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TITLE: A Recombinant Platform for Prioritizing Aerolysin Molecular Grenades
for Metastatic Prostate Cancer

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14. ABSTRACT The progression of prostate cancer (PCa) to castrate resistant metastatic disease is an ominous diagnosis. To overcome tumor cell heterogeneity based therapeutic resistance of PCa, the Isaacs/Denmeade laboratories have advocated the use of chemical engineering principles to modify potent killing toxins as "molecular grenades", which are delivered systemically and selectively "detonated"; thereby, liberating their killing toxin efficiently only within the extracellular microenvironment at cancer sites. <i>The objective for this proposal is to use a bio-engineering approach to produce recombinant pro-toxins designed for specific cleavage by a defined protease whose high expression is restricted to the tumor microenvironment at sites of metastatic castration resistant prostate cancer (CRPC).</i> Results show 1) a recombinant protein consisting of human serum albumin (HSA) and proaerolysin (PA) can be produced via a peptide linker specific to a protease specific to the tumor microenvironment. 2) Recombinant HSA/PA shows efficacy <i>in vitro</i> and low toxicity in animal studies.					
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Introduction

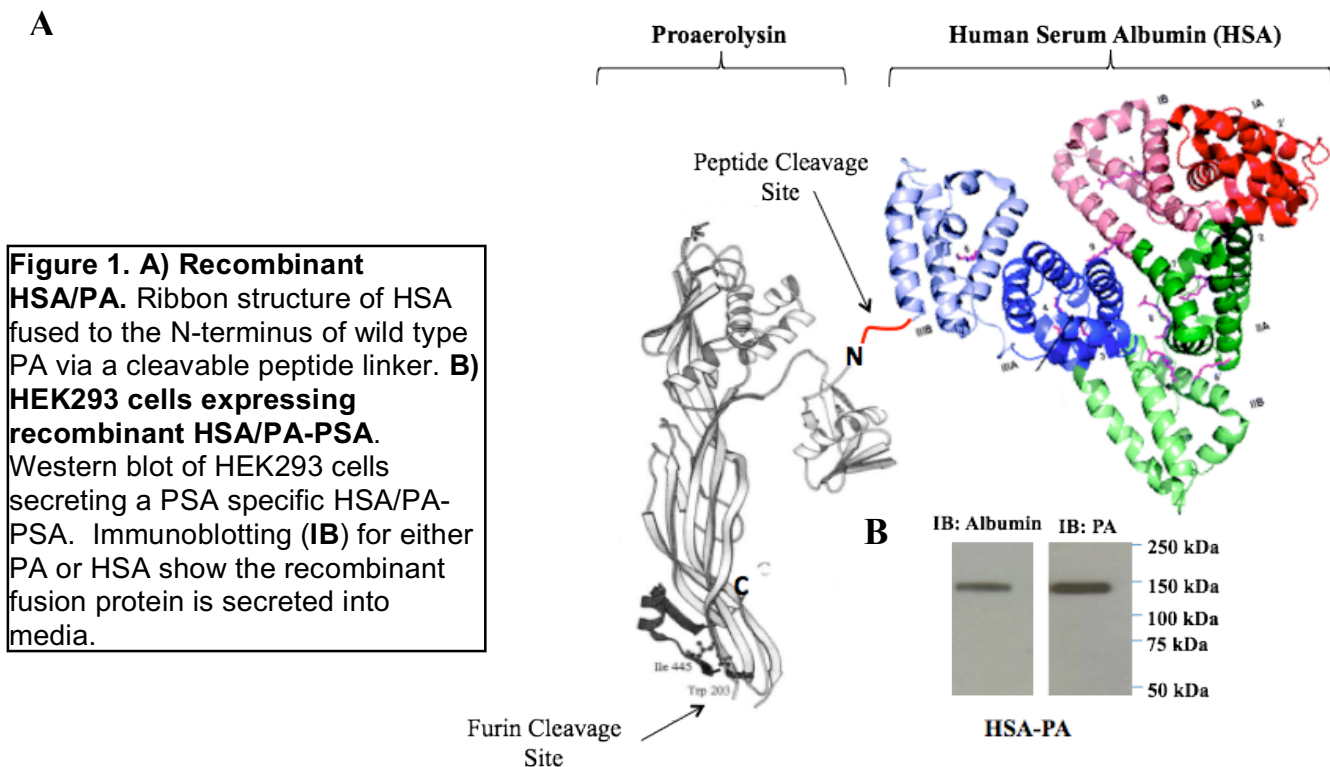
The progression of prostate cancer (PCa) to castrate resistant metastatic disease is an ominous diagnosis. ***The objective for this project is to use a bio-engineering approach to produce recombinant pro-toxins designed for specific cleavage by a defined protease whose high expression is restricted to the tumor microenvironment at sites of metastatic castration resistant prostate cancer (CRPC).*** Specifically, this application will use a recombinant molecular biology platform to produce a series of molecular grenades, each based upon the bacterial toxin proaerolysin, but varying in the activating enzyme responsible for toxin liberation. We believe the specific overexpression and hypersecretion of these active proteases specific to the tumor microenvironment in both primary and metastatic sites are ideal substrates for the targeted release of the highly cytotoxic proaerolysin (PA) in a restricted fashion.

Keywords

Molecular grenades, Tumor microenvironment, metastatic castration resistant prostate cancer, Proaerolysin (PA), Human Serum Albumin (HSA)

ACCOMPLISHMENTS

The original stated goal in the approved statement of work for Task 1 was to **Engineer enzymatically cleavable linker for the release and activation of PA from HSA with lead peptides**. Using standard molecular biology techniques to produce a platform plasmid (i.e. termed HSA/PA) which contains a c-DNA encoding an N-terminal signal sequence tagged (i.e., for extracellular secretion)-full length HSA fused to a cloning site for insertion of the appropriate protease specific activation sequence in front of the N-terminal of full length wild type PA. As a proof-of-principal, we inserted the prostate specific antigen (PSA) activation sequence HSSKLQ (1) into the cloning site of this HSA/PA vector which we transfected into HEK293 cells. This resulted in secretion of large amounts of the ~ 130kD- recombinant HSA/PA-PSA protein into the media. We developed a FPLC method for the rapid purification of the secreted recombinant protein, **Figure 1B**. We have also documented that the length of the activation sequence is critical for the efficient hydrolysis of this fusion protein releasing HSA, **Figures 2 and 3**. Based upon this validation of the platform vector, we are working on separately inserting the recognition sequences for Cathepsin B (i.e., RLVGF with hydrolysis occurring between glycine and phenylalanine (2)); Fibroblast activated protein (FAP) (i.e., ASGPAGPA] with hydrolysis occurring after the proline (3), or hK₂ (i.e., GKAFRRL with hydrolysis occurring after arginine (4)) into the HSA/PA plasmid which will be transfected into HEK293 cells, and the respective -hK₂, -CathB, and -FAP recombination proteins separately isolated from the media via FPLC.



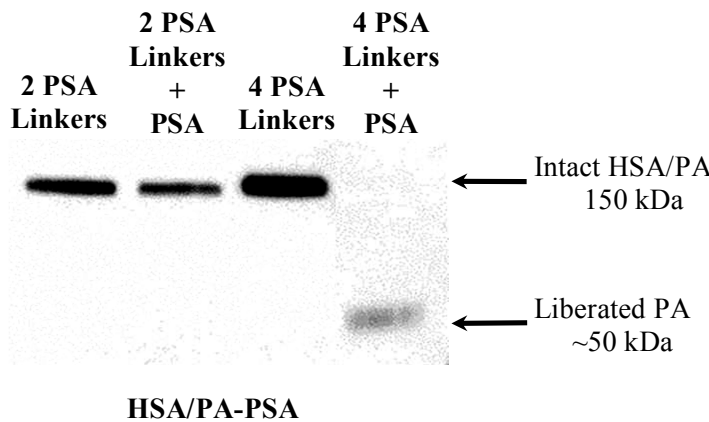
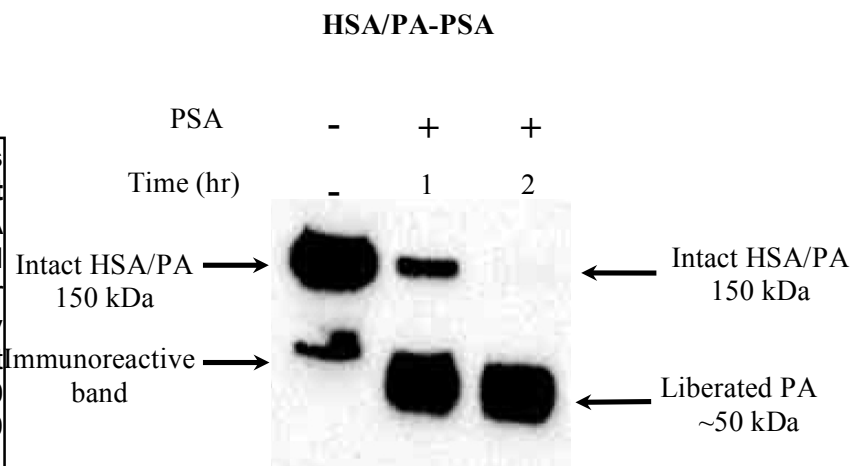


Figure 2. Length of cleavage sequence is critical for PA liberation. Recombinant HSA/PA-PSA were produced with varying number of PSA specific linkers. HSA/PA with 2 PSA specific linkers was not cleaved after treatment with PSA. However, complete PA liberation was achieved when HSA/PA was engineered with 4 PSA specific linkers.

Figure 3. Recombinant HSA/PA-PSA is completely liberated after treatment with PSA. Recombinant HSA/PA specific for PSA cleavage was incubated with purified enzyme for 1 or 2 hrs. After 1 hr, PSA is able to liberate the majority of PA from HSA. After 2 hrs of treatment with PSA, the recombinant HSA/PA (150 kDa) is completely liberated to PA (~50 kDa).



The second approved task for aim 1 was to **determine the stability of the recombinant HSA/PA in plasma**. In order to deliver the recombinant fusion proteins to sites of metastatic prostate cancer, they must be stable (i.e., non-hydrolyzed) when distributed systemically via the blood. We are currently working on developing the ELISA assay to allow for the detection of PA in pooled samples of plasma. However, we have developed a standard curve with our detection antibody which allows us to confidently detect nanogram amounts of PA in solution, **Figure 4**. We have tested the specificity and efficacy of the HSA/PA-PSA to lyse human red blood cells (RBCs). PA was discovered as a hemolytic toxin (5), so we developed an RBC lysis assay to evaluate our recombinant HSA/PA. After three hours we can achieve ~90% lysing of RBC with 100nm HSA/PA-PSA after complete proteolytic activation with purified PSA and furin. Non-specific lysing of RBCs at a higher concentration of HSA/PA and furin alone is not observed, **Figure 5**. We have also started to assess the specificity of the HSA/PA-PSA on PCa cell lines based on secretion of enzymatically active PSA. To determine specificity we transduced the C19 cell line, androgen independent derivative of LNCaP, to constitutively secrete enzymatically active PSA, and compared the toxicity of HSA/PA-PSA at various concentrations to this cell line along with the non-PSA producing PCa cell line DU145. In comparison to C19-PSA, the non-PSA producing cell line DU145 show no reduced cell viability when treated with the HSA/PA-PSA. C19-PSA cells displayed a 60% reduction in viable cells at 10nm of drug after 4 hours, **Figure 6**.

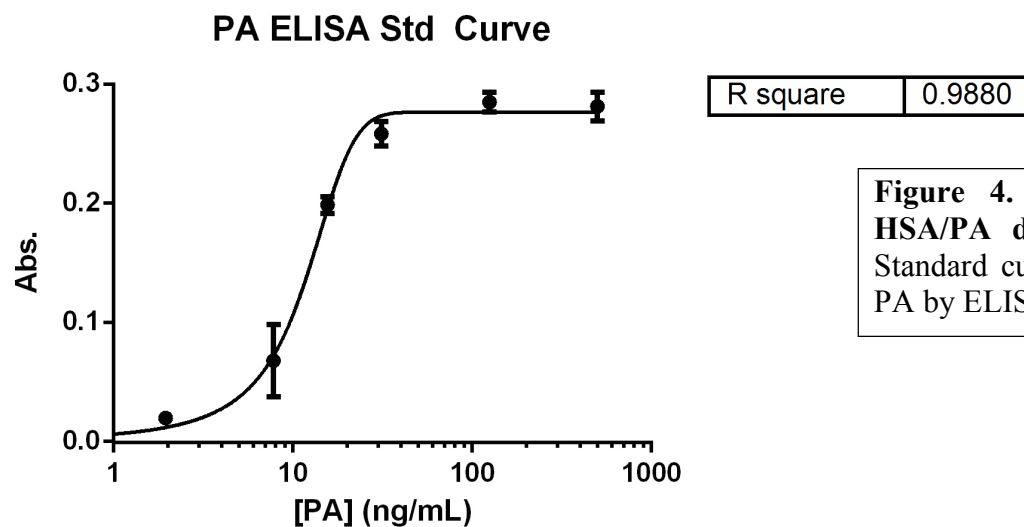


Figure 4. Standardized ELISA for HSA/PA detection in mouse tissues. Standard curve showing ability to assay PA by ELISA.

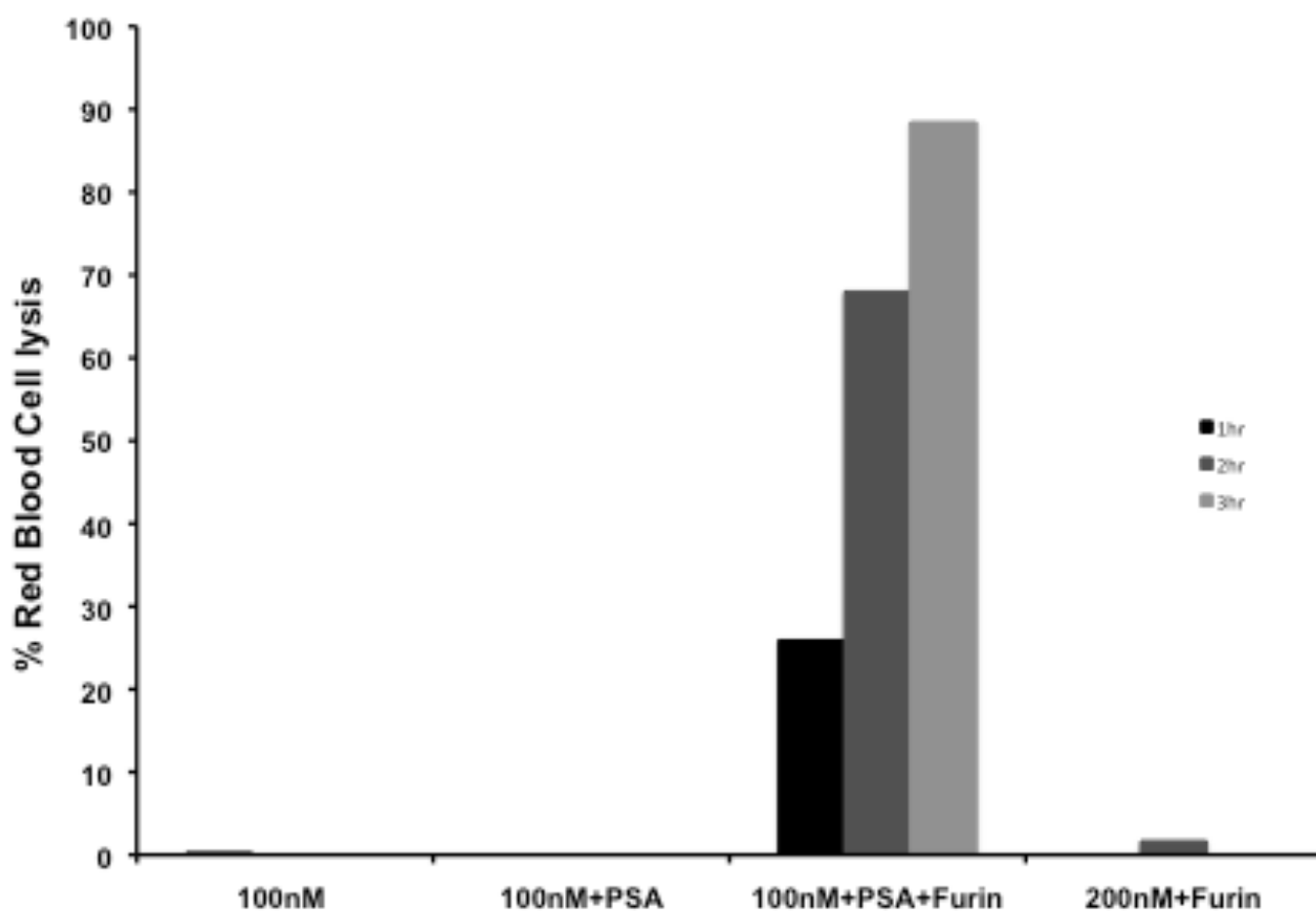


Figure 5. Hemolysis assay with HSA/PA-PSA after proteolytic activation. Human RBCs suspended in a isotonic buffer were incubated with HSA/PA-PSA at 100 nM or 200 nM in the presence/absence of PSA and PSA+Furin. After three hours RBCs incubated with fully activated HSA/PA-PSA 90% of cells are lysed by the liberated toxin.

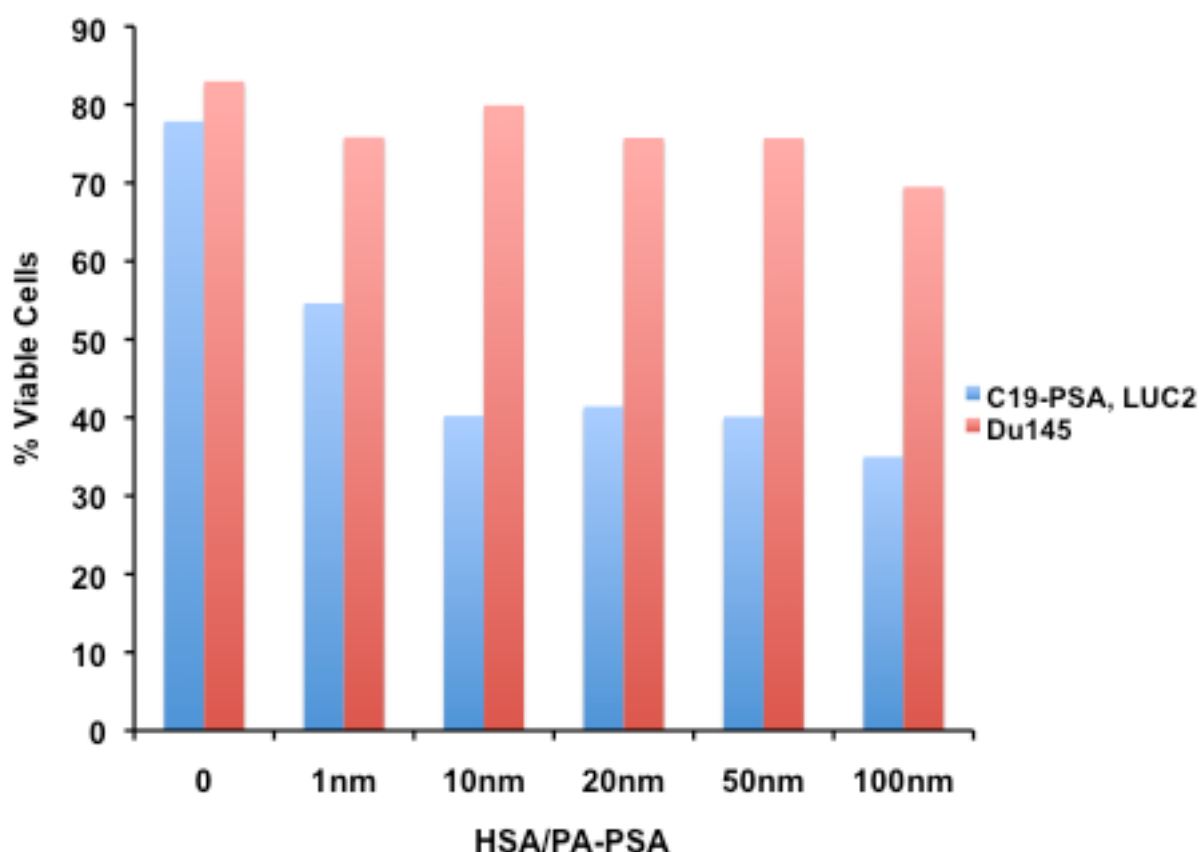


Figure 6. PSA expressing PCa cell lines are specifically targeted by HSA/PA-PSA. PSA expressing C19 cells along with non-PSA expressing DU145 cells were plated and allowed to condition the media for 48 hrs, prior to dosing with HSA/PA-PSA. Viable cells were determined by exclusion of Trypan blue after 4 hrs.

While the data from the subcutaneous tumor xenografts is an established method for evaluating pre-clinical efficacy/potency, prostate cancers do not commonly metastasize to subcutaneous sites, but grow lethally in the prostate and particularly in the bone. Therefore, the dose-response efficacy of our recombinant against prostate cancer xenografts growing either orthotopically within the prostate or within the bone will need to be determined. For these studies, the C19 derivate of the LNCaP progression model which we have shown previously grow well following either orthotopic injection into the ventral prostate or in bone following intratibial injection of immune-deficient nude mice (6). To evaluate response of these xenografts to recombinant proteins in either the prostate or the bone, we plan to use the bioluminescence imaging system from Xenogen. We have transduced the human C19 PCa cell line for dual expression of the luciferase gene (7) and high secretion of enzymatically active PSA under a constitutive promoter, **Figure 7**. Following systemic exposure to injected luciferin into animals, we are able to detect disseminated tumor cells after intracardiac (**IC**) injection and orthotopic grafting into the anterior prostates (**AP**) of athymic nude mice, **Figure 8**. The creation of this C19-PSA, LUC2 line will be a useful tool going forward towards evaluating our prodrug. The bioluminescence will allow us to monitor regression of disseminated tumors over time without having to sacrifice the animal.

We can also monitor tumor burden in the animal by collecting small volumes of blood and performing an ELISA for PSA.

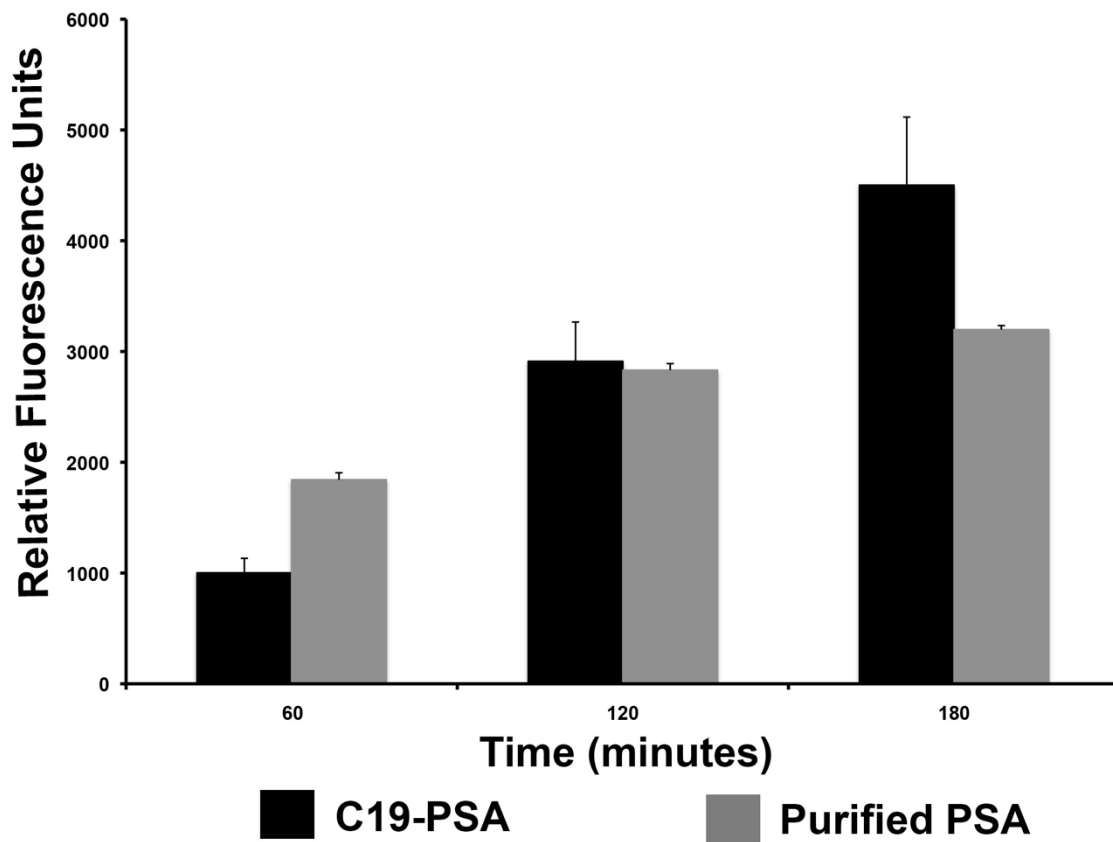


Figure 7. PSA activity assay. Conditioned media from C19 cells transduced to constitutively express PSA was collected and pooled. Activity of secreted PSA was determined using an AMC linked substrate specific for PSA. Activity of C19 secreted PSA is compared with 1ug of purified Human PSA.

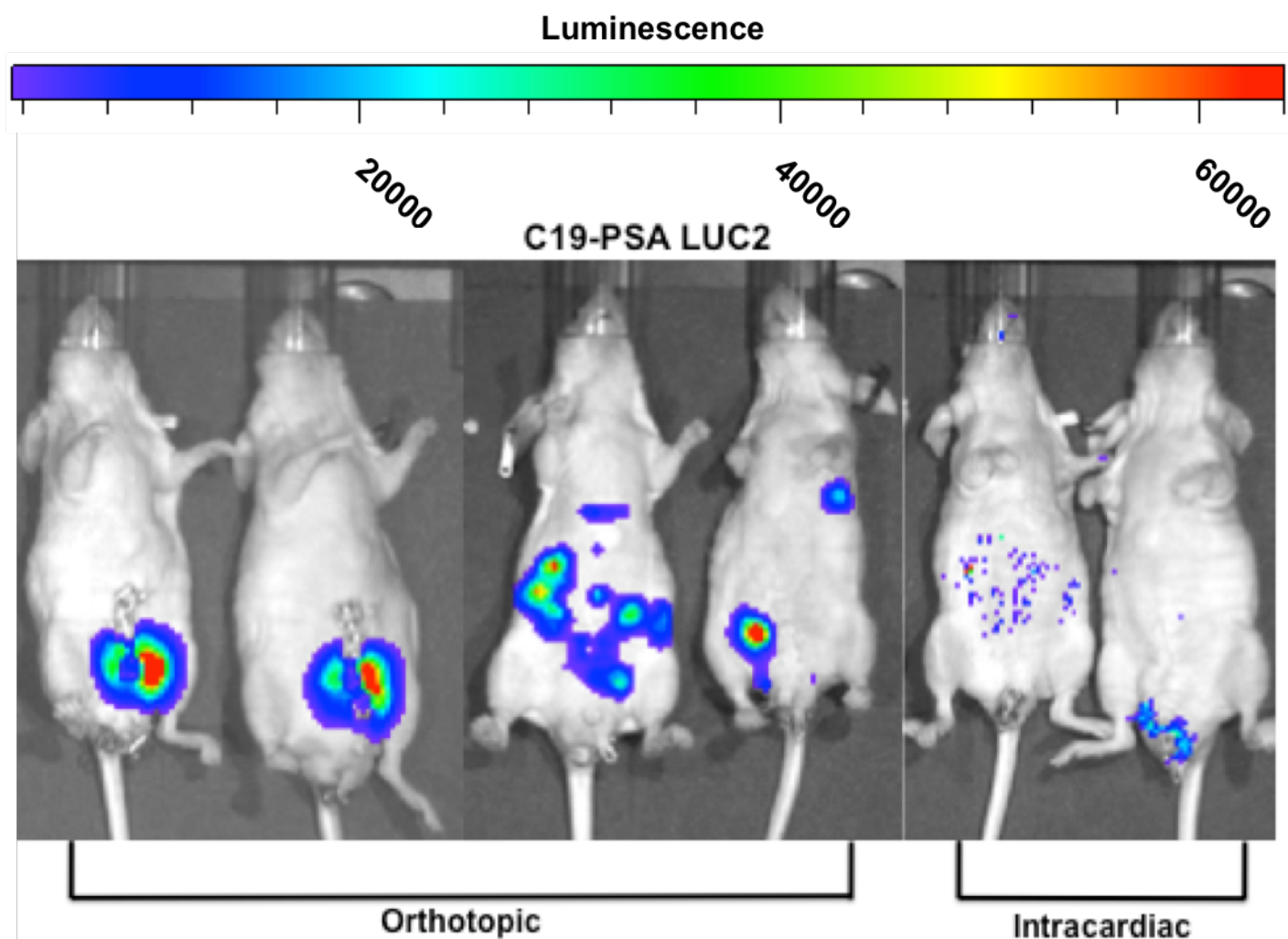


Figure 8. C19-PSA LUC2 cells form disseminated tumors when injected orthotopical or intracardiac. 2.0×10^6 cells were injected in the AP, or 2.5×10^4 cells were injected in the left ventricular of castrated nude mice. Mice were imaged two weeks after injection.

The third approved task was the Evaluation of recombinant HSA-PA conversion to cytotoxic Aerolysin *in vitro*. For this approved task we performed cell viability assays using trypan blue dye exclusion. The PSA expressing C19 and DU145 cell lines were incubated with HSA/PA at 1, 50, 100 nM doses for a total of four hours. Cell viability was determined on the ability of the cell membrane to prevent Trypan blue from being absorbed, **Figure 9A**. After a four-hour incubation, the PSA expressing C19 cells demonstrate a 40% reduction in cell membrane integrity with 50 nM HSA/PA. We also performed time lapse imaging of the same cell lines over 18 hr using 200 pM of HSA/PA. We chose this dose because the reported IC₅₀ of PA on LNCaP cells is 200 pM, and we wanted to determine if the toxicity is retained in the picomolar dose range. After 18 hours of treatment with HSA/PA, the PSA expressing C19 cells display rounded and sickly cell morphology similar to those of necrotic cells. In comparison to the DU145 cells which do not appear to be affected by the HSA/PA, **Figure 9B**. To evaluate any off-target cytotoxicity of HSA/PA in non-tumor bearing male mice were injected with varying doses of HSA/PA via the tail vein and evaluated 18 hours later. Unfortunately, all of the mice injected with the HSA/PA experienced some kind of severe toxicity after the injection. This unfortunate set back inhibited us from further evaluation of the recombinant pro-drug in *in vivo* tumor models.

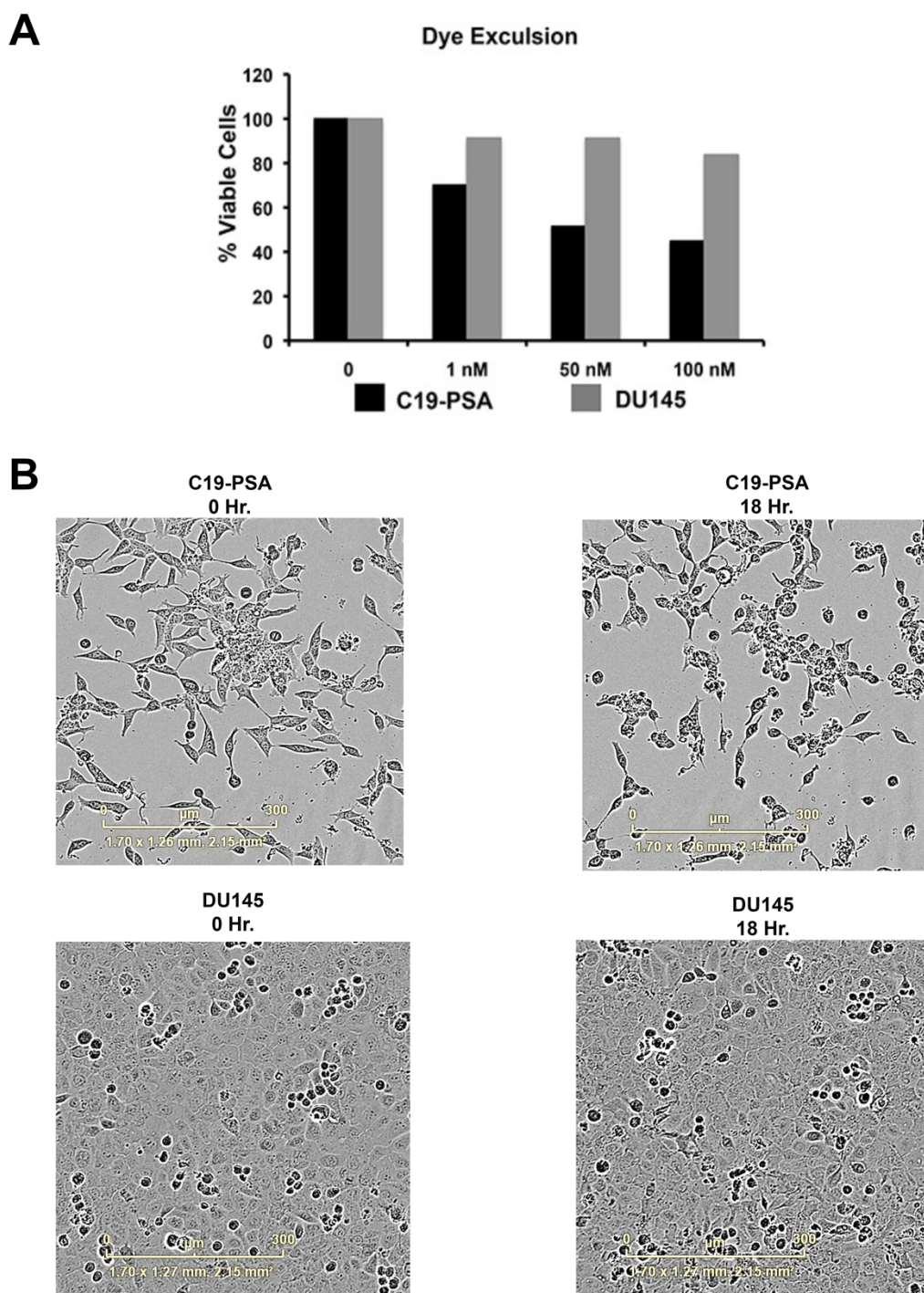


Figure 9. Recombinant HSA/PA retains cytotoxic properties and selectively activated by PSA expression cells. A) Dye Exclusion assay using Trypan blue on PSA expressing- C19 cells (black) and non-PSA expressing DU145 (grey) treated with HSA/PA at 0, 1, 50, 100nM for 4 hr. **B)** Time Lapse images of C19-PSA (top) and DU145 (bottom) treated with 200 pM HSA/PA for 18 hr.

Task 1. Engineering enzymatically cleavable linker for the release and activation of PA from HSA with lead peptides (Months 1-4): COMPLETED

1. Clone lead peptide sequences from corresponding proteases to active aerolysin produced in part 1 (Completed).
2. Engineer recombinant prodrug consisting of Human Serum Albumin (HSA) linked with active Aerolysin produced in part 2 (Completed)

Task 2. Determine specificity and stability of recombinant HSA-aerolysin in plasma (Months 5-12): Completed

1. Screen lead peptides for specificity to identified proteases, and screen for non-specific cleavage by related proteases (Completed).
2. Determine stability of recombinant HSA-aerolysin in plasma (Not completed).

Task 3. Evaluation of recombinant HSA-PA conversion to cytotoxic Aerolysin in vitro (Months 12-18): UNDER INVESTIGATION

1. Evaluate cytotoxicity of recombinant PA cleaved from HSA by purified proteases, to determine if our engineered PA retains cytotoxic characteristics (Completed).
2. Determine IC50 of PA liberated by purified proteases from HSA on prostate cancer cell lines (Completed).
3. Evaluate activation of recombinant HSA-PA by prostate cancer cell lines and benign prostate epithelial cell lines (Completed).
4. Evaluate off-target cytotoxicity of recombinant HSA-PA in non-tumor bearing mice (Completed).
5. Determine anti-tumor efficacy of recombinant HSA-PA for primary and metastatic prostate tumors (Not Completed).

KEY RESEARCH ACCOMPLISHMENTS

- Produce recombinant HSA/PA-PSA that is specifically activated by PSA.
- Recombinant HSA/PA-PSA retains nanomolar toxicity.
- Developed human PCa cell line that allows for live animal assessment of disseminated tumors.
- Established ELISA protocol for detection of PA in serum.
- Demonstrated selective activation of PA from HSA in the presence of PSA specifically.

TRAINING OPPORTUNITIES

Since receiving this award I have been afforded the opportunity to learn a very valuable technique under the direction of Donald Vander Griend, Ph.D. from the University of Chicago. Dr. Vander Griend has extensive experience with establishing disseminated tumor models using intracardiac injection. Dr. Vander Griend's demonstrations have allowed me to independently establish our C19-PSA, LUC2 lines in mouse models. Other avenues of training enrichment include attending the biannual Prostate Cancer United Kingdom meeting which arranged PCa researchers from both the United States and the United Kingdom. I have also presented my work at the Prostate SPORE meeting, which arranged researchers from Harvard, Johns Hopkins, University of California Los Angeles, University of Michigan, University of Washington.

IMPACT

The emergence of therapeutic resistant prostate cancer is an ominous clinical finding. Designing a recombinant prodrug that can be tailored for activation specifically by the tumor cells or by tumor associated stromal cells, allows for development of a combinational therapeutic targeted to the metastatic niche. Significant progress has been made towards achieving the state goals and completion of this work will allow us to push for our recombinant HSA/PA for clinical development as a systemic therapy for metastatic CRPC.

REFERENCES

1. Denmeade SR, Sokoll LJ, Chan DW, Khan SR, Isaacs JT. Concentration of enzymatically active prostate-specific antigen (PSA) in the extracellular fluid of primary human prostate cancers and human prostate cancer xenograft models. *Prostate*. 2001;48(1):1-6.
2. Stachowiak K, Tokmina M, Karpinska A, Sosnowska R, Wiczak W. Fluorogenic peptide substrates for carboxydipeptidase activity of cathepsin B. *Acta Biochim Pol*. 2004;51(1):81-92.
3. Brennen WN, Isaacs JT, Denmeade SR. Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. *Mol Cancer Ther*. 2012;11(2):257-66.
4. Janssen S, Jakobsen CM, Rosen DM, Ricklis RM, Reineke U, Christensen SB, et al. Screening a combinatorial peptide library to develop a human glandular kallikrein 2-activated prodrug as targeted therapy for prostate cancer. *Mol Cancer Ther*. 2004;3(11):1439-50.
5. Howard SP, Buckley JT. Activation of the hole-forming toxin aerolysin by extracellular processing. *J Bacteriol*. 1985;163(1):336-40.
6. Jennbacken K, Gustavsson H, Tesan T, Horn M, Vallbo C, Welen K, et al. The prostatic environment suppresses growth of androgen-independent prostate cancer xenografts: an effect influenced by testosterone. *Prostate*. 2009;69(11):1164-75.
7. Chandran SS, Williams SA, Denmeade SR. Extended-release PEG-luciferin allows for long-term imaging of firefly luciferase activity in vivo. *Luminescence*. 2009;24(1):35-8.

CHANGES/ PROBLEMS

We experienced off target toxicity which resulted in the death of non-tumor bearing animals. We could not detect any activated PA contaminating the recombinant HSA/PA preparations to explain the death of the animals. We did not observe severe necrosis at the site of injection, which would indicate immediate activation of the PA toxin from the albumin. We could only conclude that the recombinant prodrug was not stable after intravenous injection. This unforeseen problem hindered further investigation and further publication of results.

PRODUCTS

1. Recombinant Prodrug HSA/PA-PSA
2. C19-PSA LUC2 cell line

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Freddie L. Pruitt – NO CHANGE