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Induction of Cancer Cell Death by Self-assembling Nanostructures Incorporating a Cytotoxic Peptide

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Abstract

Nanotechnology offers novel delivery vehicles for cancer therapeutics. Potential advantages of nanoscale platforms include improved pharmacokinetics, encapsulation of cytotoxic agents, enhanced accumulation of therapeutics in the tumor microenvironment, and improved therapeutic structures and bioactivity. Here we report the design of a novel amphiphilic molecule that self-assembles into nanostructures for intracellular delivery of cytotoxic peptides. Specifically, a cationic α -helical (KLAKLAK)₂ peptide that is known to induce cancer cell death by membrane disruption was integrated into a peptide amphiphile (PA) that self-assembles into bioactive, cylindrical nanofibers. PAs are composed of a hydrophobic alkyl tail, a β -sheet forming peptide, and a bioactive peptide that is displayed on the surface of the nanofiber after self-assembly. PA nanostructures that included (KLAKLAK)₂ were readily internalized by breast cancer cells, in contrast to the (KLAKLAK)₂ peptide that on its own was not cell permeable. (KLAKLAK)₂ nanostructures, but not the peptides alone, also induced breast cancer cell death by caspase-independent and Bax/Bak-independent mechanisms associated with membrane disruption. Significantly, (KLAKLAK)₂ nanostructures induced cell death more robustly in transformed breast epithelial cells than in untransformed cells, suggesting a degree of tumor selectivity. Our results provide proof-of-principle that self-assembling PAs can be rationally designed to generate nanostructures that can efficiently deliver cytotoxic peptides to cancer cells.

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Keywords

Nanotechnology; Peptide Amphiphile; Drug Delivery; Cancer; Cell Death

INTRODUCTION

Nanotechnology is a multidisciplinary field providing a unique platform to create a new generation of cancer therapeutics with enhanced efficacy and diminished toxicity (1). Nanoscale drug delivery has many advantages over traditional chemotherapy, including improved drug solubility and pharmacokinetics. The enhanced permeability of tumor vasculature results in passive accumulation of nanoparticles in tumors. Therapeutic cargo, imaging agents, and tumor-targeting epitopes can be multiplexed into single nanostructures, thereby engineering controlled tumor delivery of these agents.

Recent studies have demonstrated the utility of peptides designed to activate cell death in cancer cells. One promising strategy uses cationic peptides, such as (KLAKLAK)₂ (abbreviated “KLAK”), as membrane-disrupting agents. In the presence of membranes, this peptide forms an amphipathic α -helix, with the charged and nonpolar residues on opposite faces (2). Because cells do not efficiently internalize KLAK peptide, it is typically fused with cell penetrating moieties or receptor ligands (3-6). With facilitated uptake, KLAK and other cationic peptides disrupt mitochondrial and/or plasma membranes and initiate caspase-dependent and/or caspase-independent cell death, respectively, reducing tumor burden *in vivo* (3-7).

Despite potential utility, peptides have several shortcomings, including poor cell penetration, immunogenicity, and rapid *in vivo* degradation (8). We postulated that incorporating peptides into nanostructures might be an effective strategy to overcome these deficiencies. To this end, we incorporated the KLAK peptide into a novel class of peptide amphiphiles (PAs) developed in our laboratory (9,10). PA molecules have three domains: a hydrophobic alkyl tail, a β -sheet promoting sequence, and a bioactive peptide domain. In biological fluids, the self-assembly of PAs into high-aspect ratio nanostructures is driven by electrostatic screening of charged residues, hydrophobic collapse of alkyl tails into the fiber core, and intermolecular hydrogen bonding, particularly in the β -domain. These nanofibers surface-display peptide segments at high density and have been found to be highly bioactive. PA nanofibers have been used to promote functional recovery after spinal cord injury and angiogenesis (11). Recently, the high-aspect ratio of PAs was shown to exhibit greater bioactivity relative to spherical aggregates of similar molecules (12). A major promise of PA nanostructures is the potential for drug-free, biodegradable therapeutics. We report here that a PA incorporating the KLAK peptide self-assembles into nanostructures that are readily internalized by cancer cells and induce cell death, thereby supporting the potential utility of PA-based cancer therapeutics.

METHODS

Materials synthesis and characterization

Peptides and PAs were prepared using standard fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis methods and were purified by reverse-phase HPLC. Amino acid analyses were performed by Commonwealth Biotechnologies (Richmond, VA). Circular dichroism measurements were performed on a Jasco CD Spectrometer J-715. PAs were imaged using a JEOL 1230 transmission electron microscope. A more detailed description of the materials is included in Supplementary Information.

Cell culture and reagents

SKBR-3 cells (ATCC) were grown in α -MEM with 5% FBS, 2 mM L-glutamine, 100 IU/ml penicillin-streptomycin, 1 mM sodium pyruvate, 20 mM non-essential amino acids, HEPES, and 50 μ M β -mercaptoethanol. MDA-MB-231 cells (gift of Jennifer Koblinski, Northwestern University) were maintained in DMEM/F12 with 2x non-essential amino acids, 5% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 IU/ml penicillin-streptomycin. Wild-type and Bax^{-/-}/Bak^{-/-} mouse embryonic fibroblasts (MEFs), a gift of Craig Thompson, University of Pennsylvania (13), were maintained in DMEM with 10% FBS, 2 mM L-glutamine, and 100 IU/ml penicillin-streptomycin. MCF-10A cells stably expressing H-RasV12 or empty vector (14) were maintained in DMEM/F12 with 5% horse serum, 20 ng/ml EGF (Sigma), 0.5 mg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 μ g/ml insulin (Sigma), and 100 IU/ml penicillin-streptomycin. All cells were grown at 37 °C in 5% CO₂ atmosphere. Unless otherwise specified, reagents were obtained from Invitrogen. Lexatumumab was provided by Robin Humphreys (Human Genome Sciences) (15).

Confocal microscopy and flow cytometry

Images were taken with a Nikon C1 Confocal Microscope. Flow cytometry was performed using a Becton Dickinson FACSCalibur or a DakoCyomation CyAn.

Immunoblotting

Cells were lysed in modified RIPA buffer (50 mM Tris, 0.1% SDS, 150 mM NaCl, 0.5% DOC, and 1% NP-40) containing 1 mM PMSF and protease inhibitor cocktail (Sigma). Lysates were run on SDS-PAGE gels, transferred to PVDF membranes, and proteins were detected by immunoblotting using antibodies against cleaved caspase-3 (Cell Signaling) or tubulin (Sigma).

Cell viability

Cell viability was measured using the MTS-based CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol. Trypan blue (0.04%, Sigma) exclusion was also used to identify viable cells with intact plasma membranes.

Annexin V assay

Annexin V-positive cells were identified using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience) according to the manufacturer's instructions except that DAPI was used.

Membrane potential assays

Cells were labeled with 50 nM Mitotracker Red and DiOC₅(3) (Invitrogen) to stain mitochondrial and plasma membranes, respectively according to the manufacturer's instructions. Fluorescence was measured at 25°C using a DakoCyomation CyAn.

Transmission electron microscopy

Images were obtained using a JEOL 1220 microscope. Cells were fixed in 2% glutaraldehyde/0.1M sodium cacodylate, treated with 2% osmium tetroxide, and stained with uranyl acetate/lead citrate (Electron Microscopy Sciences).

Statistical analysis

Statistical significance was assessed by two-way ANOVA with Bonferroni post-tests using GraphPad Prism 5.0b Software.

RESULTS AND DISCUSSION

The central component of this nanotechnology platform is the KLAK PA (Figs. 1A and S1). KLAK PA monomers self-assembled in PBS into nanostructures 100-900 nm in length and 8-10 nm in diameter (Fig. 1B). Because the membrane-disrupting activity of the KLAK peptide (Fig. S2) depends on its α -helical conformation (3), we examined the secondary structure of KLAK-containing molecules. The circular dichroism (CD) spectrum of KLAK PA demonstrated helical structure, while KLAK peptide was predominantly random coil (Fig. 1C). We next examined the uptake of fluorescein-labeled KLAK PA (Fig. S3) and KLAK peptide (Fig. S4) by SKBR-3 breast cancer cells. KLAK PA, but not peptide, was internalized (Fig. 1D). Hence, incorporating the KLAK epitope into a PA stabilizes its helical conformation and facilitates its intracellular delivery.

To determine the cytotoxicity of KLAK PA, we treated human MDA-MB-231 and SKBR-3 breast cancer cells for 24 h with varying concentrations of KLAK PA or KLAK peptide, and measured cell viability by MTS assay (Fig. 2A). The IC_{50} of the KLAK PA was 6.04 μ M (95% confidence interval 5.06–7.21 μ M) and 5.67 μ M (95% confidence interval 5.30–6.26 μ M) in MDA-MB-231 and SKBR-3 cells, respectively, while the KLAK peptide was inactive. A scrambled KLAK peptide sequence designed to partially disrupt the amphipathic helix was incorporated into a PA (Fig. S5), which formed nanofibers with reduced helical structure (Fig. S6) and diminished cytotoxicity compared to the KLAK PA (Fig. 2A).

To investigate cell death mechanism(s), breast cancer cells were incubated with PBS, KLAK peptide, KLAK PA, or scrambled KLAK PA for 18 h. Consistent with the cell viability results, KLAK PA, but not peptide, robustly induced annexin V positive cells, while the scrambled KLAK PA was less effective (Fig. 2B). The majority of cells treated with KLAK PA were both annexin V and DAPI positive, even when treated for only 1 h (data not shown), suggesting that these nanostructures rapidly compromise plasma membrane integrity. Within minutes of adding KLAK PA to cells, the plasma and mitochondrial membrane potentials were reduced, suggesting membrane disruption (Fig. 2C, *left panel*). To assess the role of oxidative stress in KLAK PA-mediated cell death, cells were pretreated for 1 h with 3 mM N-acetyl cysteine (NAC). NAC partly attenuated cell death induced by KLAK PA but not the proapoptotic TRAIL receptor-2 monoclonal antibody lexatumumab, which served as a positive control (Fig. 2C, *right panel*) (15). Consistent with reports demonstrating enhanced sensitivity of cancer cells to cationic peptides due to increased negatively charged membrane phospholipids (16), we observed that MCF-10A breast cells transformed by H-RasV12 were more sensitive to KLAK PA-induced cell death than untransformed MCF-10A-Vector cells (Fig. 2D). These results suggest that the KLAK PA has some tumor-selectivity.

To determine whether KLAK PA-induced cell death was caspase-dependent, we incubated MDA-MB-231 cells with KLAK PA in the presence of zVAD-fmk. Although zVAD-fmk inhibited lexatumumab cytotoxicity, it had no effect on cell death induced by KLAK PA or scrambled KLAK PA (Fig. 3A and 3B). Consistent with these findings, lexatumumab, but not KLAK PA, induced caspase-3 processing to its active subunit (Fig. 3C). Moreover, wild-type and Bax^{-/-}/Bak^{-/-} double knockout (DKO) MEFs were equally susceptible to KLAK PA cytotoxicity (Fig. 3D). Thus, KLAK PA induces caspase-independent and Bax/Bak-independent cell death.

To investigate ultrastructural changes, MDA-MB-231 cells were treated with PBS, KLAK peptide, or KLAK PA and examined by TEM. Cells treated with KLAK PA did not exhibit apoptotic morphology, but rather had distorted plasma membranes, abundant fiber-like structures (arrows), lysosome-like structures (arrowheads), and were largely devoid of mitochondria (Fig. 4A). These changes were not observed in cells treated with KLAK peptide

(Fig. 4B) or PBS (Fig. 4C). We also observed that KLAK PA was much more efficient than the scrambled PA at permeabilizing cell membranes as determined by trypan blue staining (Fig. 4D), confirming that KLAK PA nanostructures induce cell death by disrupting cell membranes.

We have shown that PAs can be rationally designed to self-assemble into nanostructures that deliver cytotoxic peptides to cancer cells. Specifically, incorporating the KLAK peptide into PA nanostructures stabilizes its bioactive α -helical conformation and renders the peptide cell-permeable. Additionally, KLAK PA nanostructures potently induce caspase-independent and Bax/Bak-independent cell death in breast cancer cells by lysing plasma membranes, a finding consistent with some reports regarding the cytotoxic mechanism of cationic peptides (6,7). Moreover, KLAK PAs induce cell death more robustly in transformed breast epithelial cells than untransformed cells, suggesting some tumor-selectivity. Although others have recently reported PA-based platforms for cancer, the cytotoxic mechanisms of these nanostructures have yet to be fully characterized (17,18). From a therapeutic standpoint, nanostructures will likely have to be PEGylated and/or tumor-targeted to maximize their efficacy and reduce systemic toxicity *in vivo*. Having already demonstrated the feasibility of co-assembling PAs (19), we will focus future studies on constructing hybrid nanofibers by co-assembly of KLAK PA, PEGylated PA and tumor-targeting PAs. The high-aspect ratio of PA nanofibers may represent a therapeutic advantage over traditional spherical nanoparticles because fibrillar micelles have prolonged circulation time *in vivo* (20). An additional advantage is that PAs are biodegradable vehicles, disintegrating into amino acids and lipids (11). Collectively, our findings suggest that PAs represent a promising nanotechnology platform for cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. KLAK PA self-assembles into nanofibers that stabilize the α -helical peptide conformation and are cell permeable

(A) Chemical structure of KLAK PA. (B) TEM of self-assembled KLAK PA nanofibers. Scale bar, 500 nm. (C) CD spectra of KLAK PA and peptide. (D) Cellular uptake of KLAK PA nanostructures. SKBR-3 cells were incubated with fluorescein-labeled KLAK PA, peptide, or untreated for 6 h. Mitochondria and nuclei were stained with Mitotracker Red CMXRos and TO-PRO-3, respectively. Scale bar, 20 μ m. Internalization of fluorescein-labeled KLAK PA or peptide in SKBR-3 cells was also determined by FACS analysis (*far right panel*).

Figure 2. KLAKE nanostructures are cytotoxic to breast cancer cells *in vitro*

(A) MDAMB-231 or SKBR-3 cells were incubated for 24 h with KLAKE peptide, KLAKE PA, or scrambled KLAKE PA. Cell viability was measured by MTS assay. Dose-response curves were used to calculate IC_{50} values. (B) MDA-MB-231 and SKBR-3 cells were treated with PBS or 10 μ M KLAKE peptide, KLAKE PA, or scrambled KLAKE PA for 18 h. Cell death was determined by annexin V labeling. (C) SKBR-3 cells were treated with 10 μ M KLAKE PA. Plasma membrane (DiOC₅(3)) and mitochondrial membrane (Mitotracker Red) potential (normalized to PBS control) were determined over 60 min (left panel). MDA-MB-231 or SKBR-3 cells were incubated for 24 h with PBS, 10 μ M KLAKE PA, or 1 μ g/ml lexatumumab (Lexa) with or without 3 mM NAC pretreatment. Cell viability was measured by MTS assay (right panel). (D) MCF-10A-Ve and MCF-10A-H-RasV12 cells were treated with 10 μ M KLAKE PA for 24 h, and the percentage of annexin V-positive cells was measured. In all graphs, data are mean $\pm$ SEM, $n=3$ or 4. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ for the indicated comparisons.

Figure 3. KLAKE PA nanostructures induce caspase-independent cell death

(A) MDA-MB-231 cells were incubated for 24 h with KLAKE PA with or without 50 μM z-VAD-fmk. Lexatumumab (Lexa, 1 μg/ml) served as a positive control. Cell viability was measured by MTS assay. (B) MDA-MB-231 or SKBR-3 cells were incubated for 24 h with PBS, 1 μg/ml Lexa, 10 μM KLAKE PA or scrambled KLAKE PA with or without 50 μM z-VAD-fmk. The percentage of annexin V positive cells was measured. (C) MDA-MB-231 cells were treated with 1 μg/ml Lexa or 10 μM KLAKE PA for 60 and 120 min. Active caspase-3 was detected by immunoblotting. (D) Wild-type (WT) and Bax^{-/-}/Bak^{-/-} double knockout (DKO) MEFs were incubated for 24 h with KLAKE PA. Cell viability was measured by MTS assay (*upper panel*). The percentage of annexin V-positive cells was determined (*lower panel*). In all graphs, data are mean ± SEM, n=3 or 4. ****P* < 0.001 for the indicated comparisons.

Figure 4. KLAK PA nanostructures disrupt the plasma membrane

TEM of MDA-MB-231 cells treated for 2 h with 10 μ M KLAK PA (A), 10 μ M KLAK peptide (B), or PBS (C) are shown at low and high magnification (scale bars indicated). Note distorted plasma membrane, fiber-like structures (arrows), lysosome-like structures (arrowheads), and absence of mitochondria in KLAK PA-treated cells. (D) Plasma membrane integrity was measured by trypan blue exclusion. MDA-MB-231 and SKBR-3 cells were treated for 1 h with PBS or 10 μ M KLAK PA, peptide, or scrambled PA. The percentage of viable cells was determined (mean $\pm$ SEM, n=3). *** P < 0.001, ** P < 0.01, * P < 0.05 versus PBS-treated cells.