Supporting Information

Transformation of an antimicrobial peptide into a plasma membrane-permeable, mitochondria-targeted peptide via the substitution of lysine with arginine

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Materials and methods

Peptide synthesis

All peptides employed in this study had a C-terminal amide structure and were synthesized using 9-fluorenylmethyloxycarbonyl solid-phase peptide chemistry on a Shimadzu PSSM-8 peptide synthesizer. TGS-RAM resin (Shimadzu, Kyoto, Japan) and a coupling system using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/N,N-diisopropylethylamine (DIEA) were employed. To prepare fluorescently-labeled peptides, a peptide resin with γ-aminobutyric acid (GABA) at its N-terminus was prepared, and the N-terminus was modified with fluorescein-5-isothiocyanete (FITC) in the presence of DIEA. Deprotection and cleavage of the peptides from the resin were performed using trifluoroacetic acid (TFA)-ethanedithiol (EDT) (95:5) at 20 °C for 3 h, followed by purification using reverse-phase high-performance liquid chromatography (HPLC). The structures of the products were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex, Bruker Daltonics, MA, USA). The exact sequences of the synthesized peptides and their characteristics are given below:

Conditions used for HPLC:

Condition I: Column, Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient, 5-70% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow, 1 mL/min; detection, 220 nm.

Condition II: Column, Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient, 5-95% B in A

(A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 40 min; flow, 1 mL/min; detection, 220 nm.

Condition III: Column, Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient, 30-90% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH).

KLA ($_D$[KLAKLAKKLAKLAK]-amide)

MALDI-TOF MS: 1524.0 [calculated for $(M+H)^+$: 1524.0]. HPLC retention time, 14.8 min (condition I). Yield from the starting resin, 40%.

RLA ($_D$[RLARLARRLARLAR]-amide)

MALDI-TOF MS: 1692.3 [calculated for $(M+H)^+$: 1692.1]. HPLC retention time, 15.2 min (condition I). Yield from the starting resin, 54%.

*RLA*GG ($_D$[RLARLAR]-GG-$_D$[RLARLAR]-amide)

MALDI-TOF MS: 1805.9 [calculated for $(M+H)^+$: 1806.2]. HPLC retention time, 13.0 min (condition I). Yield from the starting resin, 30%.

BH4 (SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 2333.6 [calculated for $(M+H)^+$: 2333.5]. HPLC retention time, 16.2 min (condition I). Yield from the starting resin, 35%.

RLA-BH4 ($_D$[RLARLARRLARLAR]-GG-SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 4122.0 [calculated for $(M+H)^+$: 4121.8]. HPLC retention time, 17.2 min (condition I). Yield from the starting resin, 29%.

R8-BH4 (RRRRRRRR-GG-SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 3697.9 [calculated for $(M+H)^+$: 3697.3]. HPLC retention time, 15.4 min (condition I). Yield from the starting resin, 43%.

FITC-*KLA* (FITC-GABA-$_D$[KLAKLAKKLAKLAK]-amide)

MALDI-TOF MS: 1998.5 [calculated for $(M+H)^+$: 1998.5]. HPLC retention time, 19.2 min (condition II). Yield from the starting resin, 70%.

FITC-*RLA* (FITC-GABA-$_D$[RLARLARRLARLAR]-amide)

MALDI-TOF MS: 2166.3 [calculated for $(M+H)^+$: 2166.6]. HPLC retention time, 8.9 min (condition III). Yield from the starting resin, 67%.

FITC-*RLA-K1* (FITC-GABA-$_D$[KLARLARRLARLAR]-amide)

MALDI-TOF MS: 2138.9 [calculated for $(M+H)^+$: 2138.6]. HPLC retention time, 20.7 min (condition I). Yield from the starting resin, 65%.

FITC-*RLA-K4* (FITC-GABA-$_D$[RLAKLARRLARLAR]-amide)

MALDI-TOF MS: 2139.0 [calculated for $(M+H)^+$: 2138.6]. HPLC retention time, 20.8 min (condition I). Yield from the starting resin, 70%.

FITC-*RLA-K7* (FITC-GABA-$_D$[RLARLAKRLARLAR]-amide)

MALDI-TOF MS: 2138.7 [calculated for $(M+H)^+$: 2138.6]. HPLC retention time, 20.7 min (condition I). Yield from the starting resin, 52%.

FITC-*RLA-K14* (FITC-GABA-$_D$[RLARLARRLARLAK]-amide)

MALDI-TOF MS: 2138.7 [calculated for $(M+H)^+$: 2138.6]. HPLC retention time, 20.5 min (condition I). Yield from the starting resin, 68%.

FITC-*RLA*GG (FITC-GABA-$_D$[RLARLAR]-GG-$_D$[RLARLAR]-amide)

MALDI-TOF MS: 2280.5 [calculated for $(M+H)^+$: 2280.7]. HPLC retention time, 16.1 min (condition I). Yield from the starting resin, 41%.

FITC-R8 (FITC-GABA-RRRRRRRR-amide)

MALDI-TOF MS: 3530.8 [calculated for $(M+H)^+$: 3530.2]. HPLC retention time, 17.8 min (condition II). Yield from the starting resin, 57%.

FITC-R8-*KLA* (FITC-GABA-RRRRRRRR-GG-$_D$[KLAKLAKKLAKLAK]-amide)

MALDI-TOF MS: 3362.1 [calculated for $(M+H)^+$: 3362.1]. HPLC retention time, 16.1 min (condition I). Yield from the starting resin, 45%.

FITC-BH4 (FITC-GABA-SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 2808.6 [calculated for $(M+H)^+$: 2808.1]. HPLC retention time, 18.4 min (condition I). Yield from the starting resin, 25%.

FITC-*RLA*-BH4
(FITC-GABA-$_D$[RLARLARRLARLAR]-GG-SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 4596.6 [calculated for $(M+H)^+$: 4596.3]. HPLC retention time, 19.5 min (condition I). Yield from the starting resin, 15%.

FITC-R8-BH4
(FITC-GABA-RRRRRRRR-GG-SNRELVVDFLSYKLSQKGYS-amide)

MALDI-TOF MS: 4172.4 [calculated for $(M+H)^+$: 4171.9]. HPLC retention time, 18.4 min (condition I). Yield from the starting resin, 27%.

Circular dichroism

Circular dichroism spectra were recorded with a Jasco 820-L UV-Vis spectropolarimeter and a Peltier PTC-423L. The spectra were obtained over 200-250

nm at a spectral bandwidth of 1 nm, a time response of 2 s, a scan speed of 20 nm/min, and a step resolution of 0.5 nm. Each spectrum was the average of five accumulations. Spectra were obtained using non-fluorescently-labeled peptides in the presence and absence of large unilamellar vesicles (100 nm) composed of phosphatidylcholine (PC)-phosphatidylserine (PS) (4:1) (150 μM) in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, and 2 mM KCl, pH 7.4). The blank was subtracted from each spectrum.

Cell culture

Human cervical cancer-derived HeLa cells were purchased from the Riken BRC Cell Bank (Tsukuba, Japan) and cultured in α-MEM containing 10% heat-inactivated bovine serum. Cells were grown on 100-mm dishes and incubated at 37 °C under 5% CO_2.

Confocal microscopy

HeLa cells (2.0×10^5 cells/well) were plated onto 35-mm glass-bottomed dishes (Iwaki, Tokyo, Japan) and cultured in α-MEM containing 10% heat-inactivated bovine serum for 24 h. After complete adhesion, the cell culture medium was removed and the cells were washed with serum-free α-MEM. The cells were incubated at 37 °C in serum-free fresh medium (200 μL) containing fluorescently-labeled peptides. After peptide treatment, the cell culture medium was removed and the cells were treated with MitoTracker Red 580 (500 nM) (Invitrogen, Eugene, OR, USA) for 15 min at 37 °C in serum-free fresh medium (200 μL). The cells were then washed with phosphate buffered saline (PBS) containing 0.5% (w/v) heparin, and fresh medium without peptides (1 mL/well) was added. Distribution of the fluorescently-labeled peptides in the cells was analyzed using an FV300 or FV1000 confocal scanning laser microscope (Olympus, Tokyo, Japan) equipped with a 60× objective without fixing the cells to avoid artifactual localization of the internalized peptides.

Flow cytometry

HeLa cells (1.0×10^5 cells/well) were plated in a 24-well microplate (Iwaki) and cultured for 24 h in α-MEM containing 10% heat-inactivated bovine serum. After complete adhesion, the cells were washed with serum-free α-MEM and incubated at

37 °C with synthesized peptides dissolved in serum-free α-MEM (200 μL) prior to washing with PBS containing 0.5% (w/v) heparin. The cells were treated with 0.01% trypsin at 37 °C for 10 min prior to the addition of PBS (200 μL), and centrifuged at 3,000 rpm (800 x *g*) for 3 min at 4 °C. After the supernatant was removed, the cells were washed with PBS (400 μL) and centrifuged at 3,000 rpm for 3 min at 4 °C. After this washing cycle was repeated, the cells were suspended in PBS (400 μL) and subjected to fluorescence analysis on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using 488-nm laser excitation and a 515-545-nm emission filter.

Cytotoxicity (WST-1 assay)

HeLa cells (5.0×10^3 cells/well) were cultured in a 96-well microplate in α-MEM with 10% heat-inactivated bovine serum at 37 °C under 5% CO_2 for 24 h. The cells were then washed with serum-free α-MEM and incubated with peptide (total volume, 50 μL) in serum-free α-MEM at 37 °C under 5% CO_2 for 30 min. Next, the medium was removed, and the cells were incubated in fresh α-MEM with 10% heat-inactivated bovine serum (100 μL/well) at 37 °C under 5% CO_2 for 24 h. After the incubation, cell viability was determined using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay, according to the manufacturer' s protocol (Roche Applied Science, Mannheim, Germany). WST-1 reagents (10 μL) were added to each well and incubated for 40 min at 37 °C. Measurement of absorbance was conducted at 450 and 650 nm, and the value obtained by subtracting A_{650} from A_{450} corresponded to viable cell number.

Cytotoxicity (trypan blue staining)

HeLa cells (1.0×10^5 cells/well) were cultured in a 24-well microplate in α-MEM with 10% heat-inactivated bovine serum at 37 °C under 5% CO_2 for 24 h. The cells were then washed with serum-free α-MEM and incubated with peptide (total volume, 200 μL) in serum-free α-MEM at 37 °C under 5% CO_2 for 1 h. Next, the samples were removed, and the cells were incubated with etoposide (200 μM) (Sigma-Aldrich) in fresh α-MEM for 15 h. After the treatment, the cells washed with PBS and treated with 0.01% trypsin (200 μL) to detach the cells. The collected cells were stained with trypan blue prior to counting the number of living and dead cells using a Countess Automated Cell Counter (Invitrogen).

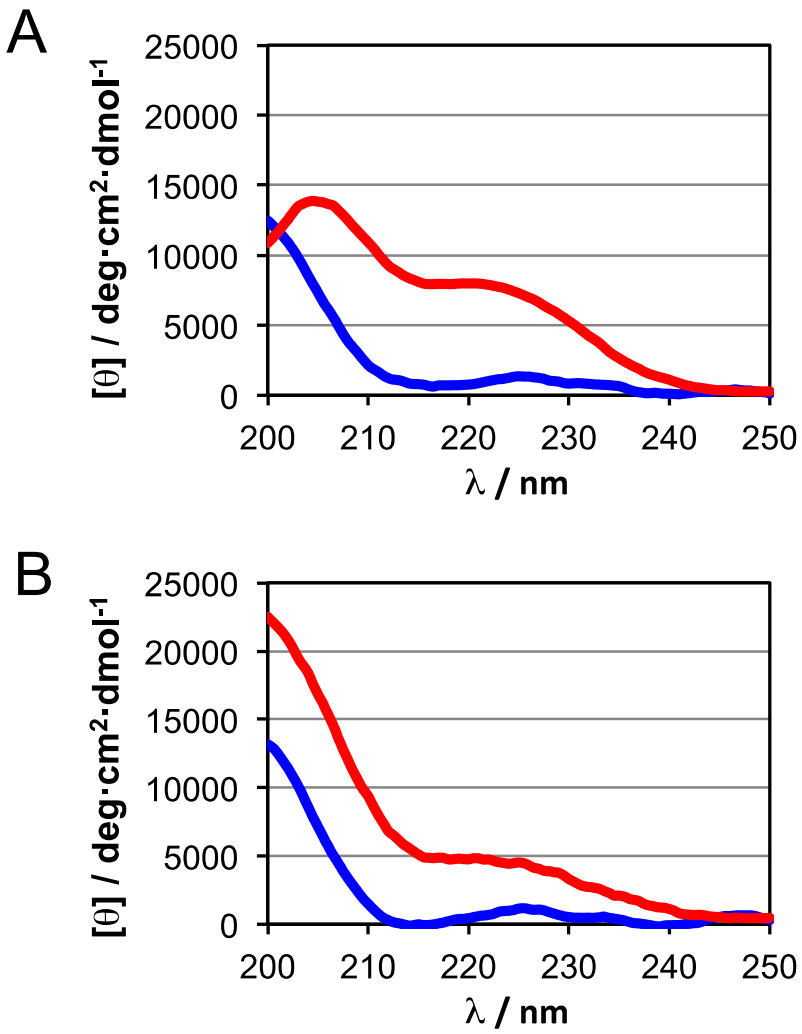

Fig. S1 Circular dichroism (CD) spectra of *KLA* (A) and *RLA*GG (B) (4.5 μM) in the presence (red) and absence (blue) of liposomes comprised of phosphatidylcholine (PC)–phosphatidylserine (PS) (4:1) (150 μM) in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4).

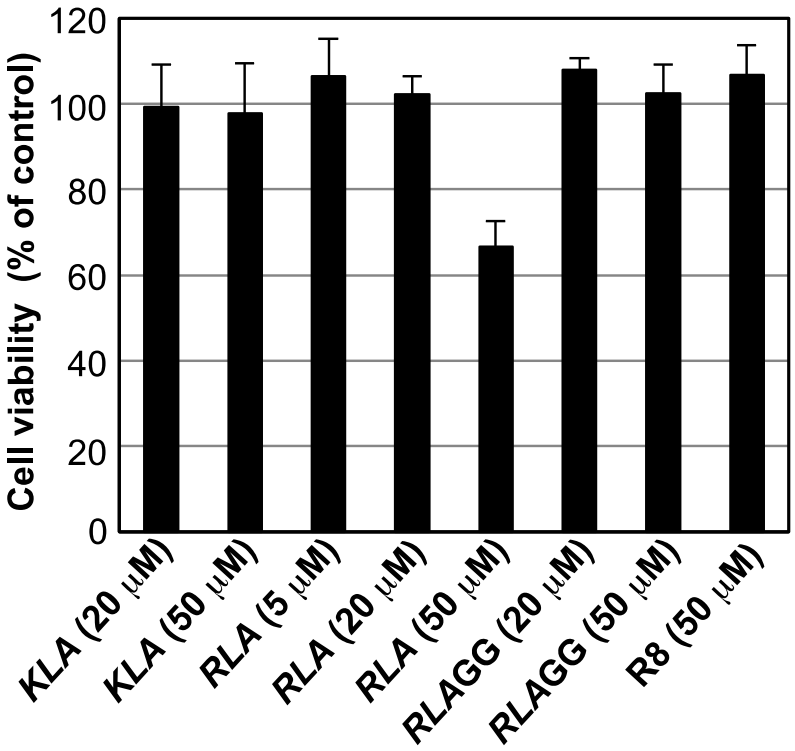

Fig. S2 Cytotoxicity of *KLA* and its derivatives. HeLa cells were treated with *KLA*, *RLA*, *RLA*GG, and R8 peptides for 30 min at 37 °C, and analyzed using WST-1 assay. The data represent the average (± SD) of four experiments.

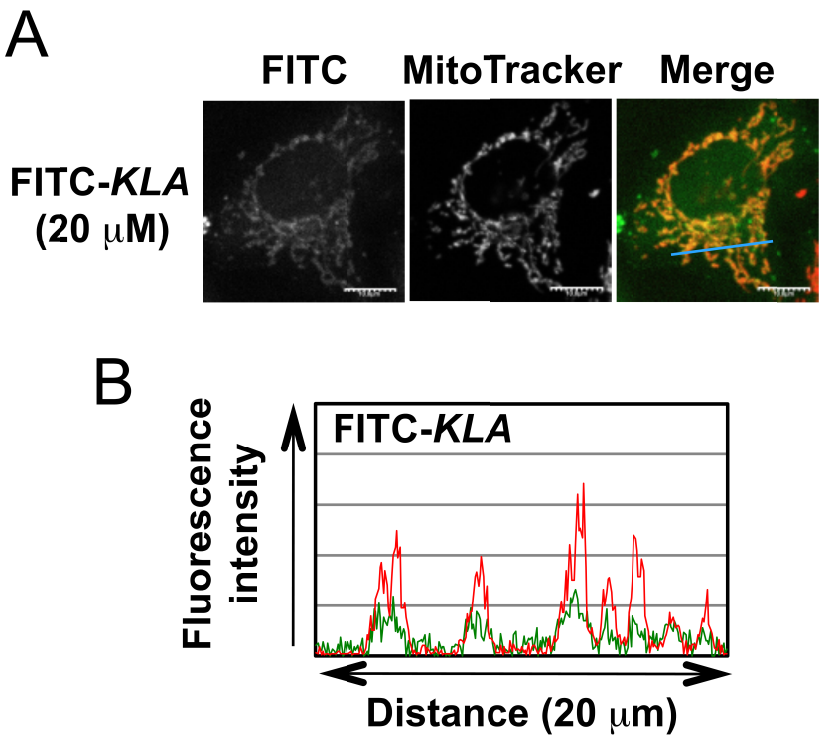

Fig. S3 (A) Confocal microscopic observation of HeLa cells treated with FITC-*KLA* peptide (20 μM) for 30 min at 37 °C. Scale bar: 10 μm. (B) Fluorescence intensity on blue line in merged picture in A (green: FITC, red: MitoTracker).

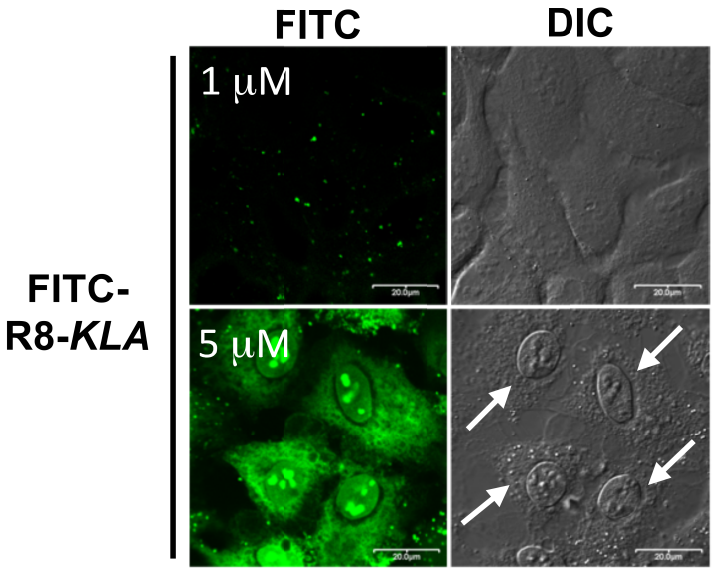

Fig. S4 Cytotoxicity observed on R8-*KLA* peptide treatment. HeLa cells were treated with fluorescently-labeled R8-*KLA* peptide (1 or 5 μM) for 30 min at 37 °C, and then analyzed by confocal microscopy. Arrows indicate dead cells.

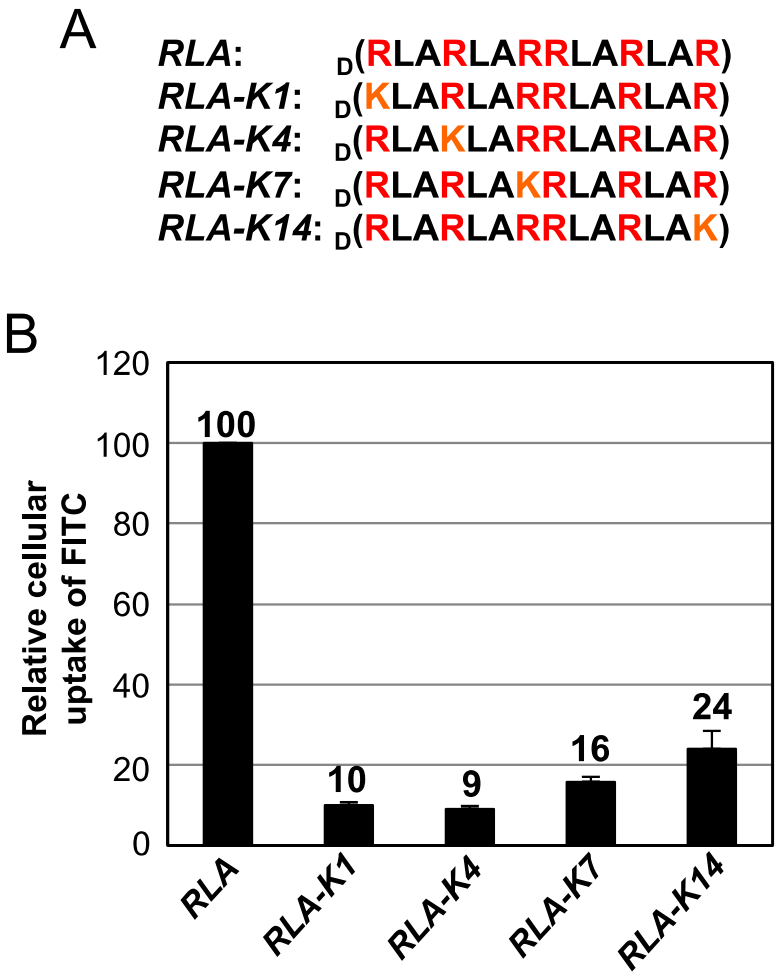

Fig. S5 (A) Amino acid sequences of *RLA* derivatives. The respective peptides have an amide structure at their C-termini. (B) Relative cellular uptake of fluorescently-labeled peptides. HeLa cells were treated with each peptide (5 μM) for 30 min prior to flow cytometric analysis. The data represent the average (± SD) of three experiments.

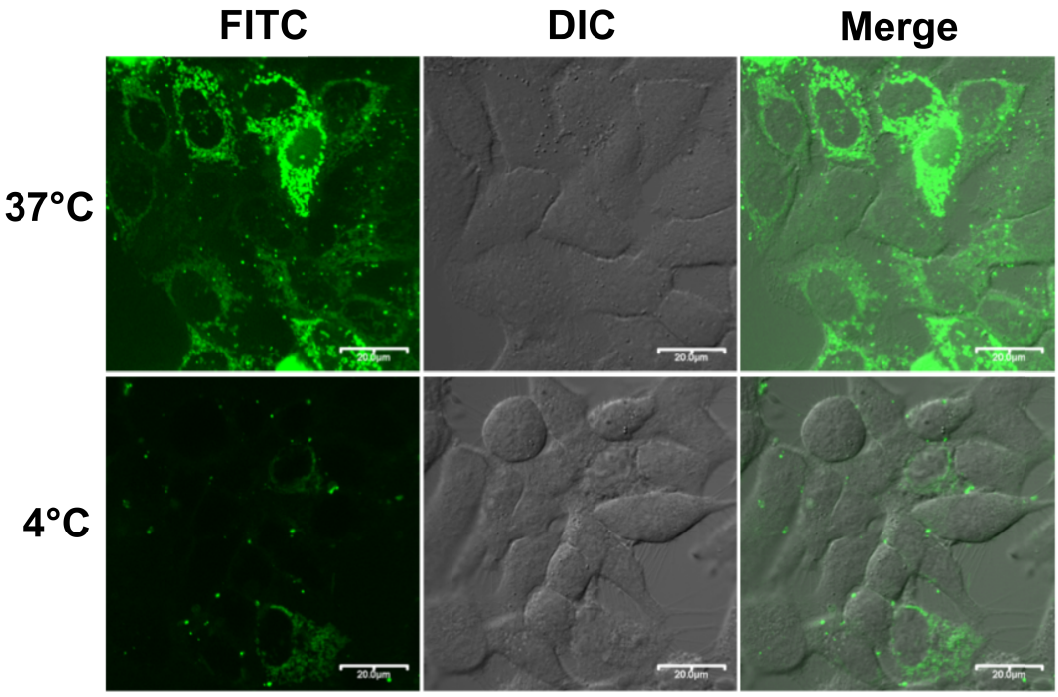

Fig. S6 Decreased cellular uptake in cells treated with FITC-*RLA* (5 μM) for 30 min at 4 °C. Note that energy-dependent cellular uptake was suppressed at 4 °C.

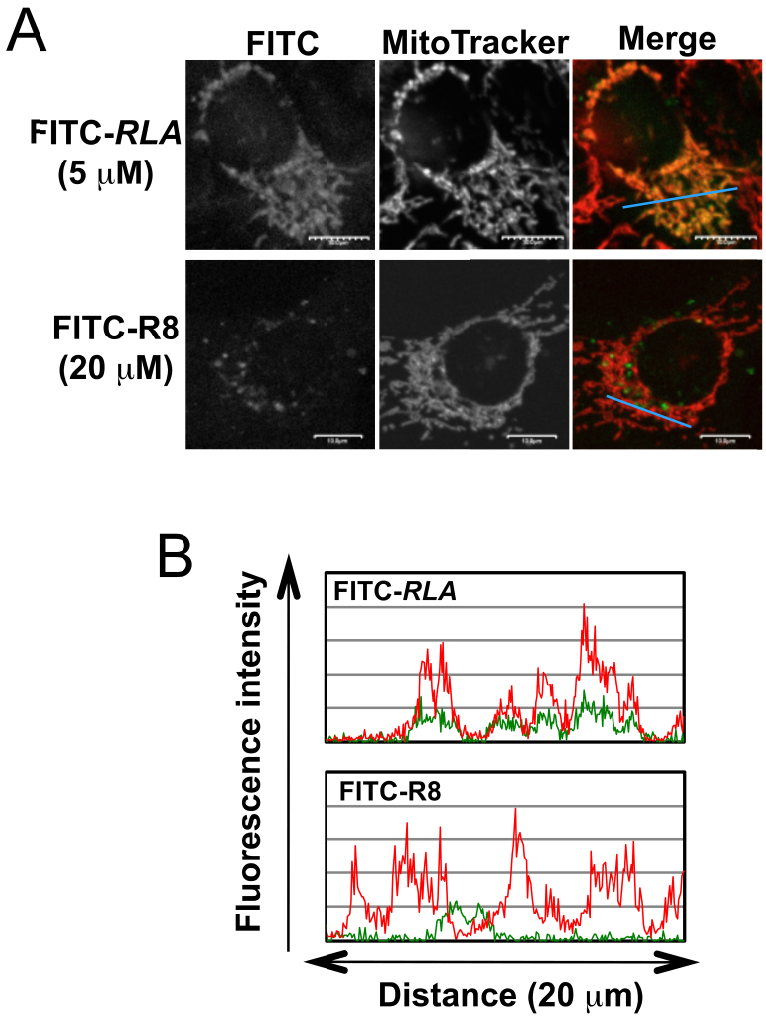

Fig. S7 Sustained cellular retention of FITC-*RLA*. (A) HeLa cells were treated with FITC-*RLA* (5 μM) or FITC-R8 (20 μM) for 30 min at 37 °C, and then washed and incubated in fresh cell culture medium without peptide for 24 h at 37 °C. Next, the cells were stained with MitoTracker and observed by confocal microscopy. (B) Fluorescence intensity along the blue lines in the merged pictures shown in (A) (green: FITC, red: MitoTracker).

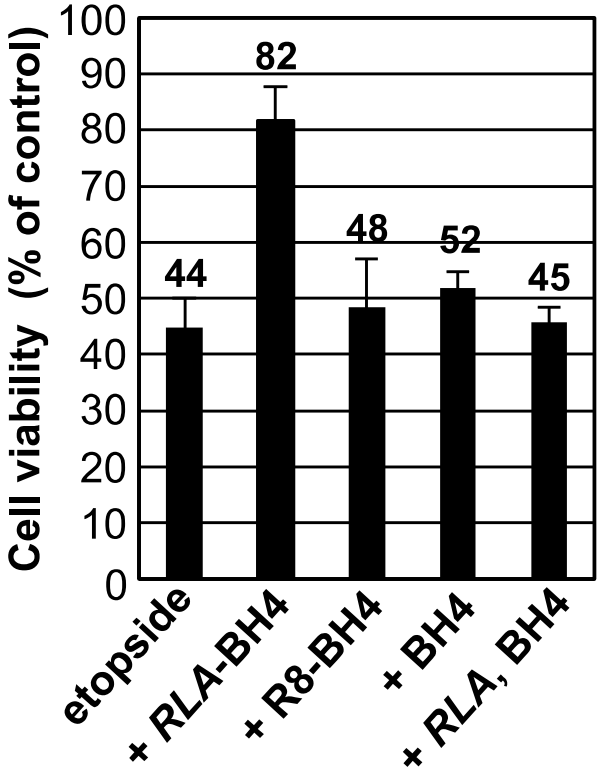

Fig. S8 Anti-apoptotic effects of the *RLA*-BH4 peptide on HeLa cells treated with an apoptosis inducer, etoposide. Cells were treated with each peptide (5 μM) for 1 h, and then treated with etoposide (200 μM) for 15 h prior to trypan blue staining and cell counter assay. The data represent the average (± SD) of three experiments.