Protease-Activated Pore-Forming Peptides for the Treatment and Imaging of Prostate Cancer

Aaron M. LeBeau¹* and Samuel R. Denmeade²

Affiliations: ¹Center for Molecular and Functional Imaging, Department of Radiology and Biomedical Imaging, University of California, San Francisco, San Francisco, California USA. ²Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland USA.

*Current address: Department of Pharmacology, University of Minnesota Masonic Cancer Center, Minneapolis, MN USA

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Abbreviations: PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; HD₅₀, 50% hemolysis; KLL, pore forming peptide, RBC, red blood cell.

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Address Correspondence to:

Aaron M. LeBeau, Ph.D.
Department of Pharmacology
University of Minnesota Masonic Cancer Center
6-120 Jackson Hall, 321 Church St. SE
Minneapolis, MN, 55455-0217
Phone: (612) 301-7231
Fax: (612) 625-8408
Email: alebeau@umn.edu
Abstract

A common hallmark of cancers with highly aggressive phenotypes is increased proteolysis in the tumor and the surrounding microenvironment. Prostate cancer has a number of proteases uniquely associated with it that may play various important roles in disease progression. In this report, we utilize the peritumoral proteolytic activity of prostate cancer to activate engineered peptide constructs for the treatment and non-invasive imaging of prostate cancer. Using a modular “propeptide” approach, a cationic diastereomeric pore-forming peptide domain was linked to an inactivating acidic peptide domain. The inactivating acidic peptide domain was engineered to be a cleavable substrate for the secreted serine protease prostate-specific antigen (PSA) or the transmembrane metalloprotease prostate-specific membrane antigen (PSMA). The propeptides were then evaluated in a direct comparison study. Both the PSA and PSMA activated propeptides were found to be cytotoxic to prostate cancer cells in vitro. In vivo, however, treatment of LNCaP and CWR22Rv1 xenografts with the PSMA propeptide resulted in a pronounced cytostatic effect when compared to xenografts treated with the PSA propeptide or the cationic diastereomeric peptide alone. The PSMA activated propeptide also proved to be an effective optical imaging probe in vivo when labeled with a near-infrared fluorophore. These data suggest that protease-activated pore forming peptides could potentially be used for both imaging and treating prostate cancer.
INTRODUCTION

The increased activity of membrane-bound and secreted proteases on the surface of cancer cells and in the transformed stroma is a common characteristic of cancer, and, specifically prostate cancer. The elevated peritumoral proteolysis associated with prostate cancer can be the direct result of protease over-expression, mislocalization or a concomitant decrease in the expression of endogenous protease inhibitors (1-3). Unregulated proteolysis results in the activation of growth factors, cytokines and dissolution of the extracellular matrix (ECM) (4-6). A number of proteases are unique to the prostate and prostate cancer. Prostate-specific antigen (PSA), a member of the kallikrein-related peptidase family of serine proteases, is expressed exclusively by normal and malignant prostate cells (7). PSA is inactivated in the serum due to binding to serum protease inhibitors. The presence of PSA covalently bound to the inhibitor α1-antichymotrypsin in the serum is commonly used as a biomarker for cancer detection and monitoring therapeutic efficacy. Other proteases, such as the kallikreins human glandular kallikrein 2 (hK2) and kallikrein 4 (KLK4) and the transmembrane metalloprotease prostate-specific membrane antigen (PSMA), have been investigated as potential biomarkers and promoters of disease progression (8-10).

With varying degrees of success, proteases have been targeted for potential therapeutic benefit using small-molecule active-site inhibitors in several cancer types (11, 12). Although they are highly destructive, the enzymatic activity of proteases can be exploited to activate targeted molecules for therapy and imaging. By harnessing their catalytic activity, molecules activated by proteases can overcome the traditional one-to-one stoichiometric binding of active-site targeted therapeutics and imaging agents to deposit limitless amounts of drugs or imaging probes at the site of the tumor. In the past, we have utilized the enzymatic activity of a number of proteases to activate prodrugs. Previously, we coupled the small molecule SERCA pump inhibitor thapsigargin to peptides carriers to create protease activated prodrugs (13). This prodrug was inactive because the carrier peptide prevented it from entering cells until the thapsigargin analog was liberated from the carrier peptide by proteolysis. Using this strategy, thapsigargin prodrugs have been developed for the proteases PSA, hK2, PSMA and the reactive stroma protease fibroblast activation protein (FAP) (14-17).
In this report, we detail the development of a protease-activated peptide technology to image and treat prostate cancer. For this “propeptide” technology, we used a modular platform consisting of a cationic diastereomeric peptide domain linked to an acidic peptide domain. The cationic diastereomeric domain was composed of D and L isomer lysine and leucine residues. Highly positively charged, this domain can disrupt the cell membrane leading to membrane depolarization and cell death. A structure function study was performed to determine the optimal size of the acidic peptide domain required for charge neutralization and inhibition of pore formation. Following optimization of the acidic inhibitory domain, the propeptides were engineered to be activated by the secreted protease PSA or the membrane-bound protease PSMA. This was accomplished by the addition of a PSA peptide substrate sequence in between the pore forming domain and the acidic inhibitor domain or by changing the acidic inhibitor domain into gamma linked glutamic acid residues to take advantage of the folate hydrolase ability of PSMA. A comparative study was then performed and the PSA and PSMA activated propeptides were evaluated for therapeutic efficacy in prostate cancer cell lines and xenografts to determine a lead candidate propeptide. The lead propeptide, which was activated by PSMA, was labeled for non-invasive near-infrared optical imaging in vivo and a biodistribution study using $^{99m}$Tc was performed. Our results suggests that engineered protease-activated propeptide technology could be potentially used for both imaging and therapy in prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostate cancer cell lines LNCaP, CWR22Rv1 and PC3 were purchased from American Type Culture Collection. The cell lines were authenticated using short-tandem repeat profiling provided by the vendor. The standard tissue culture conditions required that the cells be maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% pen/strep, and 2 mM l-glutamine (Invitrogen) in a 37°C incubator with 5% CO2 and 98% humidity. For serum free tissue culture conditions, the cells were grown in the absence of fetal bovine serum with normal supplements. The expression of androgen sensitive gene products (e.g. PSA) was induced by growing the cell lines under serum free conditions with 10nM of R1881 added to the media as previously described (18).
**Peptide Synthesis**

The reagents for Fmoc solid-phase peptide synthesis, including D-isomer amino acids, were purchased from Anaspec (San Jose, CA). All peptides were elongated using standard Fmoc solid-phase peptide conditions on an AAPPTEC Apex 396 peptide synthesizer (Louisville, KY) using Wang resin as previously described (19). AF680-γE-KLL was synthesized in the same method as the other peptides except for after the final Fmoc deprotection, Fmoc-Orn(Dde)-OH (Anaspec) was added as the final residue. After addition the side chain protecting group was removed with a 2% hydrazine/DMF solution for 10 minutes. Following deprotection and washing, 3 equivalents of AlexaFluor 680 NHS ester (Invitrogen) were added along with 2 equivalents of DIPEA in DMF. The resin solution was agitated for six hours followed by washing and deprotection of the final Fmoc. For the biodistribution study using D-KLL and AF680-γE-KLL, a single amino acid chelate group was created by chemical synthesis in multi-gram quantities using a previously described protocol and capped onto the peptides using normal coupling conditions on the peptide synthesizer (20).

The cleavage and deprotection of the peptides from the resin were carried out using a cleavage cocktail of TFA/thioanisole/water/phenol/EDT (82.5:5:5:5:2.5, v/v) for 4 hours. The peptides were precipitated from the cleavage cocktail using cold ether and dissolved in water for RP-HPLC purification. RP-HPLC purification was performed on a Waters Delta 600 semi-prep system using a Phenomenex Luna 10u C18 250 x 10 mm semi-prep column. The HPLC gradient profile was linear starting at 100% solvent A (0.1% TFA in H₂O) and changing to 100% solvent B (0.1% TFA in acetonitrile) over 25 min with a flow rate of 8 ml/min. Fractions of the desired purity (> 95% as determined using an analytical RP-HPLC) were pooled and lyophilized. The purified propeptides were mass analyzed on an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer using a matrix of 10 mg/ml 2,5-dihydroxy benzoic acid (DHB) in 50% ethanol/water. The mass spectrometer was calibrated using the ProteoMass Peptide MALDI Calibration Kit (Sigma). All spectra were acquired in the positive ion mode.

**Hemolysis Assays**

For the hemolysis assays, the peptides were dissolved in DMSO and were diluted using 1X PBS buffer to concentrations of 100 µM, 10 µM and 1µM. The peptides were incubated over a range of concentrations with washed human red blood cells (RBCs) at a concentration of 2% v/v
for 1 hr at 37°C. The control for zero hemolysis was RBCs suspended in PBS buffer alone and the 100% hemolysis control consisted of RBCs in the presence of 1% Triton X-100. Following incubation with the peptides, the RBCs were pelleted and 50 μl of each sample was transferred in triplicate to a clear flat bottom 96-well polystyrene plate. Hemolysis was assessed by measuring the absorbance of the samples at 540 nm with a Molecular Devices Spectra Max Plus automatic plate reader.

**Protease Digestion of the Peptides**

50 μg of γE-KLL was incubated with 5 μg of purified PSMA (gift from Shawn Lupold, Johns Hopkins) in 250 μl of PSMA assay buffer containing 10mM CoCl₂, 50 mM Tris, 100mM NaCl, pH 7.8 at 37°C. Aliquots of the digest were removed from the sample, desalted using P10-C18 ZipTips (Millipore), and spotted (0.5 μl) on a MALDI-TOF plate using the DHB matrix. Spectra were collected on an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer in positive ion mode.

**Cytotoxicity Assays**

Cell viability assays were performed in a 96-well plate format using approximately 3,000 cells per well. The cells were exposed to peptides over a range of concentrations for 72 hrs prior and then cell viability was determined using an MTT cell proliferation assay (Promega) as previously described and according to manufacturer’s instruction (19).

**Tumor xenograft studies**

Mouse care and treatment was approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California San Francisco. Cells maintained under standard conditions were detached by treatment with 0.25% trypsin–EDTA solution and washed in Hank’s balanced salt solution (HBSS). They were then suspended in a 60% mixture of Matrigel Matrix (BD Biosciences, San Jose, CA) in HBSS at a concentration of 2.0 x 10⁶ cells per 100μL of solution. The cells were then injected into the subcutis overlying the rear flanks of 6-week-old male nude mice (Harlan). Once the tumors were established, the animals were divided into four treatment groups per xenograft. The average tumor volumes were 98.6 ± 9.3mm³, 82.7 ± 5.1mm³ and 107.3 ± 13.1mm³ for the LNCaP,
CWR22Rv1 and PC3 xenografts respectively at day 0 of the study. The propeptides were dissolved in DMSO and diluted in sterile PBS to a final DMSO concentration of 5% v/v. The mice were then dosed via tail vein injection with 7 mg/kg of the propeptides at days 0, 3, 6 and 9. Tumor measurements were made twice weekly with calipers and the tumor volume (in mm\(^3\)) was calculated by the formula \(0.5236 \times L \times W \times H\). The endpoint of the study was either four weeks after the first treatment dose or when the tumors reached a volume of 1000 mm\(^3\) as dictated by our animal protocol.

**NIR Optical Imaging**

Mice for the optical studies were fed an alfalfa free diet of Harlan Tekland Global 2018 to minimize background fluorescence. The mice used for the imaging study had tumor volumes of 210 – 326 mm\(^3\) for the CWR22Rv1 xenografts and 280 – 420 mm\(^3\) for the PC3 xenografts. The mice were anesthetized with 2% isoflurane. 1.5 nmole of AF680-γE-KLL diluted in PBS buffer was injected via the tail vein of the mice in a volume of 150-200 µl. Three animals from each cell line xenograft were injected and imaged with optical imaging. Images were collected in fluorescence mode on an IVIS 50 using Living Image 2.50.2. Using the software, region of interest measurements were made and the fluorescence emission images were normalized to reference images and the unit less efficiency was computed.

**Biodistribution**

γE-KLL and D-KLL with N-terminal single amino acid chelate group caps were radiolabeled and HPLC purified as previously reported (21). Mice (n = 3 / peptide) bearing CWR22Rv1 xenografts between 171 – 240 mm\(^3\) in volume were tail vein injected with 10μCi of the labeled peptides. At six hours post-injection the animals were euthanized for analysis in accordance with UCSF Animal Care and Use Committee guidelines. Blood was collected by cardiac puncture. The tumor, heart, lung, spleen, kidneys, and muscle were harvested, weighed and counted in an automated γ-counter (Wizard2; Perkin Elmer). The percentage injected dose per gram (\% ID/g) of tissue was calculated by comparison with standards of known radioactivity.

**Statistical Analysis**
Data for the biodistribution were analyzed using the unpaired, 2-tailed Student t test. Differences at the 95% confidence level (P < 0.05) were considered to be statistically significant.

RESULTS

**Design, Synthesis, and Characterization of Protease Activated Pore Forming Peptides.**

Previous studies documented the discovery of a short 15-mer diastereomeric peptide composed of D, L isomer lysine and leucine amino acid residues (KLL; LKLKKLKKLKLKL-NH₂, underline residues are D-isomer) that was cytotoxic towards cancer cells *in vitro* and *in vivo* (22, 23). When administered via intratumoral or systemic injection, KLL was able to inhibit the growth of primary and metastatic tumor models with minimal toxicity observed in healthy neighboring tissues. By contrast, the fully L isomer version of the peptide had little effect on the tumor growth presumably due to the presence of degrading proteases that dramatically shortened the *in vivo* half-life. The selectivity of KLL for cancer cell membranes has been partly explained by its ability to bind phosphotidylserine exposed on the outer leaflet of cancer cell membranes. Biophysical studies with artificial membranes found that KLL adhered to phosphotidylserine containing membranes two to three fold better than non-phosphotidylserine containing membranes (24). As the KLL peptides oligomerize on the negatively charged outer leaflet of the cancer cell membrane, a threshold concentration is reached leading to pore formation, membrane depolarization, and ultimately cell death (23).

Though not specific for cancer cell membranes, the presence of proteoglycans and other glycoproteins on the surface further creates additional negative charge drawing the positively charged KLL peptides towards the vicinity of the membrane (25). This non-specific interaction with normal cell membranes decreases the population of available peptides to specifically interact with cancer cell membranes resulting in a peptide therapeutic that must be administered frequently and at high doses. By neutralizing the net positive charge of the KLL peptide with an acidic amino acid domain, derivatives of this peptide can be created to mask the non-specific cell binding potential. The acidic domain can be cleaved by cancer-associated proteases in the peritumoral environment yielding a membrane binding portion that can then be deposited selectively on the cancer cell surface for imaging or therapy (Figure 1). This “propeptide” approach could eliminate non-specific interactions with normal cell membranes resulting in a
more effective therapeutic that requires less frequent dosing at lower levels compared to the original KLL peptide (LKLKKLKKLLKLL-NH₂).

To assess the effect of charge neutralization on pore formation, KLL peptides containing all L isomer amino acids were used. Seven aspartic acid residues were added sequentially to either the amino or carboxyl termini of the KLL peptide to neutralize the positive charge (Figure 2). A hemolysis assay with 2% human red blood cells was used as a method to monitor charge neutralization. At a concentration of 100µM, the KLL peptide with a net charge of +7 resulted in the lysis of nearly 90% of the red blood cells (Figure 2A). As aspartic acid residues were added to amino terminus, the hemolytic ability of the peptides at 100µM decreased as the overall charge approached zero. The neutral peptide with seven acidic residues, D₇-KLL-NH₂, had a hemolysis of less than 20% at 100µM and had little hemolytic activity at 1µM. The addition of aspartic acid residues to the carboxyl terminus had a similar stepwise outcome (Figure 2B). The KLL peptide with an acidic carboxyl terminus and charge of +6 was equally as hemolytic as the KLL peptide with an amino carboxyl terminus at 100µM with 90% hemolysis observed. The positive charge of the peptide was neutralized by the addition of six aspartic acid residues to the carboxyl terminus, however, hemolysis for this peptide was nearly 50% at 100µM. By decreasing the charge further to -1, the detected hemolysis at 100µM was dramatically reduced to less than 10% with minimal hemolysis detected at 1µM. Amino terminus acetylated versions of the peptides were also synthesized and assayed (Figure 2C & D). Predictably, the hemolytic ability of these peptides decreased as the net charge of the peptides decreased as well. Comparison of the four groups of peptides tested showed that at least seven aspartic acid residues were required to reduce hemolytic potential and that the optimal placement for the linker region was at the carboxyl terminus (Figure 3A). Diasteriomeric peptides from the four groups were synthesized with seven aspartic acids residues and were found to demonstrate little hemolysis (Figure 3B).

For the lytic ability of the masked propeptides to be unleashed by PSA and PSMA, specific substrates for these proteases were engineered into the propeptide (Figure 1). PSA is a chymotrypsin-like protease that preferentially cleaves after large hydrophobic residues. In addition to this specificity, PSA also has the ability to cleave after glutamine residues—a characteristic highly unique for a mammalian serine protease (26). Previously, the development of a specific peptide substrate containing a P1 glutamine was reported for PSA (27). This
substrate, serine-serine-lysine-leucine-glutamine (SSKLQ), was incorporated into the peptide between the diastereomeric KLL sequence and aspartic acid linker to create the peptide PSA1 (cleavage site SSKLQ↓DD) (Figure 1). Since the presence of an acidic residue in the P’1 position might affect the ability of PSA to cleave the peptide, another peptide possessing an alanine residue in the P’1 position of the cleavage sequence was synthesized (PSA2) with a cleavage site of SSKLQ↓AD. PSA1 and PSA2 were non-hemolytic at 100µM and 10µM. Both peptides (25µM) were digested with purified PSA (15µg) for a total of 6 hours at 37°C. After 6 hours, red blood cells (2% v/v) were added to the digested peptides and allowed to incubate for an additional one hour. The digested peptides were able lyse the red blood cells with nearly 75% hemolysis observed, thus demonstrating that PSA1 and PSA2 were activated by PSA (Figure 3C).

Functionally, PSMA has been classified as a glutamate carboxypeptidase II based on its ability to hydrolyze the neuropeptide N-acetyl-l-aspartyl-l-glutamate into N-acetyl-aspartate and its bioactive component glutamate (28). PSMA also has the unique ability to function as a folate hydrolase (29). Possessing exopeptidase activity, PSMA can progressively hydrolyze γ-glutamyl linkages of poly-γ-glutamated folates (30). The design of the PSMA activated peptide (γE-KLL) took advantage of this unique characteristic of PSMA by replacing seven the aspartic acids residues in the acidic linker region with seven γ-linked glutamic acid residues. To assess PSMA cleavage, γE-KLL was assayed with purified recombinant PSMA to characterize the extent of PSMA mediated cleavage. γE-KLL (50µg) was incubated with PSMA (5µg) for 4 hours and the digestion was monitored using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Cleavage of γ-linked glutamic acid residues from the peptide sequence by PSMA was detected after four hours (Figure 3D). From the molecular ion peak at 2708.43 m/z, the stepwise cleavage of glutamic acids residues with a molecular weight of 129 m/z is observed down to the mass of the free KLL peptide at 1805.75 m/z. Incubation of γE-KLL with PSMA in the presence of EDTA to inhibit PSMA by binding the active site Zn2+ ion yielded no degradation of the γ-linked glutamic acid residues as detected by MALDI-TOF (Figure 3E).

**Evaluation of the peptides in PSA and PSMA expressing cell lines.**

After validating that the propeptides could be cleaved to the diastereomeric KLL sequence by purified or recombinant PSA and PSMA, the next step was to investigate their effect on PSA and
PSMA expressing prostate cancer cell lines. Activation of the peptides would result in cell death due to membrane depolarization caused by pore formation in the cancer cell membrane leading to potential therapeutic applications. For the PSA-activated peptides, PSA1 and PSA2, the PSA expressing human prostate cancer cell line LNCaP (high PSA-expressing) and CWR22Rv1 (low PSA-expressing) were used as positive controls and the PSA null human prostate cancer cell line PC3 as the negative control (31). When cultured using standard conditions in the presence of serum, both PSA1 and PSA2 had IC\textsubscript{50} values in these cell lines greater than >100µM (Table 1). Fetal bovine serum contains numerous proteases inhibitors making it likely that all of the enzymatically active PSA secreted by the cells was inhibited and unable to activate PSA1 and PSA2. Under serum free conditions, both PSA1 and PSA2 were activated by PSA resulting in the death of the LNCaP and CWR22Rv1 cell lines. PSA2 had lower IC\textsubscript{50} values in the three cell lines with values of 22.5µM and 27.1µM compared to PSA1 at 58.4µM and 66.1µM respectively. The values for PSA1 and PSA2 were significantly higher compared to the native KLL peptide along which possessed IC\textsubscript{50}s in the low micro molar range for the same cell lines.

The results from the serum-free media study suggested that PSA1 and PSA2 could either be degraded by the presence of other proteases or were not fully activated due to insufficient levels of active PSA. Since the transcription of PSA is under control of the androgen receptor in LNCaP and CWR22Rv1 cells, the synthetic androgen methyltrienlone (R1881) was used to stimulate increased production of PSA (31). After culturing in the presence of R1881 for five days, PSA1 and PSA2 were added to the stimulated cells. Androgen stimulation by R1881 decreased the IC\textsubscript{50} for PSA1 and PSA2 in the two cell lines. For PSA2 the most dramatic decrease was in LNCaP where the IC\textsubscript{50} deceased nearly by half from 23.5µM to 12.3µM in the presence of R1881. A less significant decrease for PSA2 were observed in CWR22Rv1 with a value of 19.6µM. Stimulation of PSA production with R1881 did affect the IC\textsubscript{50} of PSA1 in the two cell lines, but all of the values were still above 30µM. As a result of this PSA2 was the lead PSA-activated peptide for therapeutic efficacies\textit{ in vivo}. No cell death was detected in the PSA null prostate cancer cell line PC3 with PSA1 and PSA 2 under any of the conditions tested.

The ability of γE-KLL to be activated by PSMA was studied in the PSMA positive cell lines LNCaP and CWR22Rv1 under standard culture conditions containing 10% fetal bovine serum. PSMA is a metallopeptase and it retains its activity, unlike PSA, in the presence of serum due to a lack of inhibitors. When cultured with the two cell lines for 72hrs, γE-KLL was activated by
PSMA resulting in cell death. The IC$_{50}$ values were remarkably similar for the two cell lines with values near 10µM (Table 1). In the PSMA null cell line PC3, no toxicity was documented.

**The in vivo evaluation of the propeptides as therapeutics and imaging agents**

Encouraged by the results demonstrating potent and selective toxicity towards PSA and PSMA expressing cell lines *in vitro*, PSA2 and γE-KLL were next tested as therapeutics in LNCaP and CWR22Rv1 xenograft bearing mice. In a toxicity study, both propeptides were found to be non-toxic in healthy nude mice, however, at doses above 10mg/kg solubility issues became apparent with both propeptides. Mice bearing established LNCaP and CWR22Rv1 tumors were treated systemically via tail vein with 7mg/kg of PSA2, γE-KLL and KLL administered a total of four times with injections three days apart starting at day 0 of the study (Figure 4). Inhibition of tumor growth compared to the saline control was observed in the LNCaP mice treated with PSA2, γE-KLL and KLL at day 9 of the study. The difference between the tumor volume of the control arm and γE-KLL was first determined to be significant ($P < 0.05$) at day 9. After the final dose, however, tumor growth inhibition was lost in the PSA2 and KLL treated animals. By day 28, the tumor volume of the KLL group nearly equal to the saline control at day 28 (690.4 ± 93.2mm$^3$) while the PSA2 group had smaller tumors (445.7 ± 69.3mm$^3$) at the end of study. The LNCaP xenograft mice treated with γE-KLL demonstrated tumor growth inhibition throughout the study even after the final dose was administered with a final tumor volume of 220.3 ± 44.6mm$^3$ at day 28. The Treated tumor/Control tumor (T/C) ratio at day 28 for the γE-KLL was 0.3 for both LNCaP and CWR22Rv1. Additionally, in CWR22Rv1, PSA2 and KLL resulted in tumors that were double the volume of the γE-KLL treated arm by day 28. Established PC3 xenograft mice were also administered PSA2, γE-KLL and KLL using the same dosing regimen as for LNCaP and CWR22Rv1. No therapeutic effect observed in the PC3 tumors treated with γE-KLL, therefore, demonstrating that propeptide’s specificity for PSMA expressing xenografts.

Following these therapeutic trials, the most efficacious propeptide γE-KLL and the control KLL were next radiolabeled with $^{99m}$Tc using an amino hexanoic acid based single amino acid chelate group attached to the N-terminus of the peptides to compare their biodistribution in CWR22Rv1 xenografts. At 6hr post-injection, the tumor uptake of γE-KLL was significantly higher ($P < 0.05$) than the uptake of KLL (Figure 5A). Both peptides showed pronounced renal
clearance with minimal uptake in secondary organs. γE-KLL was next labeled with the near infrared (NIR) fluorophore AlexaFluor 680 for optical imaging. The propeptide was labeled with the fluorophore during solid phase peptide synthesis using ornithine with a 1-(4,4-dimethyl-2,6-dioxo-cyclohex-1-ylidene)ethyl (Dde) protecting group attached to the side chain. Following hydrazine deprotection of the Dde, the free amino side chain was labeled with an NHS ester AlexaFluor 680 derivative to create AF680-γE-KLL. The ability of AF680-γE-KLL to localize to PSMA-expressing tumors was tested in CWR22Rv1 xenograft bearing mice (n=3). Two hours post-tail vein injection a high background was detected with accumulation of AF680-γE-KLL in the tumor (Figure 5B). Over time the background decreased, the probe localized to the PSMA expressing tumor, and the signal in the kidney persisted (Figure 5B). AF680-γE-KLL was still detected in the kidney up to 24 hrs post-injection. This uptake was likely due to both clearance of the peptide imaging agent through the kidney and potential binding of AF680-γE-KLL to PSMA produced by within the proximal tubules of the mouse kidney. Remarkably, the signal from AF680-γE-KLL in the tumor was detectable up to 72hrs post-injection. No tumor localization of AF680-γE-KLL was detected in PC3 xenograft mice with only renal clearance observed (Figure 5B).

DISCUSSION

The goal of this study was to develop a platform technology that could exploit the hyper proteolytic peritumoral environment of prostate cancer to activate peptides for therapeutic and imaging applications. To accomplish this, we utilized a previously reported peptide design incorporating both D and L isomer amino acids. This peptide, which we referred to as KLL in our study, was entirely made up of lysine and leucine residues. The mode of action of KLL, and other pore forming peptides, has been thoroughly studied using various biophysical techniques (32). Electrostatic forces overwhelming attract the peptide to the surface of the cancer cell membrane. The positively charged KLL peptide, with poorly defined secondary structure, interacts with the phospholipid head groups on the outer membrane. As more and more peptides interact on the cell surface, a threshold concentration is reached and the peptides oligomerize, with defined secondary structure, to form pores that ultimately lead to membrane depolarization and cell death. Because the mechanism of cell killing targets an intrinsic structural feature, it is unlikely that the cancer cell can develop resistance making KLL an attractive candidate for drug
development. Even if a threshold concentration is not achieved to induce pore formation, the peptides would still be deposited on the cell surface. This makes KLL an ideal candidate for imaging probe development.

Previous studies found that KLL was effective at suppressing tumor growth and metastasis in several models, however, extremely high and frequent dosing of KLL were required to be effective. Since normal cell membranes also have a large amount of negatively charged components, it was possible that a significant fraction of the therapeutic dose did not reach the target cancer cells. Thus, the main factor limiting the implementation of KLL as a viable therapeutic was the inability to deliver enough of it to the site of the cancer to be effective. To overcome this problem, we developed a propeptide consisting of KLL coupled to a sufficient number of acidic amino acids to neutralize the positive charge and prevent membrane binding. Our study found that to effectively abrogate membrane lysis in an erythrocyte model, we had to add at least seven aspartic acid residues (D₇) to the peptide to produce a charge neutral compound. Working with this model we developed three protease-activated pore forming peptides. Two of the propeptides (PSA1 and PSA2) were activated by PSA by incorporating two PSA substrates in between the pore forming KLL domain and the acidic domain. A PSMA activated propeptide (γE-KLL) was created by modifying the acidic domain into a PSMA cleavable sequence consisting of seven γ-linked glutamic acid residues.

In vitro studies using LNCaP and CWR22Rv1 cells that express PSA found that PSA2 was more efficiently activated by PSA and resulted in greater cell killing. The reason for this can be explained by the different cleavage sequences used for PSA1 (SSKLQ↓DD) and PSA2 (SSKLQ↓AD). Compared to other proteases, PSA is an inefficient enzyme with poor reaction kinetics for small molecule peptide substrates. The kcat/km for the substrate sequence used in the design of PSA1 and PSA2 (SSLQ) is only $23.6 \text{ s}^{-1}\text{M}^{-1}$ (27). Additionally, analysis of the semenogelin cleavage map of PSA, from which the substrate SSKLQ was derived, found that PSA did not cleave any fragments that resulted in a P'1 acidic amino acid residue such as aspartic acid or glutamic acid (33). Phage display and other techniques have documented that serine or small aliphatic residues are the preferred amino acids in the P'1 position for PSA (34). Therefore, the PSA1 sequence was the least optimal of the two propeptide sequences as demonstrated by the IC₅₀ values in Table 1.
The PSMA peptide was easily hydrolyzed by recombinant PSMA and was uniformly lethal in vitro in LNCaP and CWR22Rv1 cells. A study by Wang et al. found that the number of PSMA molecules differed greatly between LNCaP and CWR22Rv1 cells when measured using their anti-PSMA antibody (35). They determined that LNCaP expressed nearly ten-fold more PSMA molecule per cell than CWR22Rv1. Denmeade et al. documented that the enzymatic activity of PSMA in LNCaP and CWR22Rv1 cells were remarkable similar when measured by $^3$H NAAG assay (31). Our results and the previous finding suggest that even though PSMA is more abundant on LNCaP cells, it is an efficient enough of enzyme that both cell lines work as models for PSMA activity. This trend carried over into the in vivo xenografts studies where γE-KLL prevented tumor growth in both LNCaP and CWR22Rv1 xenografts. PSA2 was largely ineffective in vivo. This finding could again be attributed back to the poor catalytic efficiency of PSA as an enzyme and a lack of enzymatically active PSA. It is known that several mouse serum protease inhibitors can bind PSA leading to decreased levels of PSA available to cleave PSA2 (36). Another factor that could explain the decreased efficacy of PSA2 compared to γE-KLL is that PSA2 is activated by a secreted protease. The activation product of γE-KLL by PSMA is immediately juxtaposed to the target cancer cell membrane, thus, reducing the possibility of the activated peptide diffusing through the extracellular matrix and binding to non-target membranes. With PSA2, activation can occur far from the target membrane resulting in a diluted peptide population that has to navigate through an environment consisting of many non-target membranes to find the target. When administered at the same dose as PSA2 and γE-KLL, KLL demonstrated little therapeutic effect in the in vivo study. The treatment used in this study was four doses of 7mg/kg over a nine days compared to the previously reported regimen (nine doses of 9mg/kg over nine days) that demonstrated therapeutic efficacy (22). Our findings suggested that γE-KLL had a longer circulating half-life compared to the native KLL peptide or it was more effective at localizing to the site of the tumor. In the NIR optical imaging experiment, high tumor localization was apparent with AF680-γE-KLL. This was corroborated by the biodistribution data of $^{99m}$Tc labeled versions of KLL and γE-KLL. Six hours post-injection the tumor uptake of γE-KLL was significantly higher than the uptake of KLL suggesting the greater bioavailability and retention of γE-KLL compared to KLL.

Other groups have used strategies similar to our propeptide approach. All of these other approaches have employed protease activated cell penetrating peptides to image tumors or sites
of thrombin activity (37-39). These previous applications have been purely diagnostic in nature with no therapeutic component. Likewise, they have relied on the use of endopeptidases to activate their molecules. Our approach is novel in that our molecules can be used as theranostic agents to both image and treat the cancer with a single agent. Additionally, the fact that γE-KLL is activated by the unique exopeptidase activity of PSMA makes it novel from other approaches in the literature for both therapy and imaging. Aside from our thapsigargin analogues, no other PSMA activated prodrugs have been reported in the literature. A number of nuclear and NIR imaging agents for PSMA have been reported. These antibody or inhibitor based PSMA targeted imaging agents bind to the active site of the enzyme in a one-to-one stoichiometry. Based on these stoichiometric limitations, it is highly plausible that many of the current PSMA-targeted imaging agents are not sensitive enough to accurately detect cancers with low levels of PSMA on the cell surface. In contrast, an advantage of the propeptide strategy is that targeted release of the imaging agent by the proteolytic activity of PSMA allows for continued accumulation of the probe in the cell membrane, thus amplifying the signal over time.

Acknowledgements

The authors would like to thank Marc Rosen, Anna Solomon and Stephanie Murphy for their technical assistance with some of the experiments. The authors would also like to dedicate this paper to the memory of Dr. Michael J. Page. This research was funded by a Prostate Cancer Foundation Young Investigator Award (A.M. LeBeau) and a Department of Defense Prostate Cancer Research Program Postdoctoral Award PC094386 (A.M. LeBeau).
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TABLES

**Table 1.** Calculated IC$_{50}$ values (μM) for PSA1, PSA2 and KLL as determined by MTT assay after a 72hr incubation. Values were calculated using stand cell culture conditions with 10% FBS (Std.), serum free (SF), and in the presence of the synthetic androgen R1881 (R1881). All values are reported are μM. For the γE-KLL Ic50 calculation, the cell lines were treated under standard cell culture conditions for 72hrs.

<table>
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<tr>
<th></th>
<th>LNCaP</th>
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<th>CWR22Rv1</th>
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<th>PC3</th>
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<tbody>
<tr>
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<td>Std.</td>
<td>SF</td>
<td>R1881</td>
<td>Std.</td>
<td>SF</td>
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<tr>
<td>PSA1</td>
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<td>58.4 ± 6.3</td>
<td>31.6 ± 1.8</td>
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<td>27.1 ± 1.7</td>
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<td>10.2 ± 0.5</td>
<td>&gt;100</td>
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</table>
FIGURE LEGENDS

**Figure 1.** Schematic of the propeptide technology developed in this technology. A propeptide consists of a positively charged pore forming domain (purple) coupled to an acidic inhibitory domain (red) that prevents pore formation. Proteolysis mediated by a cancer-associated protease liberates the pore forming domain from the inhibitory domain allowing for insertion into the cancer cell membrane and pore formation. Below are the acidic inhibitory domain structures for PSA1, PSA2, and γE-KLL. The structures of PSA1 and PSA2 take advantage of the unique endopeptidase ability of PSA to cleave after glutamine residues. The PSMA activated propeptide, γE-KLL, harnesses the folate hydrolase activity of PSMA to stepwise remove the poly gamma glutamic acid linked inhibitory domain.

**Figure 2.** Structure function study to determine the optimal length of the acidic inhibitory domain for neutralization. Above each set of bars depicting the hemolytic ability of the peptides at three different concentrations (1μM, 10μM, and 100μM) are the net charges of the peptides. Graphs showing the effect of adding aspartic acid residues to the A.) N-terminus of the peptide (D<sub>n</sub>-KLL-NH<sub>2</sub>) and B) the C-terminus of the peptide (KLL-D<sub>n</sub>-OH). Also shown are graphs showing the effect on N-terminal acetylation for C.) Ac-D<sub>n</sub>-KLL-NH<sub>2</sub> and D.) Ac-KLL-D<sub>n</sub>-OH.

**Figure 3.** A.) Comparison of the hemolytic ability of the four groups of peptides synthesized as a function of acidic inhibitory domain length. B. D-isomer peptides were synthesized with seven aspartic acids residues and were found to demonstrate little hemolysis. C.) Hemolysis of PSA1
and PSA2 after *in vitro* activation by recombinant PSA followed by incubation with 2% red blood cells. D.) The stepwise cleavage of the gamma glutamic acid inhibitory domain of γE-KLL by PSMA. γE-KLL was incubated with recombinant PSMA and degradation was monitored using MALDI-TOF mass spectrometry. E.) The stepwise cleavage of the acidic inhibitory domain was prevented by the addition of EDTA in the buffer resulting in an uncleaved molecular ion peak at 1805.75 m/z.

**Figure 4.** Therapeutic evaluation of PSA2, γE-KLL, and KLL in subcutaneous xenograft models of human prostate cancer. Nude mice bearing established LNCaP (PSA⁺, PSMA⁺), CWR22Rv1 (PSA⁺, PSMA⁺) and PC3 (PSA⁻, PSMA⁻) xenograft tumors were treated with 7mg/kg of PSA2, γE-KLL, KLL or saline control at days 0, 3, 6 and 9 by tail vein injection as denoted by the arrows on each graph. Each treatment group consisted of n = 10 mice/xenograft and tumor measurements were made out to day 28. For the PC3 xenograft experiment, the study was halted as soon as the tumors exceeded a volume of 1000mm³ as dictated by our animal protocol. Statistical significance of γE-KLL compared to control is denoted by * (P < 0.05) and ** (P < 0.01) as determined by Student’s t test.

**Figure 5.** A.) Biodistribution study of \(^{99m}\)Tc labeled γE-KLL and KLL in CWR22Rv1 xenograft mice (n = 3) at six hours post injection of 5μCi of probe (*, P < 0.05 by Student’s t test for γE-KLL versus KLL). The tumor volumes of the CWR22Rv1 xenograft mice used were 201.2 ± 26.2 mm³. Probe localization is expressed as percent injection dose per gram (%ID/g). B.) NIR optical imaging using AF680-γE-KLL in subcutaneous prostate cancer xenografts CWR22Rv1 (top) and PC3 (bottom), red arrow denotes tumor location. CWR22Rv1 xenograft before the injection of 1.5 μmoles of AF680-γE-KLL. Images collected at 2hrs, 24hrs, 48hrs and 72hrs post-injection of the probe via the tail vein. Imaging of AF680-γE-KLL in the PSMA negative PC3 xenograft at 6hrs and 24hrs post-injection.
Propeptide → Proteolysis → Pore Formation

Pore Forming Domain

Acidic Inhibitor Domain

NH₂ → PSA1: SSKLQ↓DDDDDDD → CO₂H

PSA2: SSKLQA↓DDDDDDD

γE-KLL: γE, E, E, E, E, E, E
Figure 2

A.)

B.)

C.)

D.)
Figure 3

A.)

![Graph showing percent hemolysis against number of Asp (D) with different peptides: Dn-KLL-NH2, KLL-Dn-OH, Ac-Dn-KLL-NH2, Ac-KLL-Dn-OH.]

B.)

![Graph showing percent hemolysis for Dn-KLL-NH2, KLL-Dn-OH, Ac-Dn-KLL-NH2, Ac-KLL-Dn-OH with error bars.]

C.)

![Graph showing PSA1 and PSA2 with percent hemolysis.]

D.)

![Mass spectrometry data with peaks at various m/z values: Glu1, Glu2, Glu3, Glu4, Glu5, Glu6, Glu7, 1805.73, 1934.69, 1993.66, 2063.66, 2192.63, 2321.59, 2450.51, 2580.47, 2708.43, 2708.84.]

E.)

![Mass spectrometry data with peaks at 2708.84.]
Figure 4

The graphs show the tumor volume (mm$^3$) over days for different cell lines and treatments:

- **LNCaP**
- **CWR22Rv1**
- **PC3**

- **Control**
- **KLL**
- **PSA2**
- **γE-KLL**

The arrows indicate significant differences between treatments. The y-axis represents tumor volume in mm$^3$, and the x-axis represents days from 0 to 28.
Figure 5

A.)

B.)

CWR22Rv1

Pre-injection  2hrs  24hrs  48hrs  72hrs

PC3

6hrs  24hrs
PROTEASE-ACTIVATED PORE-FORMING PEPTIDES FOR THE TREATMENT AND IMAGING OF PROSTATE CANCER

Aaron M. LeBeau and Samuel R. Denmeade

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