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

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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 noninvasive 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 with 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. Mol Cancer Ther; 14(3); 1–10. ©2014 AACR.

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 overexpression, 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; refs. 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 used 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 prostate fibroblast activation protein (FAP; refs. 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...

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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 noninvasive near-infrared optical imaging in vivo and a biodistribution study using 99mTc was performed. Our results suggest 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 the 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 mmol/L 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 10 mmol/L of R1881 added to the media as previously described (18).

**Peptide synthesis**

The reagents for Fmoc solid-phase peptide synthesis, including α-isomer amino acids, were purchased from AnaSpec. All peptides were elongated using standard Fmoc solid-phase peptide conditions on an AAPTEC Apex 396 peptide synthesizer using Wang resin as previously described (19). AF680-γE-KLL was synthesized in the same method as the other peptides except for 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/dimethylformamide (DMF) solution for 10 minutes. Following deprotection and washing, 3 equivalents of AlexaFluor 680 NHS ester (Invitrogen) were added along with 2 equivalents of diisopropylethylamine in DMF. The resin solution was agitated for 6 hours followed by washing and deprotection of the final Fmoc. For the biodistribution study using 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 (82.5:5:5:2.5:2.5, v/v) for 4 hours. The peptides were precipitated from the cleavage cocktail using cold ether and dissolved in water for reverse-phase high-performance liquid chromatography (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% trifluoroacetic acid (TFA) in acetonitrile] over 25 minutes 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 matrix-assisted laser desorption/ionization time-of-flight (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 1× PBS buffer to concentrations of 100, 10, and 1 μmol/L. The peptides were incubated over a range of concentrations with washed human red blood cells (RBC) at a concentration of 2% v/v for 1 hour 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**

Fifty micrograms of γE-KLL was incubated with 5 μg of purified PSMA (gift from Shawn Lupold, Johns Hopkins University School of Medicine, Department of Urology) in 250 μL of PSMA assay buffer containing 10 mmol/L CoCl2, 50 mmol/L Tris, 100 mmol/L 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 hours prior and then cell viability was determined using an MTT cell proliferation assay (Promega) as previously described and according to the 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 Minnesota. 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) in HBSS at a concentration of 2.0 x 10^6 cells per 100 μL 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 ± 9.3 mm^3, 82.7 ± 5.1 mm^3, and 107.3 ± 13.1 mm^3 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 growth rates were plotted and averaged.

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volume (in mm³) was calculated by the formula 0.5236 × length (L) × width (W) × height (H). The endpoint of the study was either 4 weeks after the first treatment dose or when the tumors reached a volume of 1,000 mm³ 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 to 326 mm³ for the CWR22Rv1 xenografts and 280 to 420 mm³ for the PC3 xenografts. The mice were anesthetized with 2% isoflurane. AF680-γE-KLL (1.5 nmol) diluted in PBS buffer was injected via the tail vein of the mice in a volume of 150 to 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 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 and 240 mm³ in volume were tail vein injected with 10 μCi of the labeled peptides. At 6 hours postinjection, the animals were euthanized for analysis in accordance with 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; PerkinElmer). 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, two-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; LKKLLKKLLKKLLKLL-NH₂, underline residues are D-isomer) that was cytotoxic toward 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. In 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 phosphatidylserine exposed on the outer leaflet of cancer cell membranes. Biophysical studies with artificial membranes found that KLL adhered to phosphatidylserine containing membranes two-to three-fold better than nonphosphatidylserine-containing membranes (24). As the KLL peptides oligomerize on the negatively charged outer leaflet of the cancer cell membrane, a threshold old concentration is reached leading to pore formation, membrane depolarization, and ultimately cell death (23).

Although 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 toward the vicinity of the membrane (25). This nonspecific 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 nonspecific 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 (Fig. 1). This "propeptide" approach could eliminate...
nonspecific interactions with normal cell membranes resulting in a more effective therapeutic that requires less frequent dosing at lower levels compared with the original KLL peptide (LKKLLKKLL-KLL-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 (Fig. 2). A hemolysis assay with 2% human red blood cells was used as a method to monitor charge neutralization. At a concentration of 100 μmol/L, the KLL peptide with a net charge of +7 resulted in the lysis of nearly 90% of the red blood cells (Fig. 2A). As aspartic acid residues were added to amino terminus, the hemolytic ability of the peptides at 100 μmol/L 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 100 μmol/L and had little hemolytic activity at 1 μmol/L. The addition of aspartic acid residues to the carboxyl terminus had a similar stepwise outcome (Fig. 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 100 μmol/L 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 100 μmol/L. By decreasing the charge further to –1, the detected hemolysis at 100 μmol/L was dramatically reduced to less than 10% with minimal hemolysis detected at 1 μmol/L. Amino terminus acetylated versions of the peptides were also synthesized and assayed (Fig. 2C and 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 (Fig. 3A). Diastereomeric peptides from the four groups were synthesized with seven aspartic acids residues and were found to demonstrate little hemolysis (Fig. 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 (Fig. 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.

![Figure 2.](image-url)

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 (1 μmol/L, 10 μmol/L, and 100 μmol/L) are the net charges of the peptides. Graphs showing the effect of adding aspartic acid residues to the (A) N-terminus of the peptide (D₇-KLL-NH₂) and (B) the C-terminus of the peptide (KLL-D₇-OH). Also shown are graphs showing the effect on N-terminal acetylation for (C) Ac-D₇-KLL-NH₂ and (D) Ac-KLL-D₇-OH.
to create the peptide PSA1 (cleavage site SSKLQ|DD; Fig. 1). Because 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 nonhemolytic at 100 μmol/L and 10 μmol/L. Both peptides (25 μmol/L) were digested with purified PSA (15 μg) 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 1 hour. The digested peptides were able to lyse the red blood cells with nearly 75% hemolysis observed, thus demonstrating that PSA1 and PSA2 were activated by PSA (Fig. 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 of the aspartic acid residues in the acidic linker region with seven γ-linked glutamic acid residues. To bind PSMA cleavage, γE-KLL was assayed with purified recombinant PSMA to characterize the extent of PSMA-mediated cleavage. γE-KLL (50 μg) was incubated with PSMA (5 μg) for 4 hours and the digestion was monitored using MALDI-TOF mass spectrometry. Cleavage of γ-linked glutamic acid residues from the peptide sequence by PSMA was detected after 4 hours (Fig. 3D). From the molecular ion peak at 2,708.43 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 1,805.75 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 (Fig. 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 IC50 values in these cell lines greater than >100 μmol/L (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 IC50 values in the three cell lines with values of 22.5 μmol/L and 27.1 μmol/L compared with PSA1 at 58.4 μmol/L and 66.1 μmol/L, respectively. The values for PSA1 and PSA2 were significantly higher compared with the native KLL peptide along which possessed IC50s in the low micromolar 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. Because the transcription of PSA is under control of the androgen receptor in LNCaP and CWR22Rv1 cells, the synthetic androgen methyltrienolone (R1881) was used to stimulate increased production of PSA (31). After culturing in the presence of R1881 for 5 days, PSA1 and PSA2 were added to the stimulated cells. Androgen stimulation by R1881 decreased the IC50 of PSA1 in the two cell lines, but all of the values did not affect the IC50 of PSA1 in the two cell lines, but all of the values were still above 30 μmol/L, indicating that PSA1 and PSA2 could either be degraded by the presence of serum due to a lack of inhibitors. When cultured with the two cell lines for 72 hours, γE-KLL was activated by PSMA, resulting in cell death. The IC50 values were remarkably similar for the two cell lines with values near 10 μmol/L (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 toward 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 nontoxic in healthy nude mice, however, at doses above 10 mg/kg solubility issues became apparent with both propeptides. Mice bearing established LNCaP and CWR22Rv1 tumors were treated systemically via tail vein injection with 7 mg/kg of PSA2, γE-KLL and KLL administered a total of four times with injections 3 days apart starting at day 0 of the study (Fig. 4). Inhibition of tumor growth compared with 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 ± 93.2 mm³), whereas the PSA2 group had smaller tumors (445.7 ± 69.3 mm³) at the end of study. The LNCaP xenograph 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 ± 44.6 mm³ 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. In addition, in CWR22Rv1, PSA2 and KLL resulted in

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**Table 1.** Calculated IC50 values (μmol/L) for PSA1, PSA2, and KLL as determined by MTT assay after a 72-hour incubation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PSA1</th>
<th>PSA2</th>
<th>KLL</th>
<th>PSA2</th>
<th>KLL</th>
<th>PSA2</th>
<th>KLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>&gt;100</td>
<td>58.4 ± 6.3</td>
<td>31.6 ± 1.8</td>
<td>&gt;100</td>
<td>66.1 ± 4.8</td>
<td>58.2 ± 5.4</td>
<td>&gt;100</td>
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<tr>
<td>CWR22Rv1</td>
<td></td>
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NOTE: Values were calculated using standard cell culture conditions with 10% FBS (Std.), serum free (SF), and in the presence of the synthetic androgen R1881 (R1881). All values reported are in μmol/L. For the γE-KLL IC50 calculation, the cell lines were treated under standard cell culture conditions for 72 hours.

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**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 7 mg/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 1,000 mm³ as dictated by our animal protocol. Statistical significance of γE-KLL compared with control is denoted by: *, P < 0.05; and **, P < 0.01, as determined by the Student t test.
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 pro-
peptide’s specificity for PSMA-expressing xenografts.

Following these therapeutic trials, the most efficacious propep-
tide γE-KLL and the control KLL were next radiolabeled with99mTc
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 6 hours postinjec-
tion, the tumor uptake of γE-KLL was significantly higher ($P$
< 0.05) than the uptake of KLL (Fig. 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-cyclo-
hex-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 after tail
vein injection, a high background was detected with accumula-
tion of AF680-γE-KLL in the tumor (Fig. 5B). Over time, the
background decreased, the probe localized to the PSMA-express-
ing tumor, and the signal in the kidney persisted (Fig. 5B). AF680-γE-KLL was still detected in the kidney up to 24 hours postinjection. 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 72 hours post-
injection. No tumor localization of AF680-γE-KLL was detected in
PC3 xenograft mice with only renal clearance observed (Fig. 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 used 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 thor-
oughly studied using various biophysical techniques (32). Elec-
 trostatic forces overwhelmingly attract the peptide to the surface
of the cancer cell membrane. The positively charged KLL peptide,
with poorly de

Figure 5.
A, biodistribution study of99mTc
labeled γE-KLL and KLL in CWR22Rv1
xenograft mice ($n$ = 3) at 6 hours after
injection of 5 μCi of probe ($^*$, $P$ < 0.05
by Student t test for γE-KLL versus
KLL). The tumor volumes of the
CWR22Rv1 xenograft mice used were
201.2 ± 26.2 mm$^3$. Probe localization is
expressed as percentage of 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, tumor location.
CWR22Rv1 xenograft before the
injection of 1.5 μmoles of AF680-γE-
KLL. Images collected at 2, 24, 48, and
72 hours after injection of the probe
via the tail vein. Imaging of AF680-γE-
KLL in the PSMA-negative PC3
xenograft at 6 and 24 hours after
injection.
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. Because 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 (D-) 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 PSMA 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/D) and PSA2 (SSKLQ/AD). Compared with 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 (SSKLQ) is only 23.6 s⁻¹ (mol/L)⁻¹ (27). In addition, 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 P1 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 preferred amino acids in the P1 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 and colleagues 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 10-fold more PSMA molecule per cell than CWR22Rv1. Denmeade and colleagues documented that the enzymatic activity of PSMA in LNCaP and CWR22Rv1 cells were remarkable similar when measured by ¹¹CNAAG assay (31). Our results and the previous finding suggest that even though PSMA is more abundant on LNCaP cells, it is an efficient enough 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 with 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 nontarget 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 nontarget 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 7 mg/kg over 9 days compared with the previously reported regimen (nine doses of 9 mg/kg over 9 days) that demonstrated therapeutic efficacy (22). Our findings suggested that γE-KLL had a longer circulating half-life compared with 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 ⁹⁹ᵐTc labeled versions of KLL and γE-KLL. Six hours after 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 with KLL.

Other groups have used strategies similar to our propeptide approach. All of these other approaches have used 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. In addition, the fact that γE-KLL is activated by the unique exopeptidase activity of PSMA makes its 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. LeBeau, S.R. Denmeade

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.R. Denmeade

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