SUPPLEMENTARY INFORMATION

Cellular uptake and cytosolic delivery of a cyclic cystine knot scaffold

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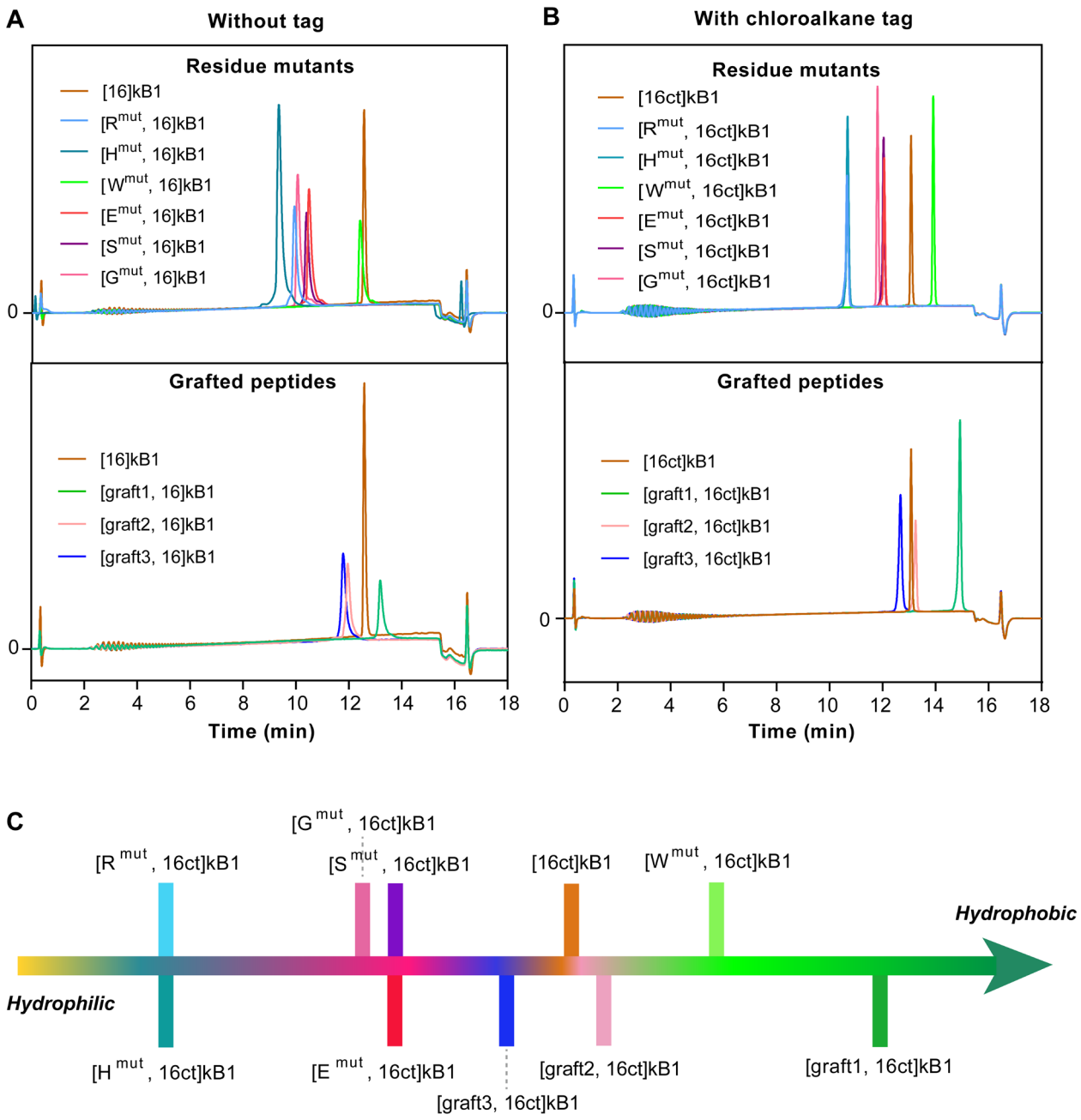
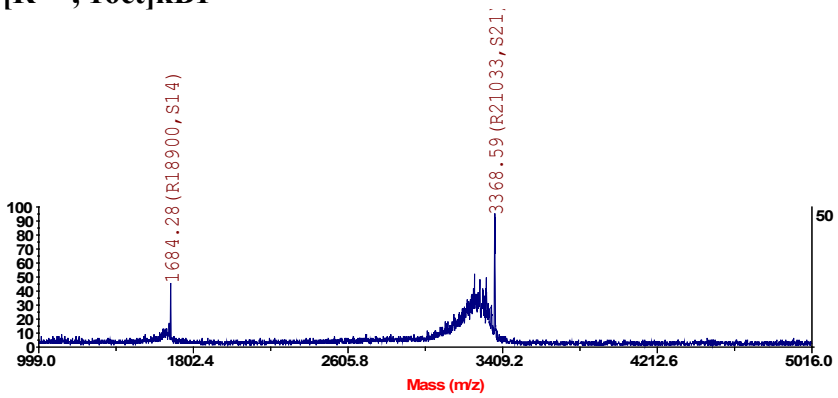
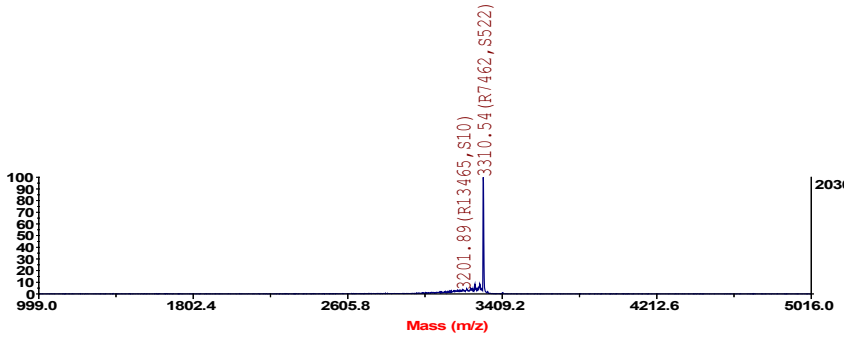


Supplementary Figure S1. Analytical UPLC chromatograms of kB1 analogues. **A**. UPLC chromatogram of kB1 analogues without a chloroalkane tag. **B**. UPLC spectra of kB1 analogues with a chloroalkane tag. For UPLC, a 4%/min gradient of 90% (v/v) acetonitrile, 0.05% (v/v) trifluoroacetic acid was used with a flow rate of 0.8 mL/min. Samples were injected on an analytical C18 column (Phenomenex Luna Omega C18, 1.6 µm, 2.1 x 50 mm). Absorbance was monitored at 214 nm. **C**. Schematic illustration of the relative hydrophobicity of kB1 analogues labeled with a chloroalkane tag based on their retention time.

[R^{mut}, 16ct]kB1



[H^{mut}, 16ct]kB1



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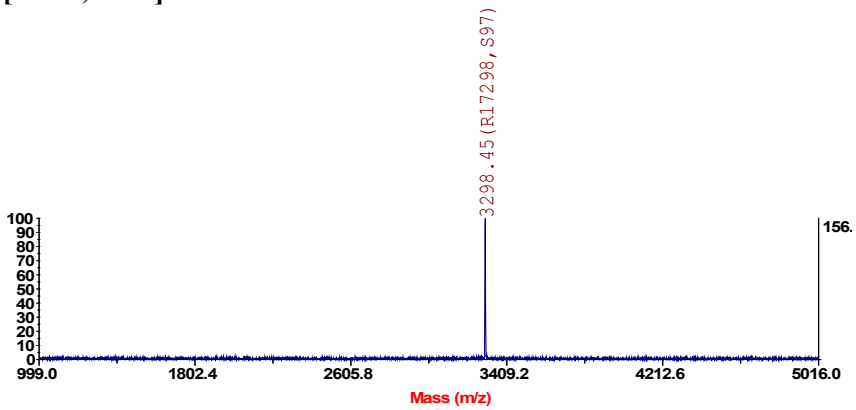
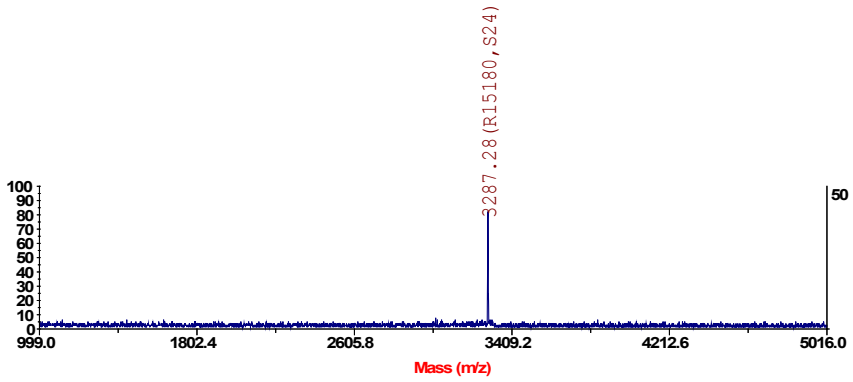
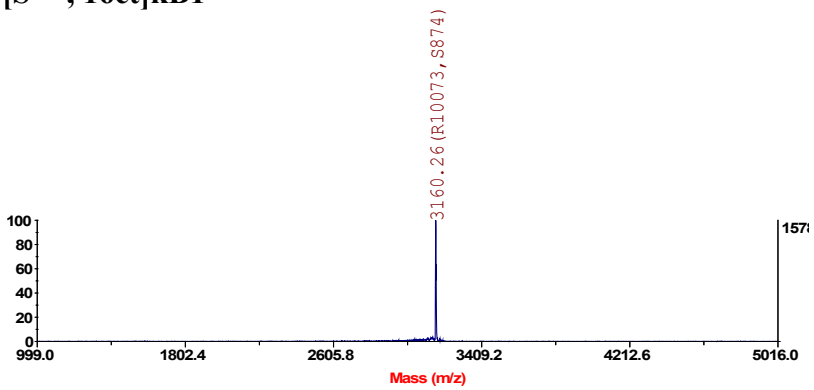


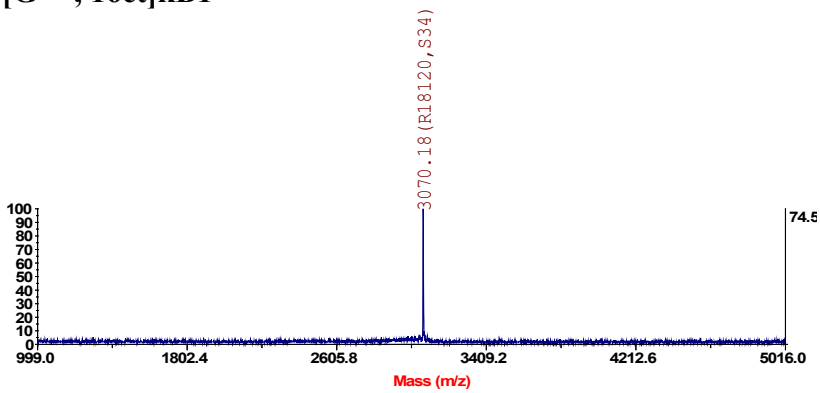
[E^{mut}, 16ct]kB1



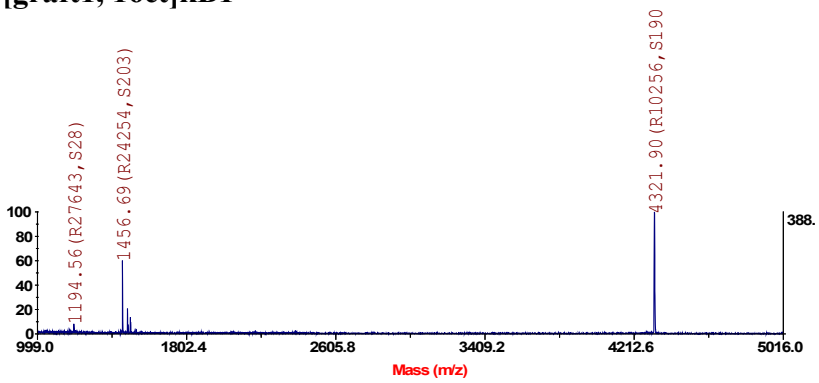
[S^{mut}, 16ct]kB1



[G^{mut}, 16ct]kB1



[graft1, 16ct]kB1



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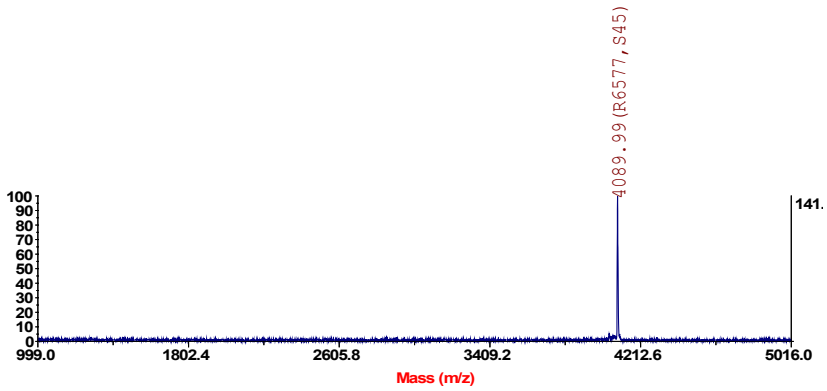

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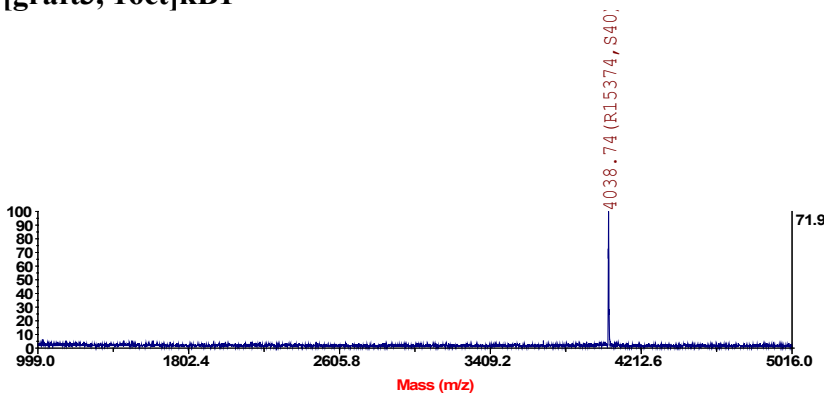
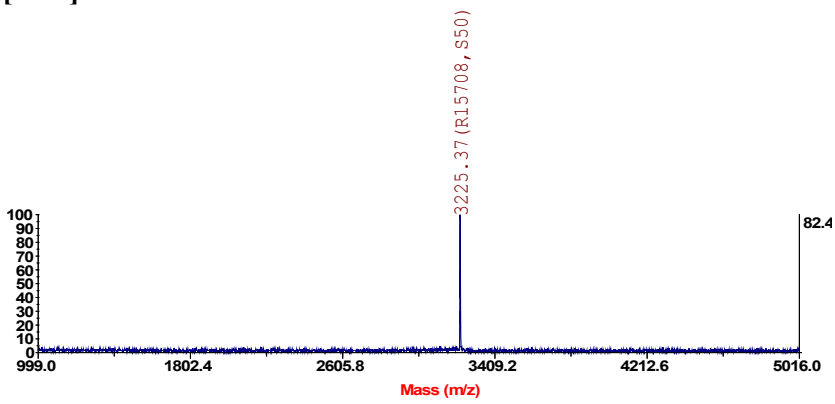
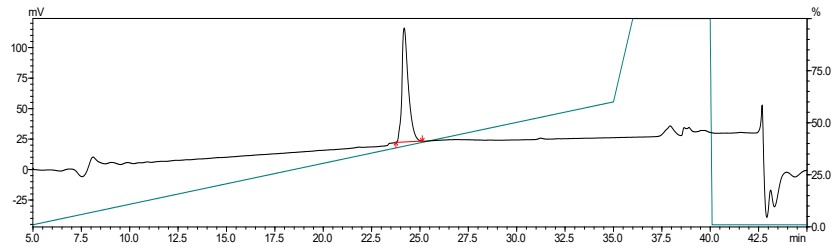
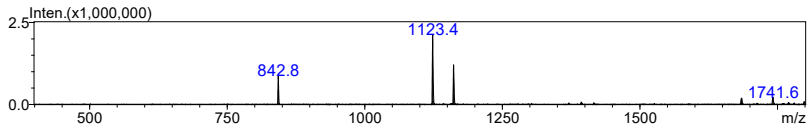


[16ct]kB1

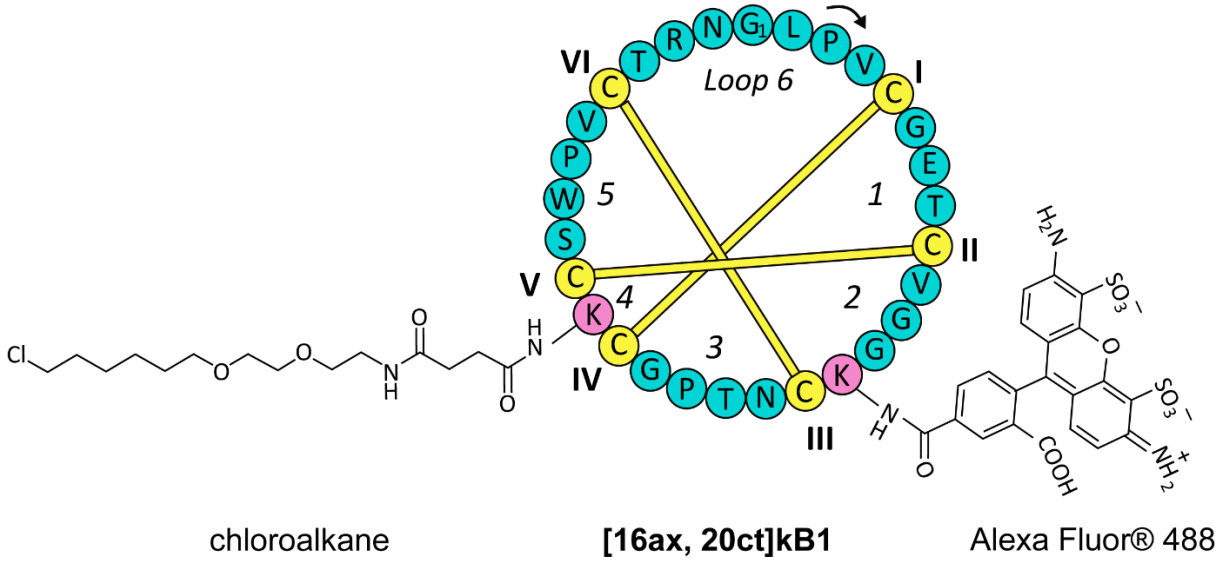


[R^{mut}, 16ct]kB1

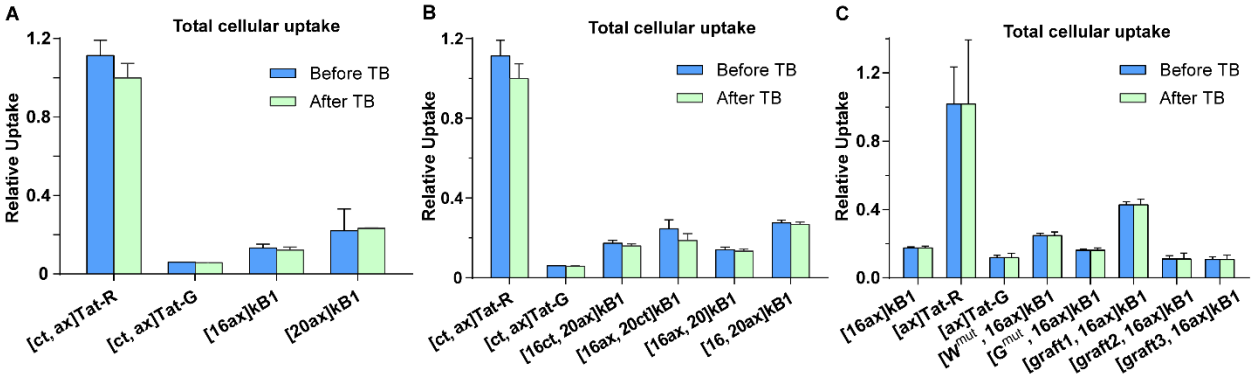




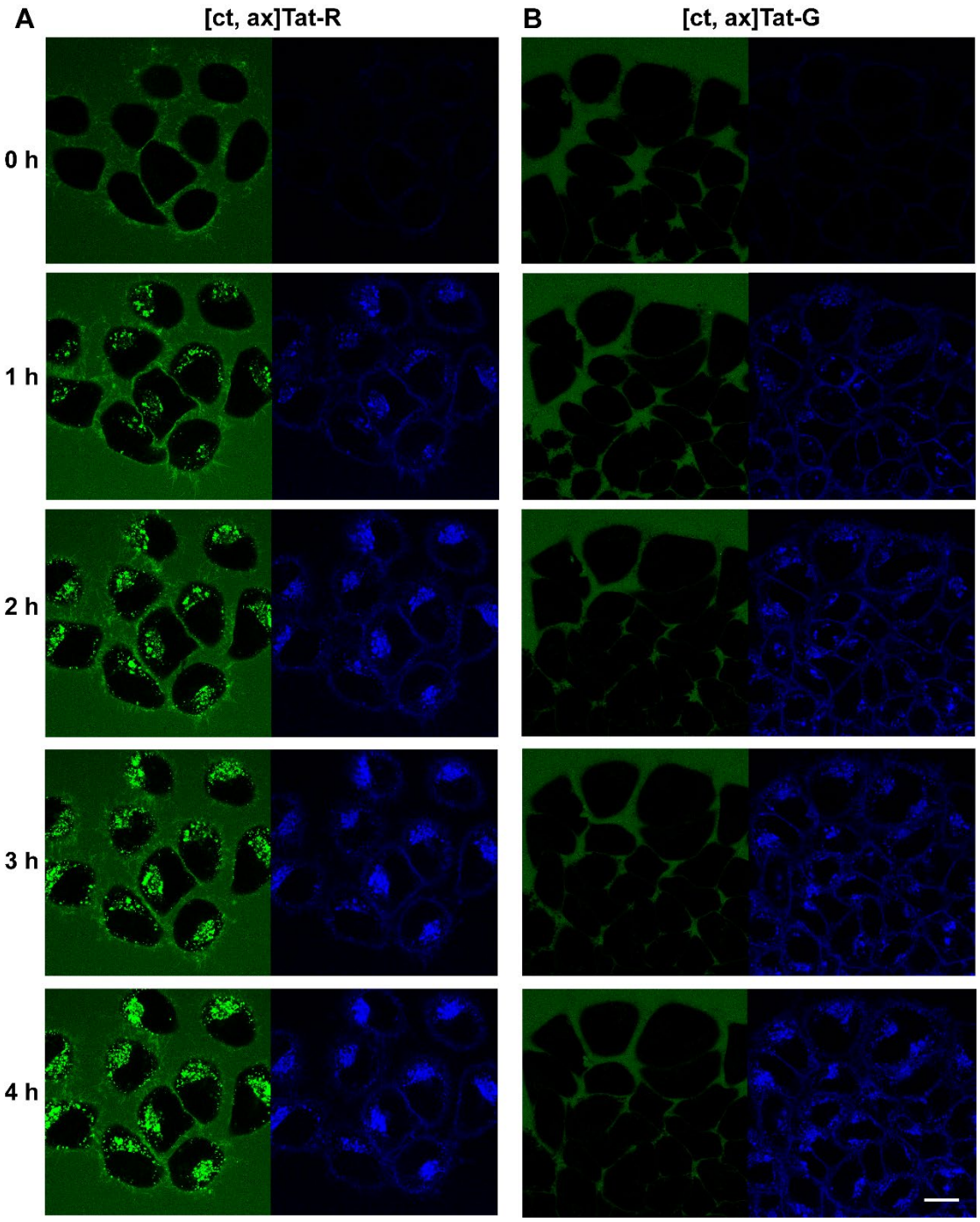
Supplementary Figure S2. Identities of kB1 analogues were confirmed using MALDI-TOF mass spectrometry. Ultra-performance liquid chromatography and electrospray ionization mass spectrometry confirmed high purity of [R^{mut}, 16ct]kB1.



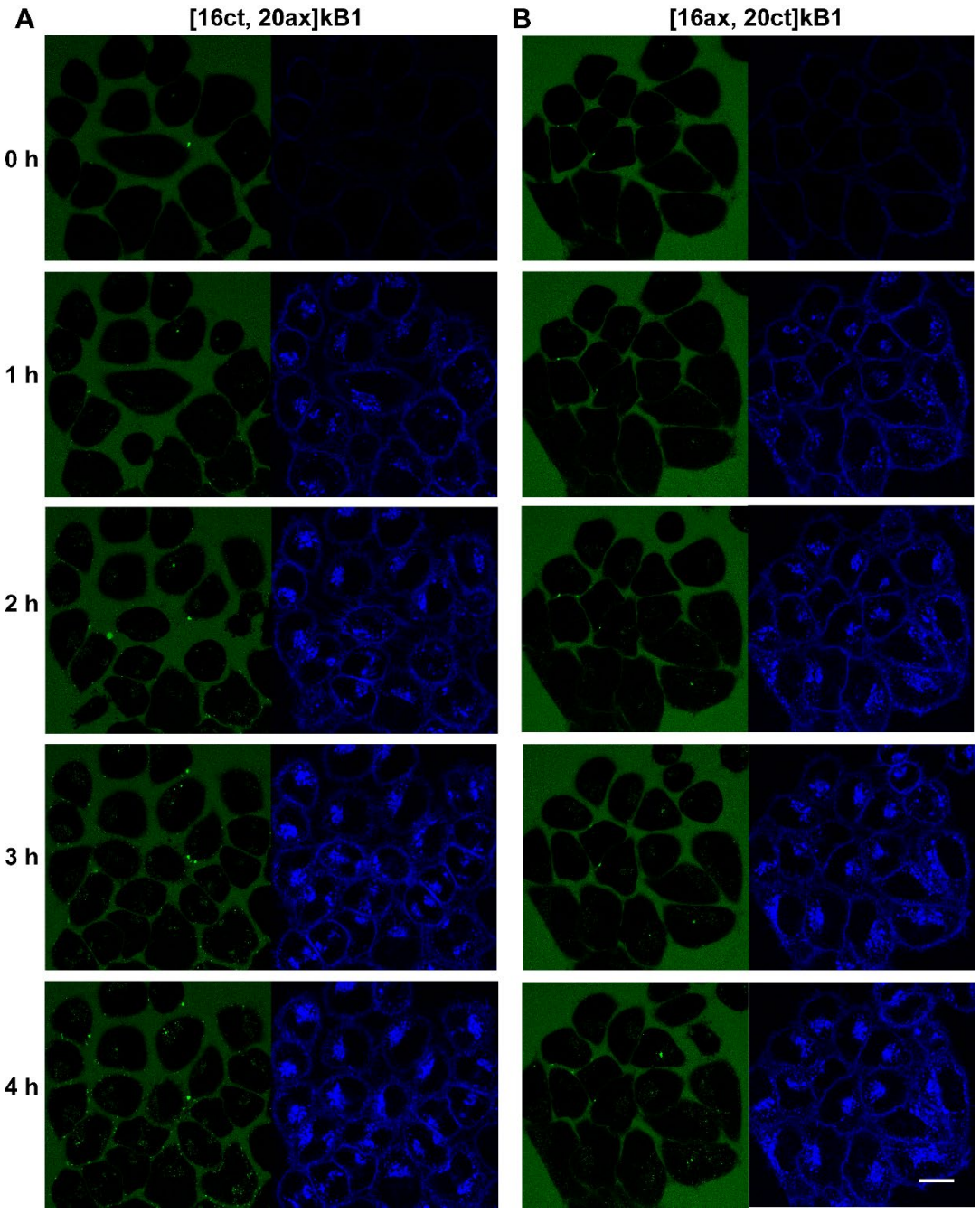
Supplementary Figure S3. Illustration of the chemical structure of [16ax, 20ct]kB1. The amino acid sequence of kB1 is illustrated as a cycle with its six cysteines highlighted in yellow and labeled using Roman numerals I to VI. Six sequence segments separated by cysteines are marked as loops 1−6. The knotted disulfide connectivity is indicated. The two threonine residues of kB1 (T16 and T20) are replaced by lysine and marked in pink. K16 is labeled with an Alexa Fluor® 488, and K20 is labeled with a chloroalkane tag.



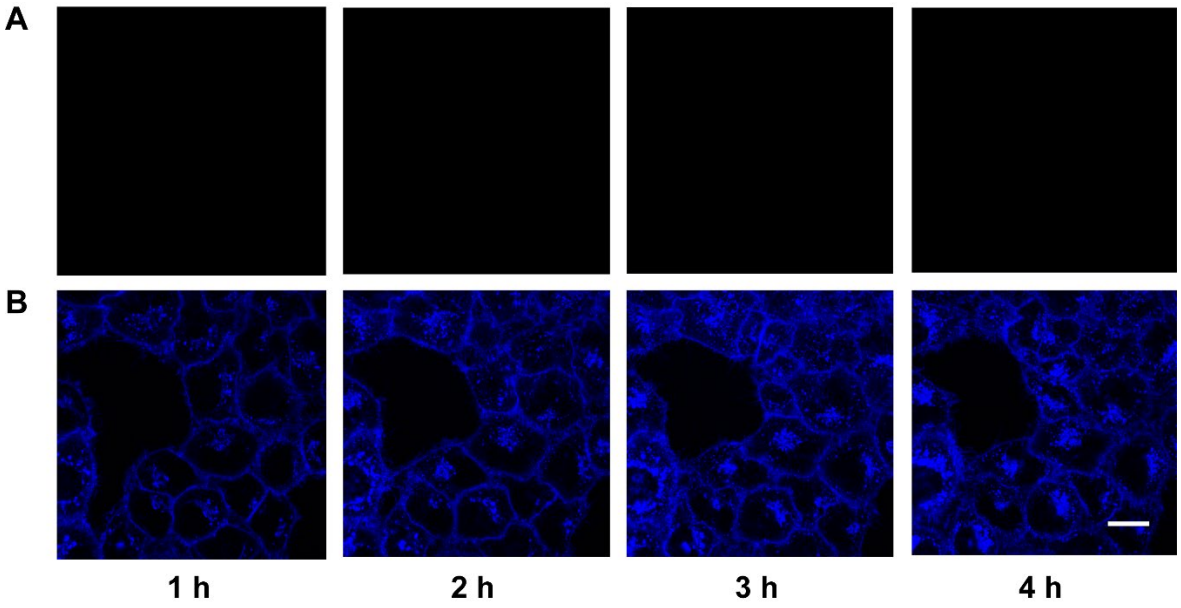
Supplementary Figure S4. Total cellular uptake of kB1 analogues. Cellular uptake was measured for Alexa Fluor® 488-tagged peptides at 8 µM using flow cytometry. (A) Total cellular uptake of kB1 with a single Alexa tag. (B) Total cellular uptake of kB1 with Alexa and chloroalkane tags. (C) Total cellular uptake of kB1 residue mutants and grafted peptides. The mean fluorescence of cells was measured by flow cytometry before (blue) and after (green) the addition of trypan blue (TB). The mean fluorescence of cells treated with [ct, ax]Tat-R or [ax]Tat-R after the addition of TB was used for normalization. All experiments were repeated in triplicate, and the results are represented as mean ± SEM.



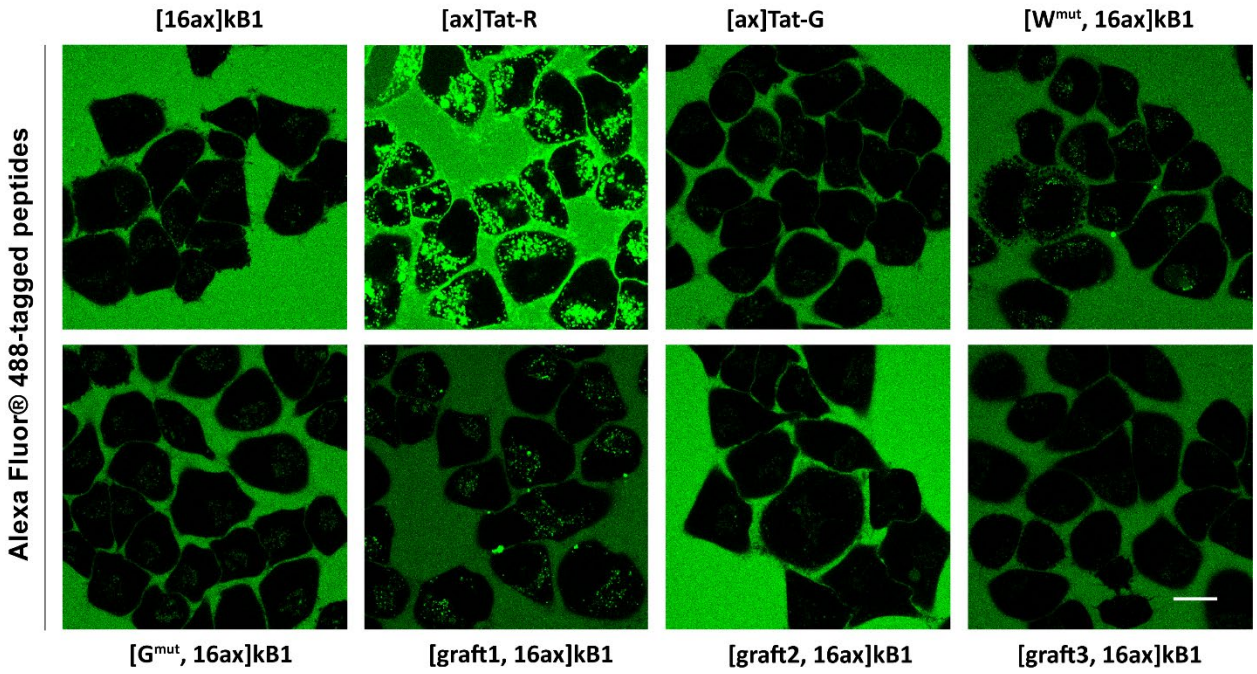
Supplementary Figure S5. Images of live cells captured using confocal microscopy of HeLa cells treated with [ct, ax]Tat-R or [ct, ax]Tat-G. Cells were co-incubated with 4 μM peptide and a Fluor® 647 conjugate of wheat germ agglutinin (WGA-A647), and the images were recorded from 0 to 240 minutes. (A) Confocal microscopy images of [ct, ax]Tat-R (left panel, green) and WGA-A647 (right panel, blue). (B) Confocal microscopy images of [ct, ax]Tat-G (left panel, green) and WGA-A647 (right panel, blue). Scale bar = 20 μm.



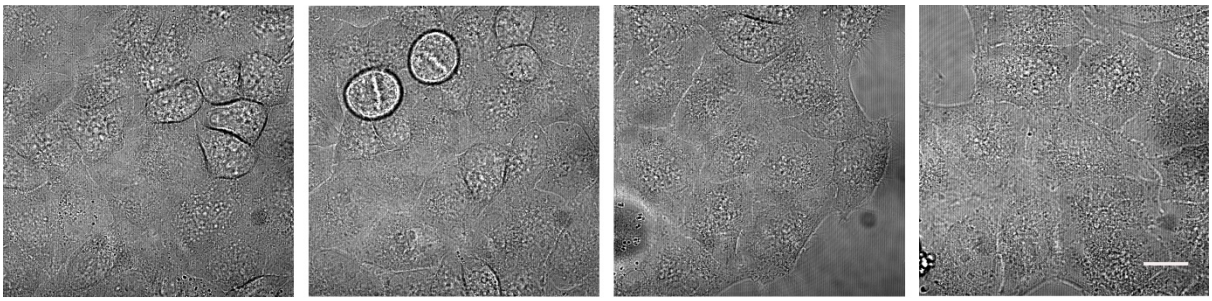
Supplementary Figure S6. Images of live cells captured using confocal microscopy of cells treated with [16ct, 20ax]kB1 or [16ax, 20ct]kB1. Cells were co-incubated with 4 μM peptide and a Fluor® 647 conjugate of wheat germ agglutinin (WGA-A647), and the images were recorded from 0 to 240 minutes. (A) Confocal microscopy images of [16ct, 20ax]kB1 (left panel, green), and WGA-A647 (right panel, blue). (B) Confocal microscopy images of [16ax, 20ct]kB1 (left panel, green), and WGA-A647 (right panel, blue). Scale bar = 20 μm.



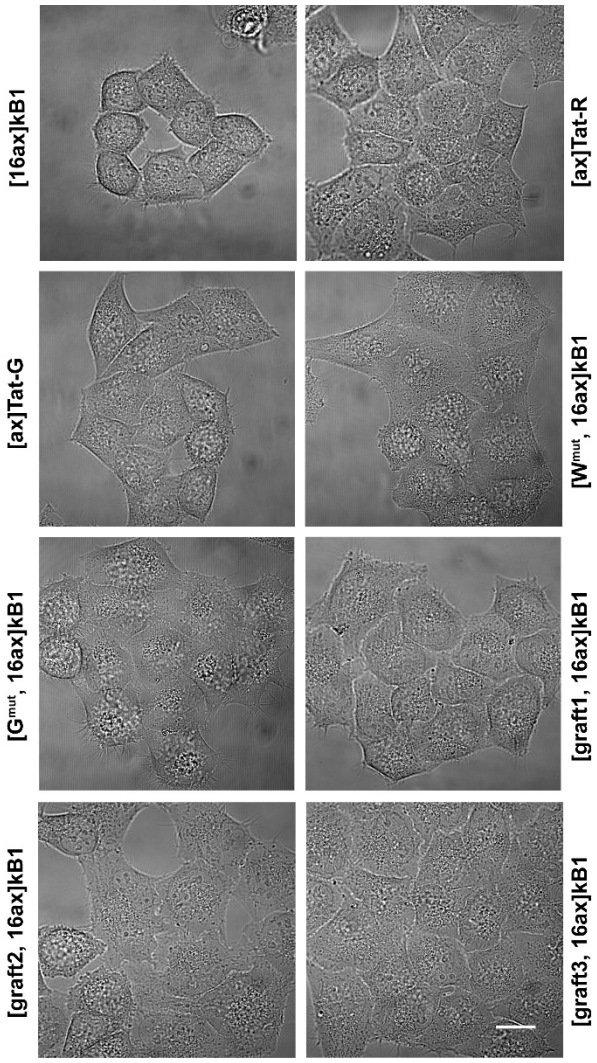
Supplementary Figure S7. Images of live cells captured using confocal microscopy of cells treated with 10 % (v/v) DMSO and WGA-A647. (A) Confocal microscopy images of 10 % (v/v) DMSO. (B) Confocal microscopy images of WGA-A647 (blue). The images were recorded from 0 h to 4 h. Scale bar = 20 μm.



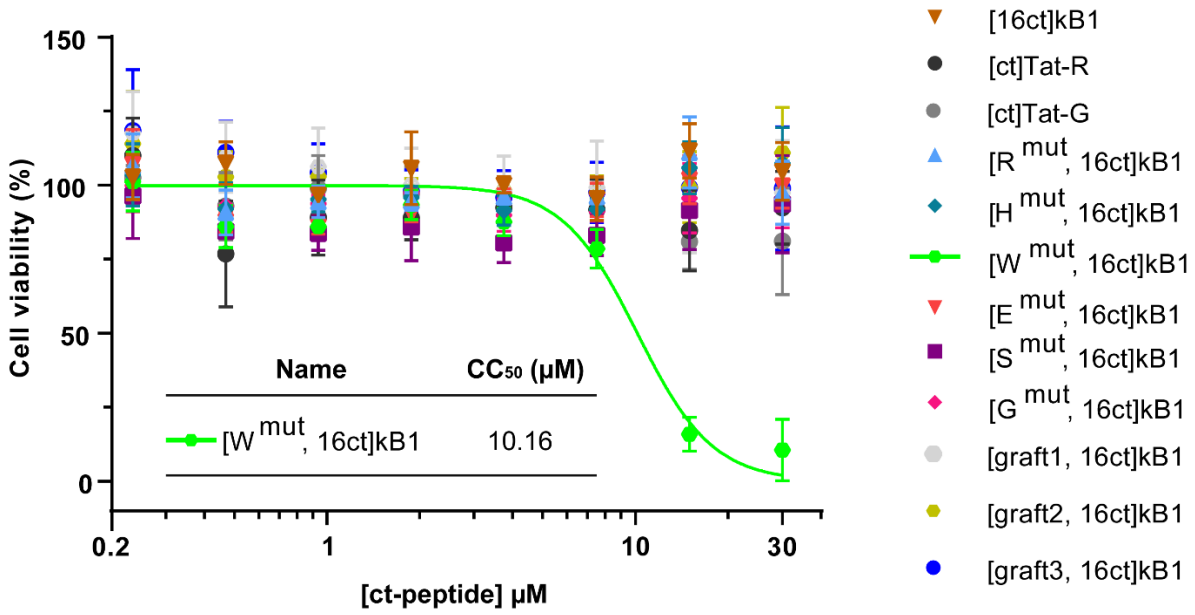
Supplementary Figure S8. Images of live cells captured using confocal microscopy of HeLa cells incubated with selected kB1 analogues. HeLa cells were treated with 4 μM of Alexa-labelled peptides (green) and the images were recorded after incubating for 240 minutes. Scale bar = 20 μm.



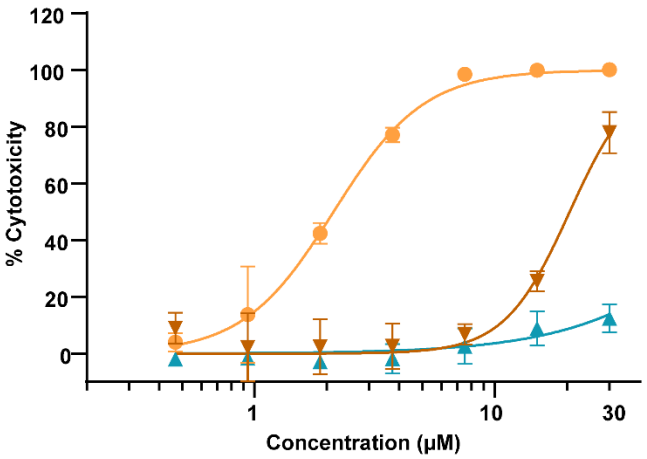
Supplementary Figure S9. Bright field images of live HeLa cells captured using confocal microscopy. Cells were incubated with peptides labeled with both Alexa Fluor® 488 and chloroalkane tags at 4 μM. No morphological changes were observed after addition of peptides and up to 4 h after treatment. Scale bar = 20 μm.



Supplementary Figure S10. Bright field images of HeLa cells captured using confocal microscopy after treatment with A488-labeled Tat-peptides and selected kB1 analogues for 4 h. No morphological changes were observed after 4 h treatment of the peptides. Scale bar = 20 µm.



Supplementary Figure S11. Quantitation of cell viability in CAPA. Hela cells were incubated with ct-peptides up to a concentration of 30 µM for 4 h. After chasing with ct-TAMRA, fluorescence intensity was measured using a flow cytometer. Live green cells (i.e. live cells expressing the HaloTag-GFP protein) were gated during detection. The percentage of live green cells from the total input population for each peptide was normalized to that of an internal small molecule positive control, a chloroalkane-labeled tryptophan (ct-W), giving an indication of the effect of each peptide on cell viability relative to the internal control. As shown, only [W^{mut}, 16ct]kB1 showed any significant signs of toxicity at higher concentrations. Other peptides tested had no significant effect on the population of cells used for detection.



Name	IC_{50} (µM)
[16ct]kB1	20.51 ± 1.08
[20ct]kB1	2.13 ± 0.10
[graft3, 16ct]kB1	>30.0

Supplementary Figure S12. Cytotoxicity of chloroalkane tagged (ct)-peptides on Hela cells in 96-well microplate (5000 cells/ well) was evaluated using a resazurin-based assay. Hela cells were incubated with ct-peptides at concentrations up to 30 µM for 4 h.

Supplementary Table S1. Summary of Tat and kalata B1 (kB1) peptides used in this study and their total cellular uptake (Uptake) and cytosolic delivery (Cytosol) efficiencies.

Name	Sequence[a]	Uptake	Cytosol
[ct]Tat-R	YGRRKRRQRRRPPQG	*	+
[ct]Tat-G	YGGGKGGQGGGPPQG	*	=
[ct, ax]Tat-R	YGRRKRRQRRRPPQG	+	+
[ct, ax]Tat-G	YGGGKGGQGGGPPQG	=	=
[ax]Tat-R	YGRRKRRQRRRPPQG	+	*
[ax]Tat-G	YGGGKGGQGGGPPQG	=	*
[16ct]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRN	*	*
[20ct]kB1	GLPVCGETCVGGTCNTPGCKCSWPVCTRN	*	=
[16ct, 20ax]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	=	+
[16ax, 20ct]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	+	+
[16ct, 20]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	*	*
[16, 20ct]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	*	=
[16ax, 20]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	*	*
[16, 20ax]kB1	GLPVCGETCVGGTCNKPGCKCSWPVCTRN	=	*
[16ax]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRN	*	*
[20ax]kB1	GLPVCGETCVGGTCNTPGCKCSWPVCTRN	+	*
[R^{mut}, 16]kB1	GRPRCGETCVGGTCNKPGCTCSWPVCTRR	*	=
[H^{mut}, 16]kB1	GHPHCGETCVGGTCNKPGCTCSWPVCTRH	*	=
[W^{mut}, 16]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRW	+	+++
[E^{mut}, 16]kB1	GEPECGETCVGGTCNKPGCTCSWPVCTRE	*	=
[S^{mut}, 16]kB1	GSPSCGETCVGGTCNKPGCTCSWPVCTRS	*	=
[G^{mut}, 16]kB1	GGPGCGETCVGGTCNKPGCTCSWPVCTRG	=	=
[graft1, 16]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRGFEWLDWEF	+	+++
[graft2, 16]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRGLDEETGEF	=	+
[graft3, 16]kB1	GLPVCGETCVGGTCNKPGCTCSWPVCTRGSFEGYDNG	=	++

[a]Tat peptides are linear and have amidated C-termini; kB1 peptides are backbone cyclic and have three disulfide bonds as depicted in Figure 1; Y denotes a tyrosine with a chloroalkane tag coupled to its N-terminus; K denotes a lysine coupled to a chloroalkane tag; and K denotes a lysine coupled to an Alexa Fluor® 488 tag.

[b]Residue differences relative to the native peptide are underlined.

[c]The cellular uptake of the peptides was compared to that of [16ax]kB1 or [16ax, 20]kB1, and the cytosolic delivery to that of [16ct]kB1 or [16ct, 20]kB1. = denotes the activity was within 1.5 times of that of the respective kB1 analogue, + denotes 1.5–5 fold higher, ++ denotes 5–10 fold higher, and +++ denotes ≥10 fold higher activity. * denotes no comparison could be made. [ct, ax]Tat-R, [ct, ax]Tat-G, [16ct, 20ax]kB1, [16ax, 20ct]kB1 and [16, 20ax]kB1 are compared with [16ax, 20]kB1; [ax]Tat-R, [ax]Tat-G, [20ax]kB1, [W^{mut}, 16]kB1, [G^{mut}, 16]kB1, [graft1, 16]kB1, [graft2, 16]kB1 and [graft3, 16]kB1 are compared with [16ax]kB1; [ct, ax]Tat-R, [ct, ax]Tat-G, [16ct, 20ax]kB1, [16ax, 20ct]kB1 and [16, 20ct]kB1 are compared with [16ct, 20]kB1; [ct]Tat-R, [ct]Tat-G, [20ct]kB1, [R^{mut}, 16]kB1, [H^{mut}, 16]kB1, [W^{mut}, 16]kB1, [E^{mut}, 16]kB1, [S^{mut}, 16]kB1, [G^{mut}, 16]kB1, [graft1, 16]kB1, [graft2, 16]kB1 and [graft3, 16]kB1 are compared with [16ct]kB1.