Amphipathic Peptide-Based Fusion Peptides and Immunoconjugates for the Targeted Ablation of Prostate Cancer Cells

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Abstract

We describe the design, generation, and in vitro evaluation of targeted amphipathic fusion peptides and immunoconjugates for the ablation of prostate cancer cells. The overexpression of the prostate-specific membrane antigen (PSMA) was exploited as means to specifically deliver cytotoxic peptides to prostate cancer cells. Cationic amphipathic lytic peptides were chosen as cytotoxic agents due to their ability to depolarize mitochondrial membranes and induce apoptosis. Specific delivery of the lytic peptide was facilitated by PSMA-targeting peptides and antibodies. Our results indicate that although the use of PSMA-targeted peptides only modestly enhanced the cytotoxic activity of the lytic peptide, peptide-antibody conjugates were two orders of magnitude more potent than untargeted peptide. In addition to quantifying the cytotoxic activities of the individual constructs, we also investigated the mechanisms of cell death induced by the fusion peptides and immunoconjugates. Although fusion peptides induced oncotic/necrotic death in cells, treatment with immunoconjugates resulted in apoptotic death. In summary, immunoconjugates based on lytic peptides are a promising class of therapeutics for prostate cancer therapy and warrant further investigation. [Cancer Res 2007;67(13):6368–75]

Introduction

Prostate cancer is the most commonly diagnosed malignancy and is the second leading cause of cancer death in men; ~30,000 deaths occur every year in the United States due to the disease (1, 2). Localized prostate cancer is generally treated with surgery (radical prostatectomy), radiation therapy, or cryotherapy (3). However, disease relapse after surgery is a common occurrence, mainly due to the outgrowth of minimal residual disease (3). Currently, patients with recurrent, locally advanced, or metastatic prostate cancer are treated by androgen deprivation alone or in combination with local therapy. Although most patients initially respond to androgen deprivation, a large fraction of patients suffer disease progression and develop androgen-independent, hormone-refractory prostate cancer. The metastasis of these androgen-independent cells, which are also resistant to conventional therapies such as radiotherapy, is the major cause of death in prostate cancer. Treatment options in these cases are fairly limited, and most available treatments rely on chemotherapeutic drugs (4), which have variable efficacies and severe side effects. As a result, there is a need to develop site-specific (targeted) therapeutics for the treatment of residual and metastatic prostate cancer.

The prostate-specific membrane antigen (PSMA) is a type II membrane glycoprotein with an intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750; refs. 5, 6). PSMA is abundantly expressed in all stages of prostate cancer disease; the expression of the protein increases in cases of hormone-refractory disease, metastasis, and advanced disease, making it an attractive target for site-specific prostate cancer therapy (7–11). Although PSMA is also expressed by cells in the small intestine, proximal renal tubules, and salivary glands, the level of expression in these tissues is 100- to 1,000-fold less than in prostate tissue, rendering a high degree of potential selectivity for site-specific therapeutic agents (5, 7–11). Furthermore, PSMA is also expressed in the neovascularature of a wide variety of malignant neoplasms, including prostate, lung, colon, breast, and others, but not in normal vascular endothelium (12). Significantly, PSMA and PSMA-antibody complexes have been shown to undergo internalization through clathrin-coated pits (12, 13), indicating that the receptor can be used for intracellular delivery of therapeutics. Consequently, different strategies, including antibodies and antibody-conjugated toxins, have been investigated for targeting PSMA-expressing prostate cancer cells (14–16).

Peptides/fusion peptides are increasingly gaining popularity as therapeutic agents for a variety of applications (17), including antiretroviral therapy (18), nucleic acid delivery (19), tumor vaccines (20), antimicrobial therapy (21), and neurodegenerative diseases (22). Peptide-based therapeutics are also attractive candidates for anticancer therapy (23–26), including prostate cancer (27, 28). Peptide therapeutics are easily produced using either recombinant or solid-phase chemical synthesis techniques and are generally less expensive than antibody-based therapeutics. In addition, as opposed to immunoconjugates, the small size of fusion peptides makes it possible to deliver them using routes other than intravenous injection (17). However, the use of peptides as targeting agents in site-specific delivery is often limited by their low binding affinities to their respective targets compared with those of antibodies. Furthermore, their small molecular size results in rapid clearance by the reticuloendothelial system and degradation by proteases in the body.

The antimicrobial peptide KLA (ref. 29; single-letter amino acid sequence: KLAKLAKKLAK) has been shown to induce apoptosis in cancer cells, presumably due to its ability to depolarize mitochondrial membranes (23). The large negative potential (~180 mV) across the mitochondrial membrane (30) promotes the accumulation of the positively charged amphipathic peptide at its surface. In time, this localization results in membrane depolarization, a membrane permeability transition, and the release of mitochondrial contents into the cytoplasm. The release of proapoptotic proteins from the mitochondria, including cytochrome c, second mitochondrial derived activator of caspase, and apoptosis-inducing factor (AIF), ultimately results in apoptosis (31).
We describe the design, generation, and evaluation of PSMA-targeted fusion peptides and antibody-peptide conjugates as site-specific therapeutics for advanced prostate cancer disease. The recent identification of peptides targeting the extracellular portion of PSMA (32) enables the development of peptide-based strategies for targeting malignant prostatic tissue. We designed a fusion peptide composed of a PSMA-targeting peptide (PTP) and the cationic amphipathic peptide KLA, and investigated the in vitro efficacy, selectivity, and mechanism of cell death induced by the PTP-KLA fusion peptide in LNCaP and PC-3 human prostate cancer cell lines. In addition, we explored the in vitro efficacy and mechanisms of cell death induced by anti-PSMA antibody-KLA immun conjugates. Our results indicate that the although the PTP-KLA fusion peptide possesses only moderate potency, the antibody-KLA conjugate potently and selectively induces cell death in PSMA-expressing LNCaP cells, thus meritng further investigating as a potential therapeutic for prostate cancer.

Materials and Methods

**Cell lines and cell culture.** Human prostate cancer cell lines LNCaP (PSMA positive) and PC-3 (PSMA negative) were obtained from the American Type Culture Collection (ATCC). LNCaP cells were grown in RPMI 1640 (ATCC) + 10% fetal bovine serum (FBS; Invitrogen Corp.) + 1% penicillin/streptomycin (Invitrogen). PC-3 cells were cultured in F-12K media (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. The cells were cultured at 37°C in a 5% CO₂ incubator. All chemicals were purchased from Sigma unless otherwise specified.

**Fusion peptides.** The following peptides were purchased from the Tufts University Core Facility, Medford, MA:

1. KLA: HHHHHKLLAKKAKLAKC (the cationic amphipathic, lytic peptide sequence is in bold font)
2. PTP: CQKHHNYLCGHHHHH (the PTP, ref. 32, is in bold font)
3. PTP-KLA: CQKHHNYLCGKLKKLAKKALKH

The peptides were chemically synthesized and purified by high-performance liquid chromatography. The molecular weights of the purified fractions were verified by mass spectrometry, and those fractions (>98% purity) that corresponded to the correct peptide molecular weight were lyophilized for use in experiments. Stock solutions of the lyophilized peptides were prepared in PBS, aliquoted, and stored at −20°C. Peptide concentrations were calculated using the ninhydrin assay (33). For cyclization of PTP (via formation of a disulfide bond between C1 and C9), 0.1 mg/mL peptide in deionized water was oxidized in air at room temperature for 4 days. The formation of the disulfide bond was verified using Ellman’s reagent (Pierce Biotechnology) and cysteine standards as described in the vendor’s protocol. The peptide was then lyophilized and resuspended in sterile PBS. All peptides were >98% pure and were sterile filtered (polyethersulfone 0.2-μm syringe filter, Nalgene) immediately before use.

**Antibody-KLA conjugation.** The anti-PSMA monoclonal antibody, MLN591, was a gift from Millennium Pharmaceuticals Inc. The MLN591 antibody was dialyzed thrice against PBS using a 3,500 molecular weight cutoff float-a-lyzer membrane (Spectrum Labs) at 4°C. The MLN591 antibody was then conjugated with the KLA peptide using an N-succinimidyl-3-(2-pyridylothio)propionate (SPDP) linker (Pierce). Briefly, MLN591 (1 mg/mL; 1 mL) was reacted with 20 μL of 5 mmol/L SPDP dissolved in DMSO at room temperature for 15 min with shaking. Unreacted SPDP was removed by gel filtration using a Bio-Rad 10 G desalting column (3 mL, exclusion limit 3 kDa) under gravity. Fractions (1 mL) were collected by flowing PBS through the column; the fractions were concentrated using a spin vacuum system and pooled to make a final volume of 1 mL. To verify the conjugation of the SPDP linker to the antibody, 100 μL of the pooled fractions were diluted to 1 mL and reacted with 10 μL DTT (15 mg/mL in PBS) for 15 min. DTT treatment results in release of for pyridine-2-thione which can be monitored at 343 nm and is an indicator of the conjugation of the SPDP linker to MLN591. The number of SPDP molecules conjugated per MLN591 molecule was determined using the SPDP conjugation protocol (Pierce). The MLN591-SPDP conjugate was then reacted with 300 μL of KLA peptide (200–300 μmol/L in PBS) at room temperature. Before the reaction, 400 μL of KLA was mixed with 10 mg of Reductaryl (EMD Biosciences Inc.) for 10 min to reduce cysteine thiols and enable conjugation to the MLN591-SPDP conjugate. Following the reduction step, 300 μL of the reduced peptide solution was recovered from the resin by centrifugation at 11,000 × g for 10 min. The kinetics of the MLN591-KLA conjugation reaction were monitored using absorbance at 343 nm; in all cases, the reaction was complete within 3 to 4 h. The number of KLA molecules conjugated to the MLN591 antibody was estimated as described previously. Using this protocol, we were consistently able to conjugate three to five molecules of KLA per molecule of MLN591. Finally, the MLN591-KLA reaction mixture was dialyzed thrice against PBS at 4°C to remove unreacted KLA, sterile-filtered through a 0.22-μm syringe filter, and stored at 4°C for further use.

**Cytotoxicity assays.** LNCaP and PC-3 cells were plated in 24-well cell culture plates (Corning Inc.) at a density of 125,000 cells per well and allowed to attach for 24 h. Cells were incubated with different concentrations of KLA, PTP-KLA, and MLN591-KLA, followed by an analysis for viability. Briefly, following individual treatments, cells were treated with 100 μL calcine AM-ethidium homodimer stock solutions (Invitrogen; final concentrations: calcine, 2 μmol/L; ethidium homodimer, 4 μmol/L) for 30 min, centrifuged (Beckman GS-6R) at 4°C, washed with 1 mL PBS, centrifuged again at 4°C, resuspended in 1 mL PBS, and immediately analyzed using flow cytometry (Beckman Coulter Epics Altra). A total of 10,000 events were recorded for each sample, and cells that stained red (ethidium homodimer positive) were considered dead. Cell viability and proliferation was also analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (ATCC) using absorbance at 570 nm. For the evaluation of antibody-based conjugates, cells were treated with buffer-exchanged MLN591 (MLN591 dialyzed thrice against PBS at 4°C), MLN591-KLA, or equivalent amounts of PBS (control) for 72 h, after which they were harvested and analyzed for viability as described above. All experiments were carried out at least in triplicate.

**Apoptosis assays.** For determination of the sub-G₀ phase population, LNCaP cells were treated with the peptides and the antibody conjugate as described above, centrifuged, fixed in 70% ethanol for 1 h at room temperature, washed twice with PBS, and stained with propidium iodide for 45 min. Cells were washed once with PBS and analyzed by flow cytometry, a total of 10,000 events were acquired. Apoptosis assays were also investigated using the annexin V–FLUOS/propidium iodide staining kit (Roche Applied Sciences). Briefly, following treatment, LNCaP cells were washed with 500 μL PBS and 20 μL annexin V–FLUOS, and propidium iodide were dissolved in the incubation buffer, and cells were incubated with 50 μL of this stock solution for 30 min. Cells were then washed with 500 μL PBS and analyzed by fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss Microimaging, Inc.). Cells stained with annexin V–FLUOS alone (green fluorescence) were considered apoptotic, whereas those that stained both green and red were considered oncocytic.

To probe the possible involvement of the caspase-mediated apoptotic pathway, cells were treated with the pan-caspase inhibitor z-VAD-Fmk (100 μmol/L; R&D Systems) for 2 h before treatment with KLA and PTP-KLA peptides and MLN591-KLA conjugates. Cell viability was determined at 24 h for the peptides and at 72 h for MLN591-KLA immun conjugates as described above; viability of cells treated with the inhibitor was compared with untreated cells to determine the involvement of the caspase-mediated apoptotic pathway. The homogeneous caspase assay (Roche Applied Sciences) was used to investigate caspase activation in LNCaP cells treated with immun conjugates, following protocols recommended by the vendor. The release of rhodamine, which is an indicator of caspase activity in the caspase pathway, cells were treated with the pan-caspase inhibitor z-VAD-Fmk (100 μmol/L; R&D Systems) for 2 h before treatment with KLA and PTP-KLA peptides and MLN591-KLA conjugates. Cell viability was determined at 24 h for the peptides and at 72 h for MLN591-KLA immun conjugates as described above; viability of cells treated with the inhibitor was compared with untreated cells to determine the involvement of the caspase-mediated apoptotic pathway. The homogeneous caspase assay (Roche Applied Sciences) was used to investigate caspase activation in LNCaP cells treated with immun conjugates, following protocols recommended by the vendor. The release of rhodamine, which is an indicator of caspase activity in the assay, was detected by fluorescence, and fluorescence intensity was used to compare caspase activation across different treatments.

**Mitochondrial depolarization.** The JC-1 dye (Invitrogen) forms red-fluorescent J-aggregates upon localization in healthy mitochondria, whereas the monomeric form of the dye fluoresces green in the cytoplasm (34).
Results

Evaluation of KLA and PTP-KLA Fusion Peptides

PTP enhances the cytotoxic efficacy of KLA in PSMA-expressing LNCaP cells. Although well documented in literature (6, 35), we first verified the presence and absence of PSMA on the surface of LNCaP and PC-3 cells, respectively, using immunofluorescence (data not shown). The cationic amphipathic untargeted KLA peptide induced significant nonspecific cell death (> 70% loss in viability) in both PC-3 and LNCaP cells at concentrations >70 μmol/L (Fig. 1A). However, although lower KLA concentrations (35 μmol/L) induced death in LNCaP cells, no PC-3 cell death was observed under these conditions. Addition of the PSMA-targeting peptide (PTP) to KLA increased its potency in LNCaP cells, but not PC-3 cells (Fig. 1A). The loss of viability in LNCaP cells was accompanied by characteristic cell detachment and clumping (Fig. 1B).

To evaluate the mechanism by which PTP enhances the potency of KLA, competitive inhibition experiments were carried out to determine if PTP-KLA is internalized via specific interaction with the PSMA receptor. Competitive inhibition experiments indicated the inhibition of PTP-KLA activity by PTP (Fig. 1C). Although 25 μmol/L PTP completely reversed the cytotoxic effects of 22 μmol/L PTP-KLA on LNCaP cells, less inhibition was observed upon raising the PTP-KLA concentration to 30 μmol/L. We verified that PTP alone was not cytotoxic under these conditions (data not shown). Preincubation with 500 nmol/L anti-PSMA antibody (MLN591) also resulted in a reduction in cell death induced by PTP-KLA (data not shown).

PTP-KLA induces oncotic/necrotic death in LNCaP cells. We then investigated the mechanism of cell death (apoptosis/oncrosis) induced by the two peptides in LNCaP cells. Preincubation with the pan-caspase inhibitor z-VAD-Fmk (100 μmol/L) did not inhibit LNCaP cell death after treatment with 30 μmol/L PTP-KLA (Fig. 2A), indicating that the caspase-mediated apoptotic pathway was not involved in cell death and further validating the observation that oncrosis was the primary mode of cell death. In addition, LNCaP cells were treated with KLA and PTP-KLA and stained with annexin V–FLUOS and propidium iodide to differentiate between purely apoptotic populations from a mixture of late apoptotic/oncotic ones. Cells treated with 30 μmol/L PTP-KLA stained positive for propidium iodide, indicating that the mode of cell death induced by this concentration of the peptide was primarily oncotic. Similar results were obtained with KLA (Supplementary Fig. S1). Inhibition of the antiapoptotic protein Bcl-2 has been shown to enhance chemotherapeutic-induced apoptotic death in prostate cancer cells (36). However, enhanced cell death was not observed when cells were preincubated with a cell-permeable Bcl-2 inhibitor (2 μmol/L; results not shown) for 90 min followed by incubation with 25 μmol/L PTP-KLA for 24 h. To further investigate the mode of cell death, LNCaP cells were treated with different concentrations of KLA and PTP-KLA for 24 h, fixed with 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. Lower PTP-KLA concentrations (10 μmol/L) induced mainly apoptotic death in a small fraction of LNCaP cells as indicated by a small increase in the sub-G0 population of cells (Fig. 2B–i). However, higher concentrations of PTP-KLA (25 μmol/L) induced onotic death; Fig. 2B–ii indicates a profile characteristic of cells undergoing G0-phase arrest. DNA laddering assays supported these results (Supplementary Fig. S2). Taken together, these results indicate that whereas low micromolar (<15 μmol/L) concentrations of PTP-KLA and KLA induce apoptosis in LNCaP cells, the percentage of dead/dying cells was significantly lower (<25%) than when high micromolar (>30 μmol/L)
concentrations were used. Use of higher peptide concentrations, however, resulted in oncotic/necrotic death. Finally, LNCaP cells were treated with KLA, PTP-KLA, or equivalent volume of PBS (control) for 24 h, stained with JC-1, and analyzed by flow cytometry to investigate mitochondrial depolarization induced by the KLA peptide. The decrease in the red/green fluorescence ratio upon treatment with 30 μmol/L PTP-KLA indicated a significant loss of mitochondrial potential (Fig. 2C).

**Evaluation of MLN591-KLA (Antibody-Peptide) Conjugates**

Conjugation of KLA to anti-PSMA antibody MLN591 enhances its potency in LNCaP cells. In addition to the fusion peptides described above, we investigated MLN591-KLA immunoconjugates as potential site-specific therapeutics for prostate cancer. Briefly, primary amines on the MLN591 antibody were reacted with the hetero-bifunctional cross-linker SPDP to obtain MLN591-SPDP conjugates. The KLA peptide was then conjugated to the activated MLN591 antibody via the thiol group of the COOH-terminal cysteine on the peptide. A ratio of three to five peptide molecules per antibody molecule was consistently obtained using this method.

The antibody-KLA conjugate was significantly more cytotoxic to LNCaP cells than KLA or PTP-KLA. Figure 3A compares the cell viability of LNCaP and PC-3 cells when treated with different concentrations of MLN591-KLA for 72 h; no loss of cell viability was observed in either cell line at 24 h. The MLN591-KLA conjugate had no effect on PC-3 cell viability but induced significant cell death in LNCaP cells.

![Figure 2. PTP-KLA induces oncotic death in LNCaP cells.](image-url)

- **A.** PTP-KLA induces cell death in LNCaP cells via a caspase-independent manner. Cells incubated without and with the pan-caspase inhibitor z-VAD-Fmk (2 h) and then with 30 μmol/L PTP-KLA (22 h). The presence of the caspase inhibitor had no effect on LNCaP cell death.
- **B.** Flow cytometry analysis of LNCaP cells fixed with 70% ethanol and stained with propidium iodide. i, Low micromolar concentrations (10 μmol/L) of PTP-KLA induce apoptosis in LNCaP cells as seen by the propidium iodide-stained sub-G₀ population. ii, Higher PTP-KLA concentrations (25 μmol/L) induced necrotic death.
- **C.** PTP-KLA induces mitochondrial damage in LNCaP cells; following treatment for 24 h, cells were stained with the mitochondria potential indicator dye, JC-1, and subjected to flow cytometry. i, A large fraction of cells untreated with PTP-KLA show intact mitochondria as seen by the high ratio of red fluorescent to green fluorescent cells. ii, A large fraction of cells treated with 30 μmol/L PTP-KLA undergo mitochondrial damage as seen by the decrease in the ratio of red fluorescent to green fluorescent cells.
LNCaP cells, indicating selectivity of the conjugate toward PSMA-expressing LNCaP cells (Fig. 3A). Unconjugated MLN591 did not result in a loss of cell viability in either PC-3 or LNCaP cells. Furthermore, we verified that LNCaP cell viability was unaffected by treatment with equivalent concentrations of either KLA or PTP-KLA for 72 h (data not shown).

Figure 3B shows phase-contrast images of the morphologic changes in LNCaP cells and the corresponding fluorescence images showing ethidium homodimer staining. LNCaP cells treated with unconjugated MLN591 (0.4 μmol/L; Fig. 3B-ii) exhibit normal morphology, similar to cells treated with PBS (Fig. 3B-i), with only a few cells in each field staining with ethidium homodimer. In contrast, cells treated with MLN591-KLA (0.4 μmol/L) exhibit extensive cell death, indicated by rounding, detachment, and extensive staining with ethidium homodimer (Fig. 3B-iii).

**MLN591-KLA induces apoptosis in LNCaP cells.** The homogeneous caspase assay was carried out to investigate caspase activation in LNCaP cells upon incubation with MLN591-KLA for 72 h. Figure 4A shows caspase activation when LNCaP cells were treated with MLN591-KLA, whereas those treated with MLN591 exhibited caspase activity comparable to cells treated with PBS.

The assay detects caspases 2, 3, and 7, indicating a role for these caspases in MLN591-KLA-mediated cell death. In addition, LNCaP cells were preincubated with 100 μmol/L of the pan-caspase inhibitor z-VAD-Fmk for 2 h before treatment with the MLN591-KLA conjugate. Inhibition of caspase activity with z-VAD-Fmk increased LNCaP viability (Fig. 4B), supporting a role for the caspase-mediated apoptotic pathway in MLN591-KLA-induced cell death. Flow cytometry analysis of propidium iodide–stained LNCaP cells after MLN591-KLA treatment indicated that a significant fraction of the cells (>80%) were apoptotic, as indicated by the sub-G0 population in Fig. 4C-i. In contrast, equivalent concentrations of the MLN591 antibody did not induce apoptosis in these cells (Fig. 4C-ii). In addition, the MLN591-KLA conjugates induced mitochondrial depolarization in LNCaP cells, which is consistent with the proposed mechanism for cell death induced by the cationic amphipathic peptide KLA (ref. 23; Fig. 4D). The mitochondrial integrity of LNCaP cells treated with the unconjugated MLN591 antibody was similar to that of the untreated control.

**Comparison of KLA, PTP-KLA, and MLN591-KLA.** Dose response studies were employed to determine the *in vitro* LC50 values (the concentration required to induce death in 50% LNCaP

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![Figure 3A](image-url)  
**Figure 3A.** A comparison of MLN591-KLA activity on LNCaP and PC-3 cells, LNCaP and PC-3 cells were treated with different concentrations of MLN591 and MLN591-KLA (3–5 KLA molecules per MLN591 molecule), and cell viability was determined 72 h following incubation and compared with control (cells treated with equal volume of PBS). **A**, PC-3 cells treated with MLN591; △, PC-3 cells treated with MLN591-KLA; ○, LNCaP cells treated with MLN591; ●, LNCaP cells treated with MLN591-KLA. The lines connecting the data points are for visualization only. **B**, phase contrast and fluorescence microscopy images of LNCaP cells treated with PBS (control), MLN591, and MLN591-KLA for 72 h. Cellular morphology (top) and ethidium homodimer staining (bottom) indicate no cell death upon treatment with (i) PBS and (ii) 0.4 μmol/L MLN591, but extensive cell death upon treatment with (iii) 0.4 μmol/L MLN591-KLA.
cells in culture) for KLA, PTP-KLA, and MLN591-KLA in LNCaP cells. The LC50 value of the MLN591-KLA conjugate for LNCaP cells was 0.28 μmol/L, which is approximately 70-fold lower than that of PTP-KLA (19 μmol/L) and more than two orders of magnitude lower than that of KLA (41 μmol/L), indicating that the anti-PSMA antibody significantly enhances the potency of the KLA peptide (Fig. 5). In addition, whereas PTP-KLA and KLA induced oncotic death in LNCaP cells, MLN591-KLA induced caspase-mediated apoptotic death.

Discussion

A number of chemotherapeutic strategies have been investigated for the ablation of advanced prostate cancer cells (4). Of these, targeted biologicals represent an emerging class of therapeutics that can potentially reduce nonspecific cytotoxicity in collateral organs such as the urethra and bladder, especially in cases of residual disease and secondary metastasis. The transformation of androgen-dependent prostate cancer disease to a highly tumorigenic, metastatic, and androgen-independent phenotype is a result of the accumulation of significant genetic changes (2, 37). Several studies have indicated that the overexpression of antiapoptotic proteins plays a direct role in the abnormal growth of prostatic tissue and correlates with the poor response to radiotherapy (38). For example, overexpression of the antiapoptotic protein Bcl-2 inhibits the mitochondrial pore transition, contributing to the resistance of the transformed cell to apoptosis. Therapeutic strategies that induce mitochondrial depolarization, therefore, are attractive because they can bypass apoptosis resistance mechanisms that act upstream of the mitochondria.

Energy from respiration is stored as a potential and pH gradient across mitochondrial membranes in mammalian cells (chemiosmotic theory; ref. 39). Once internalized, cationic amphipathic molecules preferentially localize at the mitochondria due to the large negative potential gradient across the mitochondrial membrane. Accumulation of a significant concentration of these molecules at the mitochondrial membrane leads to its disruption and, consequently, depolarization, ultimately leading to apoptosis.
We therefore investigated the cationic amphipathic peptide KLA as a potential therapeutic candidate in prostate cancer.

We exploited the overexpression of the PSMA on the surface of prostate cancer cells as means to selectively deliver KLA to these cells. We investigated the potency and mechanism of cell death in human prostate cancer cell lines treated with untargeted, peptide-targeted, and monoclonal antibody–targeted KLA constructs. Peptides targeting the extracellular portion of PSMA have recently been identified from a phage display library and have been shown to possess micromolar (μmol/L) binding affinities to the extracellular portion of the receptor (32). In addition, aptamers (40–42) and monoclonal antibodies (43, 44) have also been used to target the PSMA receptor.

In vitro investigation indicated that the LC_{50} value was 41 μmol/L for untargeted KLA and 19 μmol/L for the targeted PTP-KLA peptide. The use of the PSMA-targeting peptide (PTP) sequence, therefore, modestly enhanced the efficacy (2-fold) of the resulting PTP-KLA fusion compared with the untargeted KLA peptide. Coincubation of PTP-KLA with PTP resulted in lower LNCaP cell death, indicating that the fusion peptide acted by interacting with the PSMA receptor. However, this interaction is in addition to the nonspecific uptake of the fusion peptide mediated by the cell-penetrating activity of the KLA segment.

Contrasting mechanisms of cell death have been proposed for cationic amphipathic peptide-based cancer therapeutics (23, 25, 45, 46). Although some reports indicate that these peptides induce apoptosis due to their ability to depolarize mitochondrial membranes (23, 25, 45), others have reported that these peptides induce plasma membrane lysis leading to oncosis/necrotic death in cancer cells (47). We therefore investigated the mechanism of cell death induced by the peptide- and antibody-targeted KLA constructs.

At concentrations less than 15 μmol/L, PTP-KLA induced apoptotic death in a small fraction of LNCaP cells as indicated by propidium iodide staining/flow cytometry. However, higher concentrations (30 μmol/L) of PTP-KLA induced oncosis in a larger percentage (ca. 80%) of LNCaP cells. These results indicate that plasma membrane permeabilization and cell lysis contribute to necrotic cell death and are similar to those previously reported in the literature (47–49). Thus, in the case of PTP-KLA, the relative affinities of PTP-PSMA interaction and the KLA-plasma membrane interaction may represent competing processes underlying cellular uptake and death. This observation has implications for the design of targeted peptide therapeutics: to engineer fusion peptide therapeutics with high selectivities, the affinity of the targeting peptide (e.g., PTP) must be significantly greater than the plasma membrane lytic activity of the cytotoxic peptide (e.g., KLA). We also verified that the treatment with both KLA and PTP-KLA resulted in mitochondrial depolarization. However, it is not clear whether mitochondrial depolarization was responsible for the cell death induced by KLA and PTP-KLA. It is more likely that the observed depolarization is a consequence of oncosis.

Antibody-based conjugates have been explored as potential therapeutics for a number of malignancies, including prostate cancer. We conjugated KLA to MLN591, a monoclonal antibody that targets the extracellular portion of the PSMA. This antibody has nanomolar affinity for the receptor and can therefore deliver therapeutic cargo with high selectivity to prostate cancer cells that overexpress the receptor. In contrast to the peptides (KLA and PTP-KLA), no cell death was observed when LNCaP cells were treated with the MLN591-KLA conjugate for 24 h. Cells typically entered apoptosis 36 to 48 h after treatment, necessitating measurement of cell viability at 72 h. Significantly lower (submicromolar) concentrations of the MLN591-KLA conjugate were required to induce death in LNCaP cells when compared with PTP-mediated delivery; the antibody enhanced the efficacy of the conjugate by two orders of magnitude compared with the untargeted peptide. Both MLN591 and MLN591-KLA were not toxic to PSMA-negative PC-3 cells under the described experimental conditions. Although the decrease of the LC_{50} of the MLN591-KLA conjugate are in agreement with previous reports on immunoconjugates (45), the exact mechanism by which conjugation of KLA to MLN591 results in the decrease of the LC_{50} value of the MLN591-KLA conjugate is not known. It is hypothesized that the high binding affinity of MLN591 to the receptor results in the localization and subsequently, internalization of KLA molecules inside LNCaP cells. In addition, the presence of a cleavable disulfide bond in the linker region between MLN591 and KLA can play a role in the release of the KLA peptide into the cytoplasm which ultimately results in LNCaP cell death.

Investigation of cell death mechanisms confirmed the role of caspase-mediated apoptosis in the ablation of LNCaP cells treated with MLN591-KLA. A significant proportion (>80%) of fixed LNCaP cells stained with propidium iodide showed characteristics of a sub-G_0 population when compared with the control (cells treated with equivalent PBS), indicating that these cells underwent apoptosis. In contrast, cells treated with an equivalent concentration of the unconjugated antibody did not exhibit the presence of a sub-G_0 population, indicating that the antibody alone did not induce apoptosis in these cells. These results are consistent with the cell death results (ethidium homodimer staining) in Fig. 3B and indicate that apoptosis is the primary mechanism of cell death induced by MLN591-KLA conjugates. Furthermore, caspase activation was observed in LNCaP cells treated with the MLN591-KLA conjugate. In addition to the enhanced targeting affinity due to the MLN591 antibody, it is possible that steric effects also play a role in preventing cell membrane lysis by the lytic KLA peptide. Conjugation of the (small) lytic peptide to the (large) antibody can keep the former away from the cell membrane, whereas this is not possible in case of the much smaller PTP-KLA fusion peptides. Inhibition of caspase activity resulted in an increase in cell viability.

Figure 5. Comparison of the in vitro LC_{50} values of untargeted, peptide-targeted, and antibody-targeted fusions/conjugates of KLA with LNCaP cells.
indicating a role for the caspase-mediated apoptotic pathway. Finally, we verified that the MLN591-KLA conjugate induced mitochondrial depolarization in LNCaP cells after 72 h, in accordance with the previously proposed mechanism of KLA. However, whereas it is tempting to speculate that mitochondrial depolarization is the cause of cell death induced by MLN591-KLA in LNCaP cells, our results do not conclusively prove this. To do so will necessitate the correlation of mitochondrial depolarization kinetics with apoptosis kinetics in LNCaP cells treated with MLN591-KLA, studies that are now ongoing in our laboratory. Future work will also focus on evaluating the antibody-KLA construct in vivo, with orthotopically implanted LNCaP tumors in mice.

Conclusions

We have designed, generated, and evaluated targeted fusion peptides and immunoconents based on the cationic amphipathic peptide KLA as potential targeted therapeutics for prostate cancer. Our results indicate that antibody-KLA conjugates have promise as potential therapeutics and warrant further investigation. We also investigated the mechanism of cell death induced by these molecular therapeutics. In cases where cationic amphipathic peptides are used, the affinity of the targeting sequence to the receptor must be significantly higher than the membrane-permeabilizing affinity of the lytic peptide to minimize nonspecific death. Thus, whereas lower concentrations of MLN591-KLA are sufficient to induce apoptosis in LNCaP cells due to the enhanced affinity of MLN591 antibody to the receptor, further engineering of peptides targeting the PSMA is required in order for them to be useful in potential therapeutic applications.

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