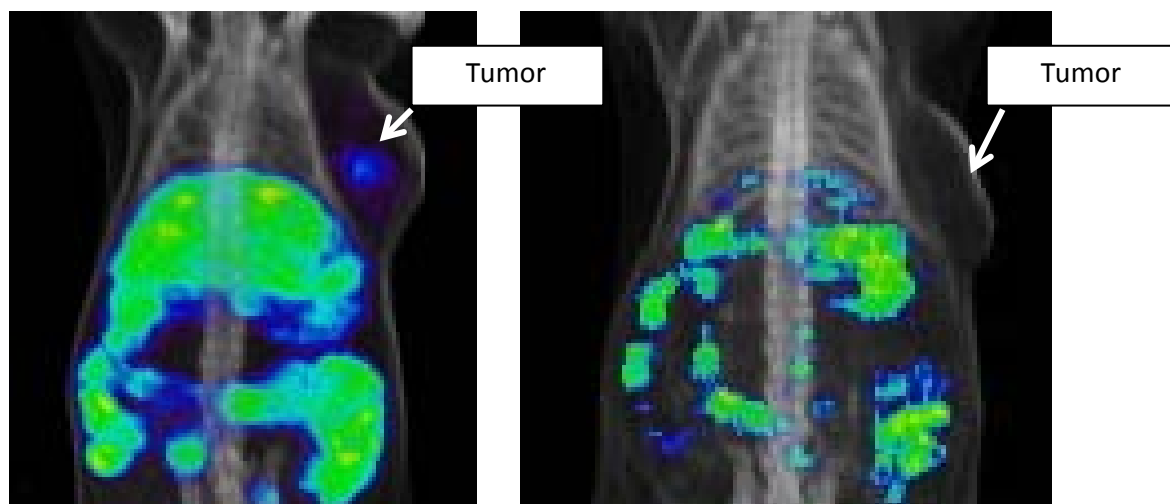
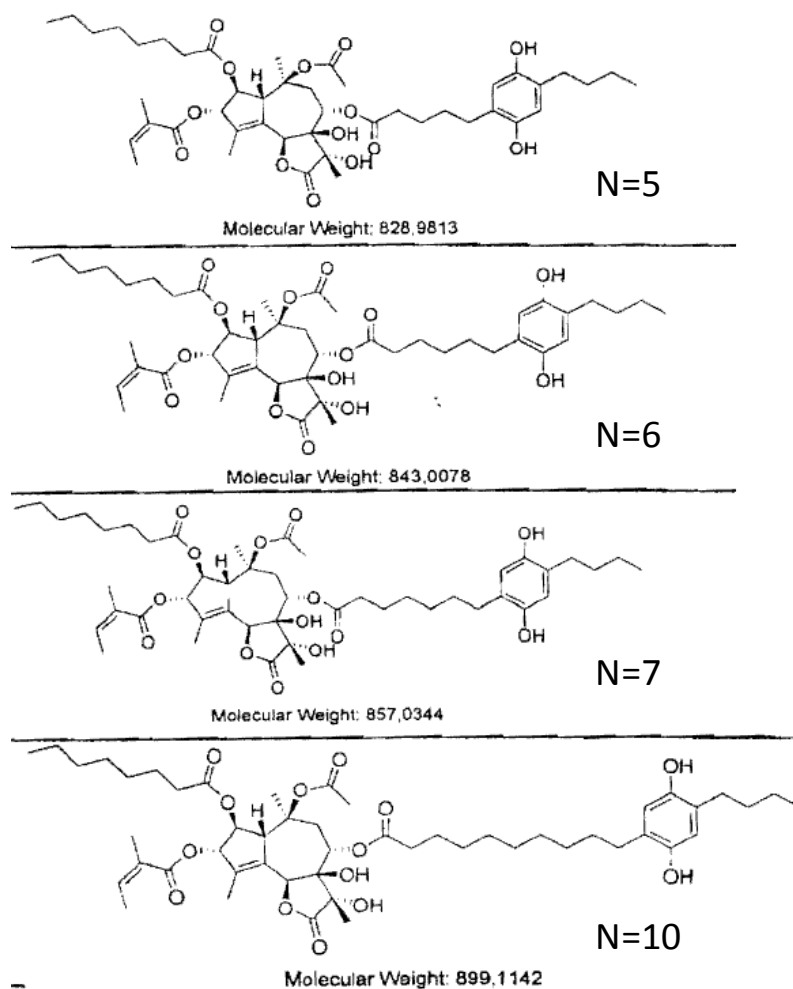
PSMA-14**PSA-15**

Figure 6. SPECT/CT Imaging of ^{125}I -PSMA-14 compared to ^{125}I -PSA-15 in LNCaP xenografts. Animals were injected with 500 μCi of each compound and then imaged at 20 hrs post injection.

In the final set of studies over the funding year we also synthesized several other phenolic ring containing thapsigargin analogs. These analogs contain side chains of varying length (i.e. N=5,6,7,10 carbon spacer) connected to the phenyl ring, figure 7. We are currently working out conditions for coupling to the PSMA peptide and will assess these compounds for stability, PSMA hydrolysis, toxicity and imaging potential.

Figure 7. New Phenolic ring containing Thapsigargin analogs



Key Research Accomplishments

- Developed a 14 step method to synthesize a radioiodinateable TG analog
- Developed methods to synthesize a PSMA-cleavable prodrug
- Synthesized 65 mg of PSMA prodrug precursor for iodination
- Demonstrated cleavage of PSMA-activated compound by PSMA producing prostate cancer cells
- Demonstrated cleavage of PSA-activated compound by PSA producing prostate cancer cells
- Documented cytotoxicity of the PSMA- and PSA-activated compounds
- Developed methods to synthesize and purify 125-I labeled PSMA and PSA compounds
- Completed in vivo studies on distribution of the 125-I labeled PSMA and PSA compounds
- Performed initial imaging studies demonstrating tumor uptake of the 125-I labeled PSMA compound
- Generated a series of phenolic ring containing thapsigargin analogs for future studies

Reportable Outcomes

- “PSMA-Activated Imaging Agents” Presented at Tri-Institutional SPORE meeting Newport, RI, 2008 Podium Presentation:
- “Targeted imaging of prostate cancer: smart-bomb pro-drug mechanism” JP Michiel Sedelaar, John T Isaacs, Samuel R Denmeade, Moderated Poster Session European Urological Association Annual Meeting, Stockholm, Sweden, 2009.
- “Targeted imaging for prostate cancer: the smart-bomb pro-drug mechanism”, JP Michiel Sedelaar, John T Isaacs, Samuel R Denmeade, Poster Presentation American Association for Cancer Research Annual Meeting, Denver CO, 2009
- Training: Provided support for training program of Post-Doctoral Fellow J. Michiel Sedelaar
- Radiolabeled Prostate cancer protease activated agents for imaging sites of prostate cancer. Sedelaar, M., Isaacs, JT, Christensen, SB, Denmeade, SR. Manuscript in preparation.

Conclusion

The goal of these studies is to develop a PSMA-Activated Imaging Agent by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times allowing PSMA positive prostate cancers to be imaged. Over the 3 years of funding our major accomplishment was to develop a synthetic method to generate phenolic ring containing TG analogs that can be readily iodinated. We also developed methods to rapidly radiolabel and purify the compound. Having achieved these goals, we completed studies to assess the biodistribution and imaging potential of a PSMA-activated 125-I labeled compound. We demonstrate that the compound is cleared through the liver and has considerable tumor uptake compared to other normal organs. We also compared this PSMA-activated compound to a compound that can be activated by a second prostate cancer specific protease, PSA. We documented that a PSA-activated compound can be cleaved in vitro and is more cytotoxic than the PSMA-activated compound. However, imaging studies suggest that the PSMA-activated compound is preferentially taken up in tumors compared to the PSA-activated compound. We are currently applying for other funds to continue this project. We would like to compare the activity and uptake of other thapsigargin analogs linked to the PSMA-peptide.

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