Engineered Apoptosis-Inducing Peptides with Enhanced Mitochondrial Localization and Potency

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Apoptosis-inducing peptides that trigger mitochondrial disruption are a popular tool in pharmaceutical and anticancer research. While useful, their potencies are low, which impedes further development of drugs based on these sequences. Here, we describe an effort to engineer the intracellular localization and activity of a peptide with known anticancer activity, D-(KLAKLAK)$_2$, to improve potency by increasing the specificity of the peptide for mitochondria and enhancing disruption of this organelle. The engineered peptides are significantly more toxic to a wide variety of cancer cell lines, with the best analogue exhibiting a LC_{50} value 100-fold lower than the parent compound. Importantly, the peptides maintain their potency when made cell-type specific.

Introduction

Peptides capable of invading mitochondria of mammalian cells and triggering apoptosis (so-called "killer" peptide domains) have been implemented in numerous studies directed at the development of new targeted therapeutics.[1−10] Their utility is derived from the fact that mitochondria-disrupting peptides can be fused easily to tissue- or tumor-specific peptides or antibodies, permitting the facile testing of new targeted and highly specific therapeutics. However, the killer peptides developed to date suffer from low potencies,[2] limiting the practicality of clinical implementation. The origin of the weak activities observed has remained unexplained. These peptides are typically based on the sequences of membrane-disrupting anitmicrobial peptides (AMPs[a]), and thus structure−activity studies of AMPs toward microbial targets offer some insights into the determinants of membrane-disrupting activity and selectivity.[11−16] However, the disruption of mitochondria within the context of eukaryotic cells poses additional and unique challenges—peptides must nondisruptively cross the plasma membrane, navigate the intracellular milieu, and then localize to the mitochondria and disrupt the mitochondrial membrane.[17]

Recently, we demonstrated that physiochemical parameters have a strong influence on the extent of mitochondrial localization achieved by peptide sequences and that an interplay between charge and lipophilicity dictates the specificity of this type of subcellular targeting.[18] This prior work suggests that mitochondrial localization of synthetic peptides can be systematically manipulated to optimize organellar activity.

Here, we describe studies engineering the physiochemical properties of a widely used mitochondria-disrupting peptide, D-(KLAKLAK)$_2$,[2−10,19] so that it is mitochondria-specific and therefore more concentrated at its site of action. Our efforts to "engineer" the scaffold focused on synthetically manipulating the peptide to change its intracellular accumulation and its activity. By altering the hydrophobicity of this peptide sequence, more specific organellar localization and increased membrane-disrupting activity were achieved and dramatic increases in potency were observed. The activity was preserved in peptide constructs that are known to be tissue-specific, indicating that these new killer peptides could be of significant utility for the development of new targeted therapeutics.

Results and Discussion

To produce mitochondria-disrupting peptides with optimal subcellular localization, we introduced sequence modifications of the D-(KLAKLAK)$_2$ peptide that might favor mitochondrial localization and mitochondria-disrupting activity. Prior studies indicate that if the charge of a peptide sequence is kept constant, but the overall sequence is made more lipophilic, mitochondrial localization will increase, with strong cellular uptake maintained.[18] Thus, we designed analogues of the D-(KLAKLAK)$_2$ anticancer peptide[2] where the hydrophobicity of the structure was modified but retained a constant charge. To achieve this, we exchanged the leucine residue within the sequence for three different amino acids: phenylalanine (F), cyclohexyl-alanine (F_X), or a 6-carbon alkyl chain residue (Hex) (Figure 1A). The effect of these substitutions on the hydrophobicities of the peptides was monitored by HPLC (Figure 1B,E). While D-(KFAKFAK)$_2$ exhibited a similar retention time on a C-18 column to D-(KLAKLAK)$_2$, both D-(KF$_X$AKF$_X$AK)$_2$ and D-(KHexAKHexAK)$_2$ exhibited higher levels of hydrophobicity. The relative hydrophobicities of the L, F, Hex, and F_X residues in the context of the KLAKLAK scaffold were confirmed by octanol/water partitioning (i.e., LogP) to increase according to F < L < F_X < Hex (Figure 1E).

Changes in subcellular localization (Figure 2) for the engineered peptides were investigated using confocal fluorescence microscopy. The relative shifts in localization effected by the F, F_X, and Hex substitutions were analyzed with monomer analogues (i.e., KXAKXAK, where X = F_X, F, Hex, or L) to enable imaging in the absence of toxicity. As shown in Figure 2, the fluorophore-labeled F-, F_X-, and Hex-substituted analogues (green channel) all exhibited significant increases in mitochondrial localization in comparison to the leucine-containing parent compound. The staining patterns indicated a qualitative increase

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[a] Abbreviations: AMPs, antimicrobial peptides; F_X, cyclohexylalanine; Hex, amino acid with 6-carbon "hexyl" alkyl side chain; CD, circular dichroism; *to*, thiazole orange; Rr, Pearson's correlation coefficient.

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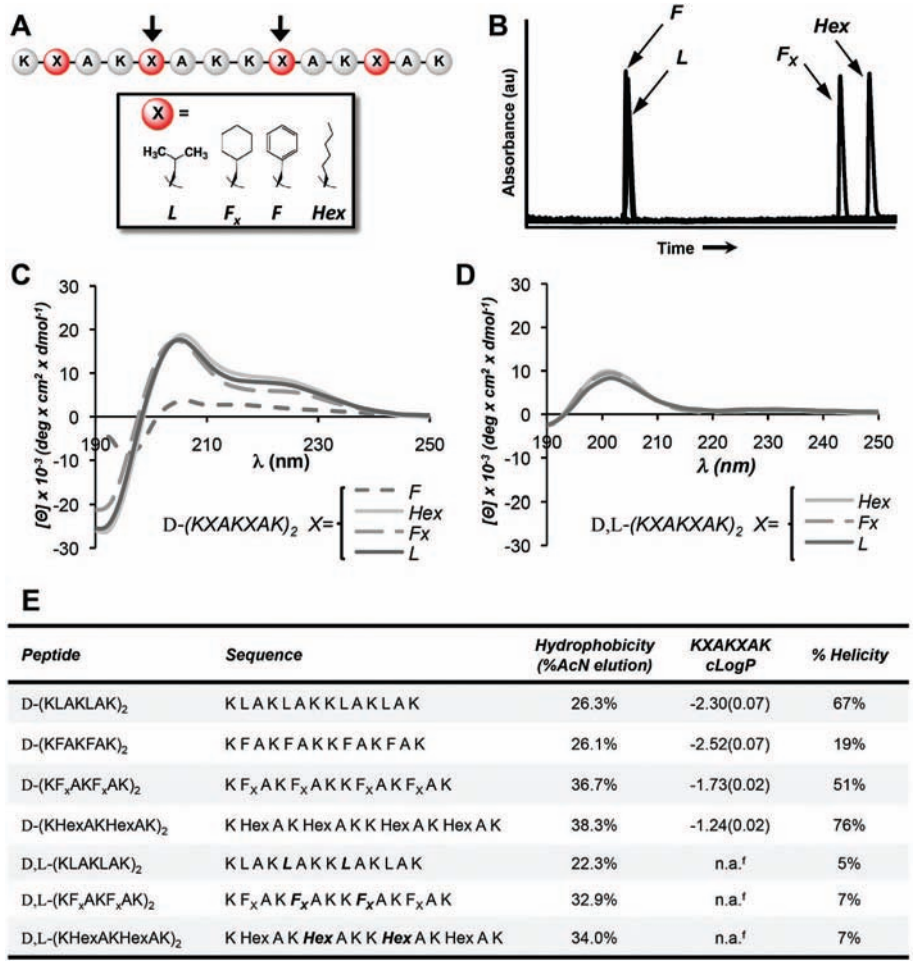

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Peptide	Sequence	Hydrophobicity (%AcN elution)	KXAKXAK cLogP	% Helicity
D-(KLAKLAK)$_2$	K L A K L A K K L A K L A K	26.3%	-2.30(0.07)	67%
D-(KFAKFAK)$_2$	K F A K F A K K F A K F A K	26.1%	-2.52(0.07)	19%
D-(KF$_X$AKF$_X$AK)$_2$	K F$_X$ A K F$_X$ A K K F$_X$ A K F$_X$ A K	36.7%	-1.73(0.02)	51%
D-(KHexAKHexAK)$_2$	K Hex A K Hex A K K Hex A K Hex A K	38.3%	-1.24(0.02)	76%
D,L-(KLAKLAK)$_2$	K L A K ***L*** A K K ***L*** A K L A K	22.3%	n.a.[f]	5%
D,L-(KF$_X$AKF$_X$AK)$_2$	K F$_X$ A K ***F$_X$*** A K K ***F$_X$*** A K F$_X$ A K	32.9%	n.a.[f]	7%
D,L-(KHexAKHexAK)$_2$	K Hex A K ***Hex*** A K K ***Hex*** A K Hex A K	34.0%	n.a.[f]	7%

Figure 1. Structures and comparison of hydrophobicity and helicity of the D-(KXAKXAK)$_2$ analogues. (A) Peptide structures. The D-(KXAKXAK)$_2$ peptide scaffold was synthesized with X = L, F, F$_X$, and Hex residues. Diastereomer peptides were substituted with L-amino acids where marked with arrows. (B) Comparison of hydrophobicities among D-(KXAKXAK)2 peptides by RP-HPLC retention times on a C18 column. (C,D) Assessment of helicity using circular dichroism (CD) spectroscopy. CD spectra of the (C) all D-isomer peptides and (D) diastereomer peptides at 25 mM were obtained in the presence of 25 μM SDS. (E) Summary of peptide attributes. L-Stereoisomers are denoted by bolded, italicized text (e.g., **Hex**). Hydrophobicity measured as % acetonitrile elution from RP-HPLC C18 column. LogP (average and sd of three trials) of partitioning between 1-octanol and 10 mM Tris pH 7.4 measured for the thiazole orange-labeled KXAKXAK peptides. Helical content was calculated from observed ellipticity at 222 nm from above conditions.[24,25]

in mitochondrial localization of the peptides and were further confirmed by quantitation of colocalization with mitotracker (red channel) through the calculation of Pearson's correlation coefficients (Rr). Mitochondrial localization increased relative to the parent sequence for the F$_X$- and Hex- substituted analogues but, unexpectedly, also increased for the KFAKFAK peptide, which possessed a slightly lower degree of hydrophobicity relative to the parent sequence when measured by RP-HPLC and LogP.

To determine whether the changes in subcellular localization could be correlated with the toxicities of the peptides, we evaluated the cell-killing capacity of the conjugates in multiple cell lines of human origin. Representative toxicity curves for HeLa cells exposed to D-(KLAKLAK)$_2$, D-(KFAKFAK)$_2$ D-(KF$_X$AKF$_X$AK)$_2$, and D-(KHexAKHexAK)$_2$ are shown in Figure 3A. Dramatic increases in potency were observed with the engineered sequences, with LC$_{50}$ values decreased by 2 orders of magnitude for the best engineered sequences relative to the parent compound (Figure 3C). This pattern of increased activity was conserved in all cell lines tested (Supporting Information Table 3), confirming that the improved activity of the engineered analogues is not a cell type-specific phenomenon. The heightened activity of the D-(KFAKFAK)$_2$ peptide in comparison with D-(KLAKLAK)$_2$ was especially interesting because this peptide displayed less hydrophobicity but increased mitochondrial localization in comparison with the parent peptide.

The utility of these peptides is derived from the fact that they can be easily functionalized with targeting sequences that are cell-type specific.[2−7] As discussed above, the peptides were not inherently cell-type specific, and so we wished to confirm that they could be altered to become specific in the same manner as previous analogues. Thus, we tested conjugates made by appending the CNGRC homing domain that is specific for cells expressing α_v integrins.[2] Indeed, the CNGRC-D-(KF$_X$AKF$_X$AK)$_2$ peptide exhibited a significant enhancement in activity (Figure 3B) relative to CNGRC-D-(KLAKLAK)$_2$ in KS1767 cells, a cell line known to bind the homing domain. In this cell line, the CNGRC-D-(KF$_X$AKF$_X$AK)$_2$ peptide displayed an LC$_{50}$ of 7 $\pm$ 1 μM compared with 63 $\pm$ 4 μM for the parent peptide. The activity is affected by cell type, as the control cell line MDA-MB-435[2] exhibited less sensitivity to CNGRC-D-(KF$_X$AKF$_X$AK)$_2$. The levels of specificity for the parent peptide and the engineered analogue were comparable; the LC$_{50}$ for the engineered analogue was 17 $\pm$ 4 μM (2.7-fold specificity) and 144 $\pm$ 4 μM for CNGRC-D-(KLAKLAK)$_2$ (2.3-fold specificity). These experiments indicate that the engineered peptides are suitable for targeting studies and maintain the same therapeutic

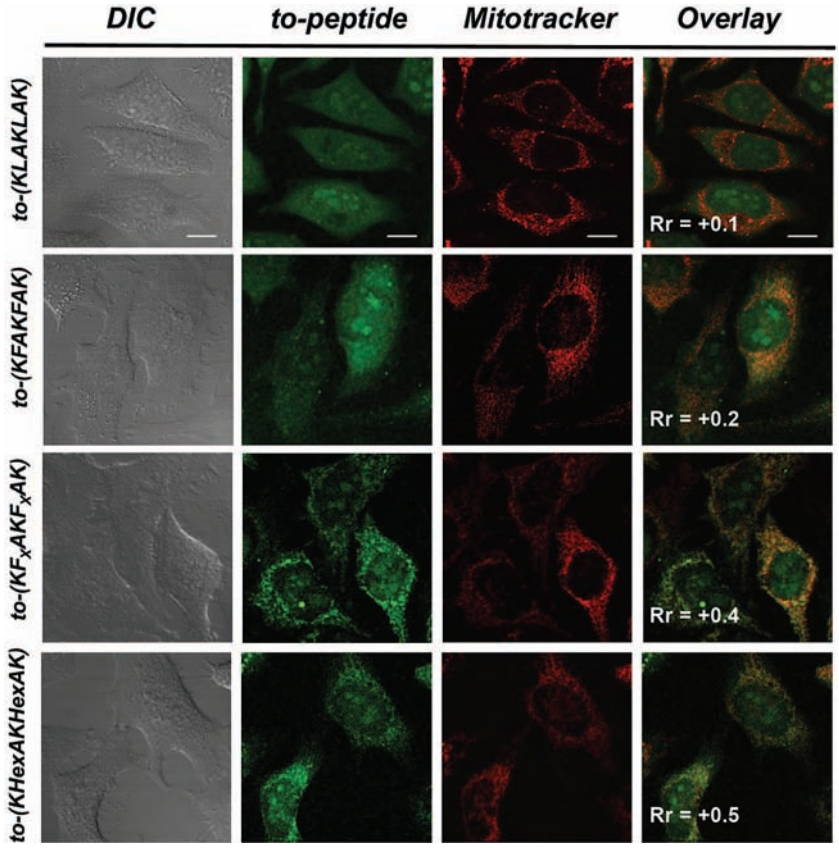


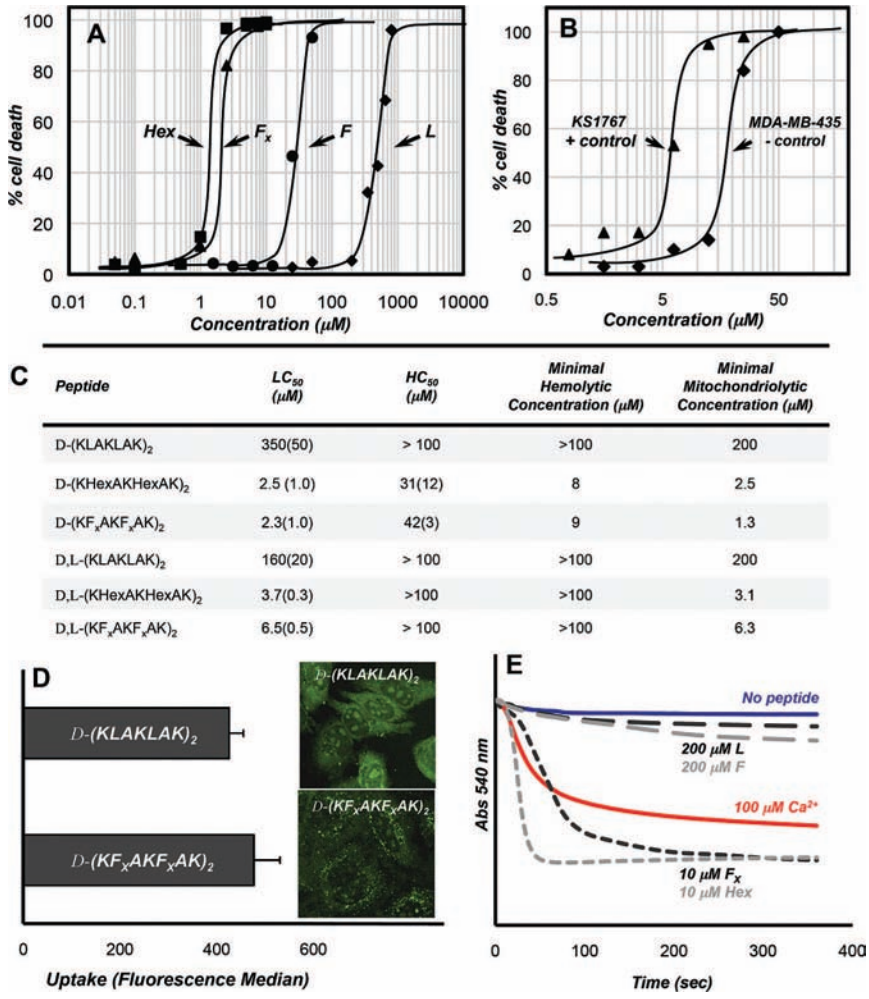
Figure 2. Subcellular localization of the KXAKXAK peptides. Mitochondrial localization of KXAKXAK peptides. Mitochondrial localization was assessed by quantitative colocalization with Mitotracker in HeLa cells. Columns from left to right: differential interference contrast image (DIC); thiazole orange N-terminus-labeled peptides (*to*-peptide); mitotracker; overlay. Pearson's correlation coefficient (Rr) expressing degree of colocalization was calculated from groups of >50 cells collected in three separate trials. Scale bar is 1 μm.

window as the parent peptide with a specificity index of ~3. However, the 10-fold improved potency of D-(KF$_X$AKF$_X$AK)$_2$ means the effect can be realized with lower dosages of drug. For experiments using mouse models, this can translate into a 10-fold reduction in research costs, permitting many more trials to be run or targeting domains to be tested.

Increased cellular accumulation could contribute to the observed increase in potency among the engineered conjugates. To explore this possibility, cellular uptake was monitored using flow cytometry, and it was confirmed that the uptake of the D-(KLAKLAK)$_2$ was not strongly affected by the F$_X$ substitution (Figure 3D). In addition, uptake of the dimers was also monitored using confocal microscopy to be similar or even slightly higher for D-(KLAKLAK)$_2$ compared with D-(KF$_X$AKF$_X$AK)$_2$ (Figure 3D). These images and uptake data were obtained at early time points and low concentrations to avoid toxicity. Under these conditions, healthy cells exhibited lysosomal sequestration of the peptide, particularly in the case of D-(KF$_X$AKF$_X$AK)$_2$. These data clearly show that the parent and engineered peptides are internalized within living cells with comparable efficiency and that enhanced uptake is not the source of the improved activity for the new analogues.

The activity of the D-(KLAKLAK)$_2$ peptide has been linked to its ability to disrupt mitochondria, leading to the release of pro-apoptotic factors and initiation of apoptosis.[2−4] To confirm that toxicity of the engineered analogues originated from the same type of activity, mitochondrial disruption and the mechanism of cell death was studied. Disruption of mitochondria within HeLa cells upon treatment with the peptides was assessed by monitoring mitochondrial morphology using fluorescence imaging, TEM, and a calcein release and quenching assay (Figure 4A). All three techniques indicated that mitochondrial swelling and disruption occurring at the LC$_{50}$ of D-(KF$_X$AKF$_X$AK)$_2$ (2.5 μM). In comparison, little mitochondrial disruption was present in D-(KLAKLAK)$_2$-treated cells at concentrations up to 350 μM. The cytotoxicity of the engineered analogue, therefore, is intimately linked with mitochondrial disruption. This hypothesis was further supported by the observation that loss of mitochondrial integrity precedes loss of plasma membrane integrity upon treatment with D-(KF$_X$AKF$_X$AK)$_2$ (see Supporting Information Figure 5). This disruption then induces apoptosis, as evaluated using the TUNEL assay (Figure 4B) and by monitoring caspase activation (see Supporting Information Figure 6).

A key feature of pro-apoptotic anticancer peptides is the ability to disrupt mitochondrial membranes *preferentially*, leaving the plasma membranes of targeted and untargeted cells intact. To evaluate the specificities of the engineered peptides toward mitochondrial membranes, we monitored the disruption of isolated mitochondria as a function of peptide concentration and compared the minimal concentrations of peptides needed to lyse mitochondria with those required to cause lysis of the plasma membranes of red blood cells (Figure 3C,E). The two engineered analogues exhibiting the highest potencies, D-(KF$_X$AKF$_X$AK)$_2$ and D-(KHexAKHexAK)$_2$, both exhibited mitochondrial disruption at concentrations close to those inducing toxicity. Some lysis of red blood cells was observed (Figure 3C and Supporting Information Figure 5) but at concentrations 3−4-fold higher than the LC$_{50}$ values, indicating that the peptides do indeed exhibit preferential targeting and disruption of the mitochondrial membrane.



Peptide	LC_{50} (μM)	HC_{50} (μM)	Minimal Hemolytic Concentration (μM)	Minimal Mitochondriolytic Concentration (μM)
D-$(KLAKLAK)_2$	350(50)	> 100	>100	200
D-$(KHexAKHexAK)_2$	2.5 (1.0)	31(12)	8	2.5
D-$(KF_xAKF_xAK)_2$	2.3(1.0)	42(3)	9	1.3
D,L-$(KLAKLAK)_2$	160(20)	> 100	>100	200
D,L-$(KHexAKHexAK)_2$	3.7(0.3)	>100	>100	3.1
D,L-$(KF_xAKF_xAK)_2$	6.5(0.5)	> 100	>100	6.3

Figure 3. Cytotoxic, mitochondriolytic, and hemolytic activities, and cellular uptake of $(KXAKXAK)_2$ peptides and derivatives. (A) Cytotoxicity induced by engineered peptides in HeLa cells. Cell death measured by flow cytometry after 24 h of treatment with the D-$(KXAKXAK)_2$ peptides. (B) Activity of integrin-targeted CNGRC-GG-D-$(KFXAKFXAK)_2$ peptide. The toxicity of the peptide against KS1767 (positive control for overexpression of CNGRC-targeted integrin) and MDA-MB-435 (negative control) cells was measured after 48 h. LC_{50} values for this and other targeted conjugates are reported in the text. (C) Summary of activities. LC_{50} values measured using HeLa cells. HC_{50} denotes concentration at 50% hemolysis. Minimal hemolytic concentration denotes concentration of peptide at 10% hemolysis (raw hemolytic data Supporting Information Figure S5). Minimal mitochondriolytic concentration represents the lowest concentration of peptide at which significant mitochondrial disruption occurred in a mitochondrial swelling assay. (D) Uptake of D-$(KXAKXAK)_2$ thiazole orange conjugates into HeLa cells. Assessed after 30 min incubation with 1 μM conjugate (average of >3 trials exhibited with sd). Images at right are representative of dimer uptake at 30 min. (E) Mitochondrial disruption. Swelling of isolated mitochondria monitored at 540 nm. Peptides traces shown are D-analogues.

Many membrane-active peptides are reliant on an α-helical amphipathic structure for activity,[11,19,20] while for others, a secondary structure that sequesters positive charges and hydrophobic residues onto opposite sides of an α helix is not a necessary feature for activity against microbial targets.[13,16] Whether the activities of mitochondria-disrupting peptides are influenced by this factor has never been addressed. We evaluated the effect of helicity on the activities of our engineered peptides by measuring % helicity using circular dichroism (CD) spectroscopy. While the spectra displayed the features expected for peptides made of D-amino acids exhibiting helical character, there was no correlation between toxicity and helicity (Figures 1C, 3B); the helicity increased in the order X = Hex > L > F_X > F, while the activities increased as X = Hex > F_X > F > L. We further investigated this issue by synthesizing diastereomeric analogues of the peptides featuring L-amino acid substitutions at the two central hydrophobic residues (the fifth and ninth residues) that would inhibit the formation of helical structures (Figure 1A). Measurements of % helicity again made using CD spectroscopy confirmed that these structures lacked helicity (Figure 1D,E). The toxicities of the diastereomers were examined in HeLa cells and, interestingly, were found to be very similar to the activities of the all D-analogues, providing further evidence that cell-killing activity is not contingent on the presence of α-helical structure (Figure 3C). Furthermore, the hemolytic activities of the engineered peptides were effectively abolished when helicity was disrupted (Figure 3C and Supporting Information Figure 5). This finding indicates that *decreases* in the helical structure of peptides *increases* specificity for mitochondrial membranes. The retention of activity toward anionic mitochondrial membranes (Figure 3C), but not zwitterionic plasma membranes, in the absence of helicity suggests that these peptides may act on mitochondria through a mechanism that does not depend on formation of an amphipathic α helix that sequesters positively charged residues from hydrophobic residues, i.e., a carpet or detergent-like mechanism that does not involve the formation of structured pores.[21,22] Furthermore, the integrin homing domain conjugate of D,L-$(KF_XAKF_XAK)_2$ was tested to determine whether this conjugate could also be used in targeted studies. An LC_{50} of 7 ± 1 μM was observed in the positive control cell line KS1767, compared with 17 ± 1 μM in the negative control cell line MDA-MB-

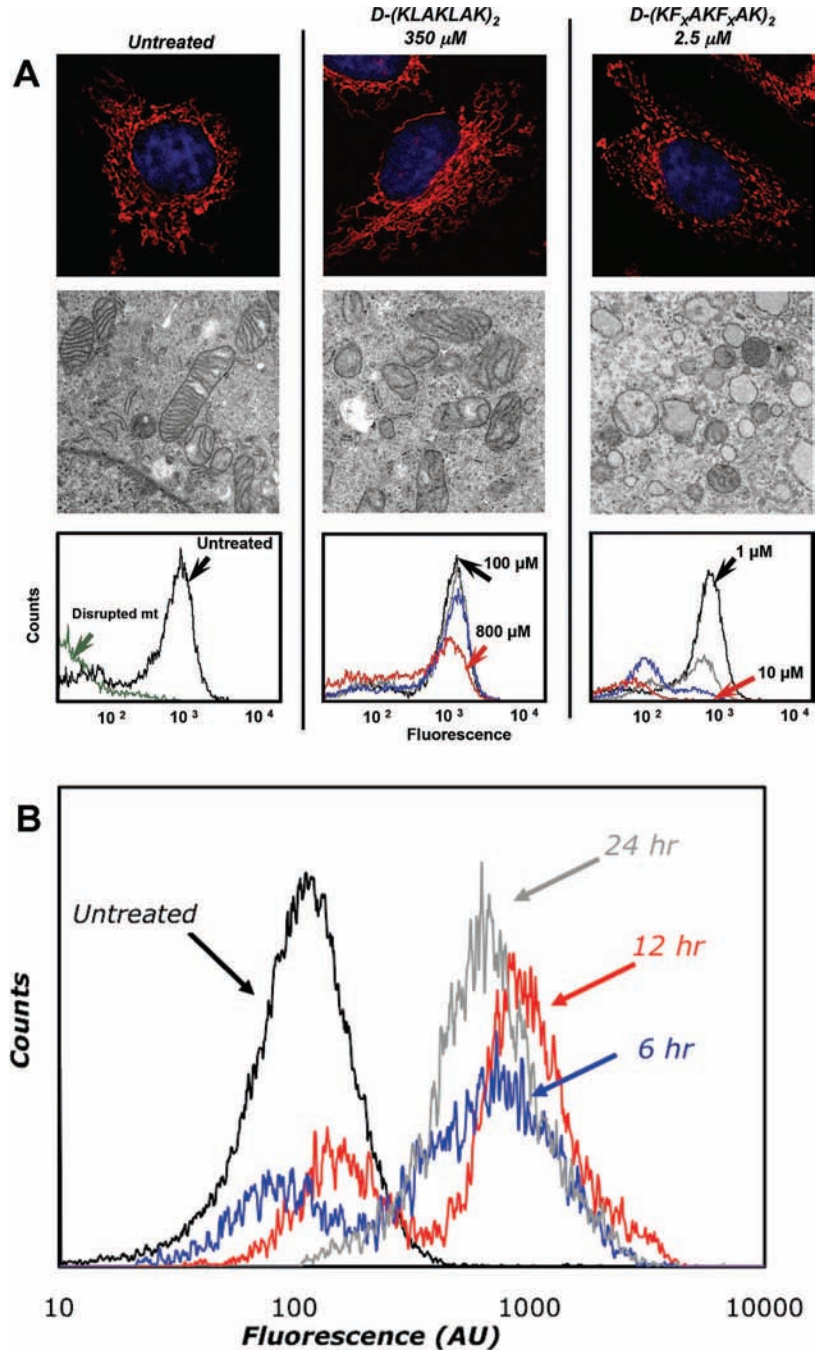


Figure 4. Mitochondrial disruption and activation of apoptosis by D-(KXAKXAK)$_2$ peptides in vivo. (A) Visualization of mitochondrial disruption. Fluorescence imaging of mitochondrial morphology with mitotracker and DAPI nuclear staining (top); transmission electron microscopy (middle); calcein release and quenching assay (bottom) for D-(KLAKLAK)$_2$ (100−800 μM) and D-(KF$_X$AKF$_X$AK)$_2$ (1−10 μM). (B) Induction of apoptosis. DNA fragmentation as measured by TUNEL assay increases over time in HeLa cells treated with 2.5 μM D-(KF$_X$AKF$_X$AK)$_2$.

435 (2.6-fold specificity). The highly similar level of specificity observed among the conjugates indicate that the sequence alterations made to the more potent engineered peptides have not affected the ability of the (KXAKXAK)$_2$ scaffold to be targeted in this manner. In conclusion, the low hemolytic activity, high therapeutic index, and targeting ability of D,L-(KF$_X$AKF$_X$AK)$_2$ suggests that this conjugate be pursued for future biological testing.

The peptides described here were engineered to be more hydrophobic than D-(KLAKLAK)$_2$, as it is known that modifications that make sequences less hydrophilic can facilitate obtaining specific localization to mitochondria.[18] The desired shifts in subcellular localization were achieved with the substitutions made, and as well, dramatic increases in toxicity were observed. The improved potencies can therefore be correlated with more complete delivery of the peptides to their site of action—the mitochondria. However, increases in hydrophobicity can also enhance the activity of membrane-disrupting peptides, presumably by favoring interactions with the hydrocarbon core of the lipid bilayer and subsequent introduction of membrane instability.[11−13,22] Thus, it is likely that the increased potencies observed result both from improved localization and also more effective membrane lysis once the peptides arrive in the mitochondria. In sum, altering hydrophobicity in this way likely increases potency both due to better subcellular targeting and better membrane lysis activity and is clearly beneficial for the generation of

toxic agents as long as membrane-type specificity and cell-type specificity can be maintained.

Conclusion

The sequence of a widely used anticancer peptide, D-(KLAK-LAK)$_2$, was engineered to display enhanced accumulation at the subcellular site of activity, increased membrane-disrupting activity, and high selectivity. There was little relationship between helicity and activity: diastereomeric analogues devoid of helical content closely mimicked the activity of the all D-stereoisomer counterpart and demonstrated the highest therapeutic index. The activity was preserved in peptide constructs that are known to be tissue-specific, indicating that these engineered peptides could be of significant utility for the development of new targeted therapeutics. The increased potency exhibited by these agents will significantly reduce the cost of work with targeted toxic peptides and will facilitate testing of such agents in animal models. This system may also provide an important precedent for the optimization of other mitochondrially targeted drugs that may require more specific localization for optimal activity.

Experimental Details

Preparation and Characterization of Peptides. Unlabeled D-(KXAKXAK)$_2$ (X = L, F$_X$, Hex) (ChemPep, Inc.) and CNGRC peptides (AnaSpec, Inc.) were obtained at >95% purity. Unlabeled D-(KFAKFAK)$_2$, diastereomers, and thiazole−orange (*to*) labeled peptides were synthesized in house using standard Fmoc chemistry, purified by RP-HPLC, and >95% purity and identity confirmed by analytical HPLC as previously described.[23] *to*-Labeled peptides were quantified as described;[18] unlabeled peptides were quantified with the QuantiPro BCA Assay Kit (Sigma).

Analysis of Peptide Hydrophobicity. RP-HPLC was conducted on unlabeled peptides using a C18 column (Varian C18, 5 μm, 250 mm × 4.6 mm) with MeCN/0.1%TFA and H_2O/ 0.1% TFA mobile phases, 1.0% MeCN/min gradient. LogP analysis was performed on thiazole orange-labeled KXAKXAK peptides as previously described.[18]

Cell Culture. HeLa, MRC5, DU145, and FaDu cells were cultured in MEM (ATCC) supplemented with 10% (v/v) FBS in a humidified incubator at 37 °C with 5% CO_2. MCF7 cells were additionally supplemented with 0.01 mg/mL insulin. KS1767 and MDA-MB-435 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS; MDA-MB-435 was additionally supplemented with 2 mM glutamine and 1 mM pyruvate.

Circular Dichroism (CD) Spectroscopy. CD spectra were acquired using a Jasco J-720 CD spectropolarimeter. CD scans of the peptides were performed at 25 μM peptide in 25 mM SDS in 10 mM PBS, pH 7.4. Average of three scans with background scans of 25 mM SDS in PBS. Scans from 190 to 250 nm with 0.2 nm resolution were obtained at 20 nm/min with a bandwidth of 1.0 nm. Helical content was calculated from observed helicity at 222 nm as previously described.[24,25]

Hemolytic Assay. The assay was adapted from Schmitt, et al.[16] Percent lysis was calculated using 400 mg/mL melittin (Sigma) and PBS as the 100% and 0% lysis controls.

Mitochondrial Swelling Assay. Mitochondria were isolated from fresh mouse liver as previously described.[26] Protein was quantified with the QuantiPro BCA Assay Kit (Sigma). Mitochondria were used at ~0.25 mg/mL for disruption experiments. Upon addition of peptide, absorbance at 540 nm was monitored for 6 min at 25 °C. 100 μM calcium chloride and PBS were used as the positive and negative control for mitochondrial disruption, respectively.

Localization of *to*-Peptide Conjugates. HeLa cells were incubated with 3 μM to 5 μM *to*-peptide conjugate for 90 min. Mitotracker CMXRos (Invitrogen, 25 nM) was added for the last 15 min of the incubation. Cells were washed and images were taken with an inverted Zeiss LSM 510 confocal microscope and Colocalizer Pro used to calculate Pearson's coefficient (Rr) as previously described.[18] Rr calculated for >50 cells over multiple experiments. The background was subtracted from the images using a manually selected region of interest (ROI).

Analysis of Peptide Uptake. For flow cytometry, cells were incubated with 1 μM *to*-labeled peptide for 30 min. Following the incubation, cells were washed with PBS, trypsinized, pelleted, stained with the viability dye Sytox Red (1 nM), and analyzed by flow cytometry (BD FACS Canto) as previously described.[18] For microscopy, cells were incubated with 250 nm *to*-labeled peptide for 30 min, then washed with PBS and imaged by confocal microscopy (see *Localization*).

Determination of D-(KXAKXAK)$_2$ LC$_{50}$. Incubations were performed in OPTI-MEM (Invitrogen) containing 2% FBS. Cells were incubated with the unlabeled peptides over a range of concentrations for 24 h (note 48 h incubations were used with homing domain peptides). Cell death was assessed by monitoring loss of plasma membrane integrity through staining with the viability dye Sytox Red (Invitrogen) and by measuring cell dehydrogenase activity with the CCK-8 assay (Dojindo Laboratories, Inc.)

Assessment of Mitochondrial Disruption by Mitochondrial Morphology. All techniques performed after incubation of HeLa cells for 30 min with peptide. Fluorescence imaging of mitochondria: Cells were incubated with 50 nM Mitotracker CMXRos and 500 nM DAPI nuclear stain for 20 min, washed three times with PBS, and then imaged. Transmission electron microscopy (TEM): Cells were washed with PBS and prepared for TEM according to a technique modified from Pietschmann, et al.[27] Calcein release and quenching: Prior to incubation with the peptides, the cells were incubated for 15 min with 1 μM Calcein-AM (Invitrogen) and 1 mM cobalt chloride. For positive controls of mitochondrial disruption, cells were treated with 1 μM staurosporine for 24 h. Cells were trypsinized and collected by centrifugation and then analyzed by flow cytometry. Sytox Red was used for viability staining. Both live and dead cells were included in the analysis.

Apoptosis Assays. Following peptide treatment, cells were trypsinized, collected by centrifugation, washed, and then stained with antibodies for activated caspase 3 (FITC conjugate, BD Biosciences) or activated caspase 9 (Santa Cruz Biotechnology)/ antigoat AlexaFluor 555 conjugate (Invitrogen). Cells were washed again and analyzed by flow cytometry. Positive controls were performed with staurosporine-treated cells. The TUNEL assay was performed on the fixed, washed cells using the APO-BRDU kit (BD Biosciences) as directed by the manufacturer.

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Supporting Information Available: Additional experimental details, chemical structures and characterization, toxicity in other cell lines, mitochondriolytic and hemolytic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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