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Supporting Information

BH3 Helix-derived Biophotonic Nanoswitches Regulate Cytochrome c Release in Permeabilised Cells

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1 Peptide design

The amino acid sequences of the peptides were modified from the wild-type BH3 regions of Bak and Bid in order to introduce appropriately spaced cysteine residues and to eliminate sidechains that might cause steric clashes with the crosslinker. For Bak$^{i,i+7}$ additional changes were made to remove an asparagine reside whose bulky sidechain might clash with the crosslinker (Q73A) and add a phenylalanine sidechain (I81F) proposed to improve binding to Bcl-x_L^{S1} to potentially improve the potency of the activated form. For Bak$^{i,i+11}$ a different asparagine sidechain was exchanged for a less hindering alanine (Q77A). For Bid$^{i,i+11}$ a methionine was changed to isoleucine to improve stability towards oxidation (M97I) and two changes to remove bulky sidechains (N92S, Q100S) whilst maintaining polarity to improve solubility.

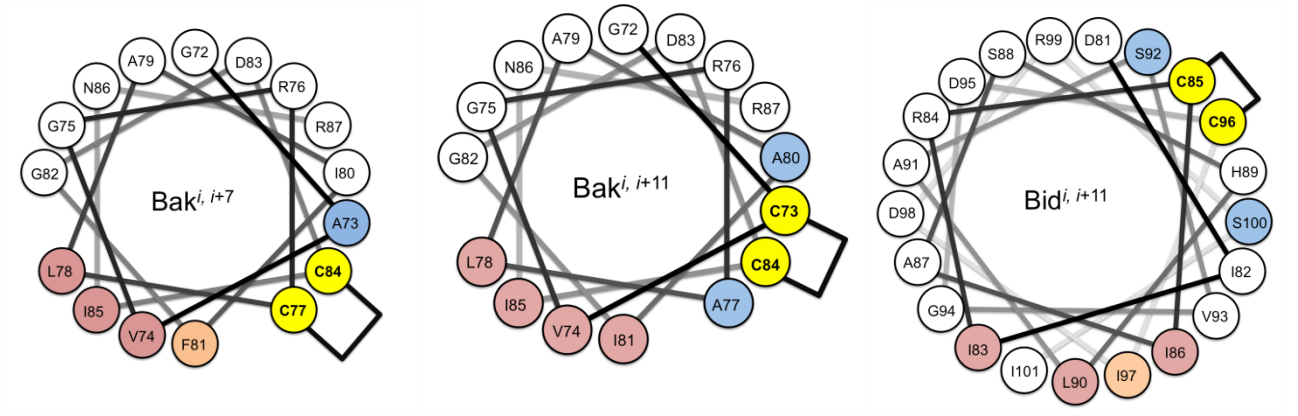

Fig. S1 Residues are picked out in the following colours: Red residues form hydrophobic interactions with the partner protein, orange residues interact with the protein and deviate from the wild type sequence (see above), yellow indicates cysteine residues crosslinked by azobenzene and blue indicates residues changed to avoid sidechain clashes with the azobenzene crosslinker.

2 Peptide synthesis

All peptides were synthesized according to standard fluorenylmethylcarbamoyl (Fmoc) solid phase synthesis protocols using a CEM Liberty microwave-assisted peptide synthesizer. Protected amino acids with trityl (Trt), tert-butyl (^{t}Bu), butoxycarbonyl (Boc) or 2,2,4,6,7- pentamethyldihydrobenzofurane (Pbf) sidechain protecting groups as required, O-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), N- methylpyrrolidinone (NMP) and dimethylformamide (DMF) were purchased from AGTC Bioproducts. Dichloromethane (DCM), trifluoroacetic acid (TFA) and diethyl ether were sourced from Fisher. Piperidine, acetic anhydride, triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA), 4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid (HEPES) and triscarboxyethylphosphine (TCEP) were purchased from Sigma Aldrich. Rink Amide resin (0.72 mmol/g) and 5(6)-carboxyfluorescein were purchased from NovaBiochem.

Activator	0.45 M HBTU in DMF
Deprotection Mix	20% Piperidine, 0.1 M HOBt in DMF
Activator Base	2 M DIEA in NMP 20%
Capping Solution	Acetic acid anhydride in DMF
Amino Acid Solutions	0.1 M solutions of Fmoc-protected amino acids in DMF: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH,Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH
Cleavage Cocktail	95% TFA, 2.5% TIS, 2.5% water

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2.1 Peptide synthesis procedures

Resin: Fresh resin (140 mg, 0.1 mmol scale) was transferred to the reaction chamber in a 1:1 mixture of DMF and DCM and allowed to swell for 15 minutes.

Deprotection: Deprotection mix (7.0 mL) as described above was added to the washed resin followed by microwave-assisted deprotection. Heating was provided for 180 seconds using 20 W to achieve a maximum temperature of 75 °C with 3 seconds of nitrogen bubbling every 7 seconds. The resin was then cooled and washed with DMF (3 × 5.0 mL).

Arginine: No heating was provided for 30 minutes, then heating was provided for 5 minutes using 20 W to achieve a maximum temperature of 75 °C. The resin was agitated throughout with 3 seconds of nitrogen bubbling every 7 seconds. The resin was then cooled and washed with DMF (3 × 5.0 mL). Due to the hindering nature of the Pbf sidechain protecting group, arginine couplings were followed by a capping step. Capping solution (7.0 mL) was added and allowed to react for 5 minutes without additional heating. The resin was then washed with DMF (2 × 8.0 mL).

Cysteine: No heating was provided for 5 minutes, then heating was provided for 10 minutes using 20 W to achieve a maximum temperature of 50 °C. The resin was agitated throughout with 3 seconds of nitrogen bubbling every 7 seconds. The resin was then cooled and washed with DMF (3 × 5.0 mL).

All other amino acids: Heating was provided for 10 minutes using 20 W to achieve a maximum temperature of 75 °C with 3 seconds of nitrogen bubbling every 7 seconds. The resin was then cooled and washed with DMF (3 × 5.0 mL).

Cleavage: Final deprotection of the resin-bound peptide chain utilized a two-part deprotection. Deprotection mixture (7.0 mL) was added and heated using 20 W to achieve a maximum temperature of 75 °C for 3 minutes. The solvent was then drained and the resin was washed with DMF (3 × 5.0 mL). A second addition of the deprotection mix (7.0 mL) was added and heated for 3 minutes using 20 W to achieve a maximum temperature of 75 °C with 3 seconds of nitrogen bubbling every 7 seconds. The resin was then cooled and again washed with DMF (3 × 5.0 mL). When required, FAM labeling was achieved as previously reported, otherwise acetyl capping as described under the arginine protocol above was used to terminate the peptide. The resin was transferred to a 50.0 mL Falcon tube, drained on a sintered glass filter, washed thoroughly with DCM, then suspended in cleavage cocktail (20 mL). The cleavage reaction was carried out at room temperature for 1 hour with shaking, then the resin was filtered and the filtrate was evaporated under nitrogen flow until the volume was reduced to approximately 1.0 mL. This suspension was diluted with 49 mL of cold diethyl ether to precipitate the product. After chilling overnight at -20 °C, the resulting suspension was centrifuged (5000 x g), the supernatant was removed and the pellet was resuspended in water (+0.5% TFA), filtered and purified by HPLC.

2.2 Peptide purification

Peptides were purified by reversed phase HPLC using a Phemonenex Gemini 10 µm, 110 Å, 10×250 mm C18 column with gradients from 100% water (+ 0.1% TFA) to 100% acetonitrile (+ 0.1% TFA) over 50 or 100 minutes at a flow rate of 5 mL/minute. Peptide purity was checked by analytical HPLC using a Dionex Acclaim 3 µm, 120 Å, 4.6×150 mm C18 column with a gradient from 100% water (+ 0.1% TFA) to 100% acetonitrile (+ 0.1% TFA) over 50 minutes at a flow rate of 1 mL/minute.

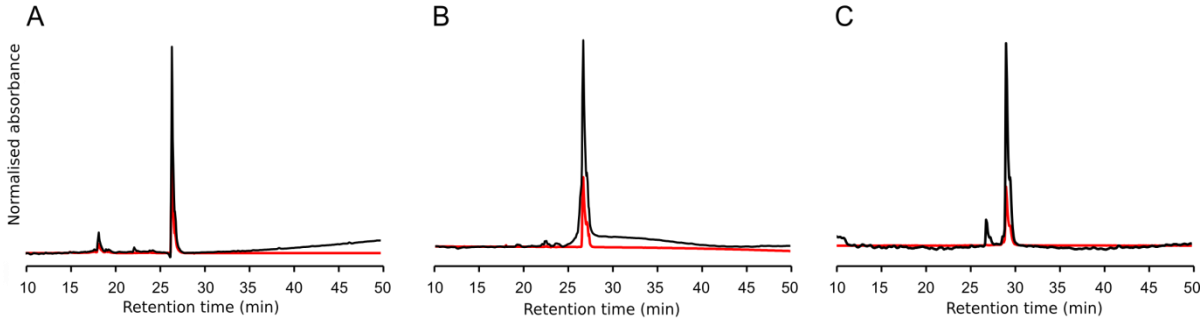

Fig. S2 Representative analytical traces of A) Ac-Bak$_{I81F}^{i,i+7}$-XL (note *cis* isomer at 18 min) B) FAM-Bak$^{i,i+11}$-XL C) FAM-Bid$^{i,i+11}$-XL (note *cis* isomer at 27 min).

2.3 Peptide identification

Peptides were identified by MALDI mass spectrometry (Micromass MALDI Micro MX) by co-spotting with α-cyano-4-hydroxycinnamic acid matrix dissolved in 50% acetonitrile-water.

Table S1 Predicted and observed peptide masses.

Peptide[a]	Sequence	RT[b] (min)	Obs m/z		Calc m/z
Ac-Bak$_{I81F}^{i,i+7}$	GAVGRCLAIFGDCINR	22.8	1075	$[M+H]^+$	1705
Ac-Bak$_{I81F}^{i,i+7}$-XL	GAVGRCLAIFGDCINR	20.4	2157	$[M+H]^+$	2157
FAM-Bak$^{i,i+11}$	GCVGRALAAIGDCINR	24.3/24.7	1945	$[M+H]^+$	1945
FAM-Bak$^{i,i+11}$-XL	GCVGRALAAIGDCINR	26.4/26.8	2397	$[M+H]^+$	2397
FAM-Bid$^{i,i+11}$	DIIRCIASHLASVGDCIDRSI	19.7/20.0	2614	$[M+H]^+$	2613
FAM-Bid$^{i,i+11}$-XL	DIIRCIASHLASVGDCIDRSI	28.5/28.9	3067	$[M+H]^+$	3065

[a] XL: Crosslinked with azobenzene crosslinker, FAM N-terminal fluorescamide modification.

[b] Retention times for both 5- and 6-carboxyfluorescein isomers are given where appropriate.

3 Peptide characterisation

3.1 Peptide secondary structure

CD spectra of peptides dissolved in 5 mM potassium phosphate buffer at pH 7.5 to a concentration of 50 μM were recorded on an Applied Photophysics Chirascan spectrophotometer with a Quantum Northwest temperature controller using a 1 mm path length cuvette.

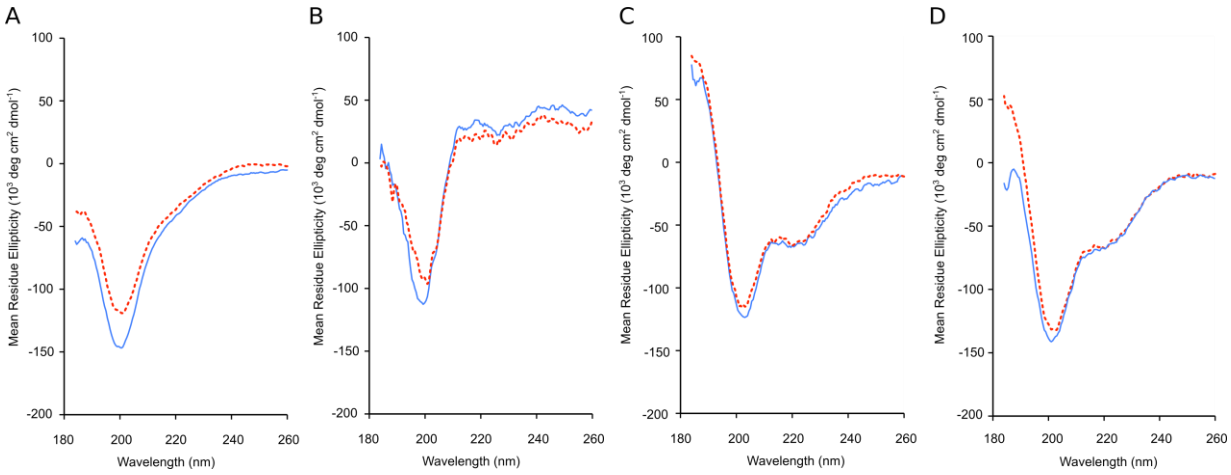

Fig. S3 A) Ac-Bak$_{I81F}^{i,i+7}$-XL [20 ºC] B) Ac-Bak$_{I81F}^{i,i+7}$-XL [5 ºC] C) FAM-Bak$^{i,i+11}$-XL [20 ºC] and D) FAM-Bid$^{i,i+11}$-XL [20 ºC] peptides in their dark adapted (solid blue) and irradiated (dotted red) states.

3.2 *In vitro* peptide binding affinities

Fluoresence anisotropy titrations were used to determine binding constants for N-terminally fluorescamide labelled peptides in the dark (*trans*) state and apparent binding constants for the irradiated (*cis*) state. Freshly prepared peptide solutions (5 nM) in buffer (100 mM potassium phosphate, 10 mM sodium chloride, pH 7.5) were equilibrated 15 ºC to minimise *cis* to *trans* relaxation during measurements and titrated with Bcl-x_L. Successive additions of Bcl-x_L caused an increase in fluorescence anisotropy as the smaller peptide molecule tumbles more slowly when bound to the protein. The sample was excited at 495 nm and the fluorescence emitted detected at 525 nm using a Perkin-Elmer LS-55 fluorescence spectrometer. Excitation and emission slits were set to 10 nm and 15 nm respectively to maximise signal intensity. The G-factor was calculated to be 1.16.

Table S2 *In vitro* binding affinities of peptides to Bcl-x_L.

	K_D (nM)

Peptide	Dark State	Irradiated
FAM-Bak$^{i,i+11}$-XL	15 ± 1[a]	75 ± 22
FAM-Bid$^{i,i+11}$-XL	43 ± 2	65 ± 7
FAM-Bak$_{I81F}$$^{i,i+7}$-XL	825 ± 157 [b]	134 ± 16 [b]

[a] See reference S2 [b] See reference S3

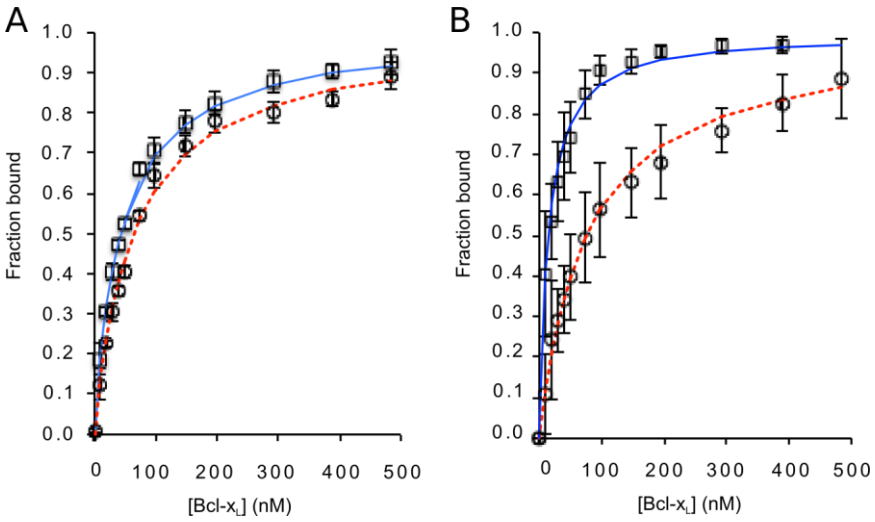

Fig. S4 Binding curves in the dark adapted (solid blue) and irradiated (dashed red) states for A) FAM-Bid$^{i,i+11}$-XL and B) FAM-Bak$^{i,i+11}$-XL.

3.3 Azobenzene relaxation rates

Peptides (50 μM) in sodium phosphate buffer (5 mM) were irradiated with a UVP-280 light source and a 360 ± 10 nm filter glass for 3 minutes to achieve a photostationary state. The increase in absorbance at 360 nm was then recorded every 2 minutes and resulting curves were fitted to first order kinetics to calculate thermal reversion half-lives.

Table S3 Relaxation rates of peptides.

Peptide	$t_{1/2}$ (min) at 37 °C
FAM-Bak$^{i,i+11}$-XL	18 ± 3
FAM-Bid$^{i,i+11}$-XL	15 ± 2
Ac-Bak$_{I81F}$$^{i,i+7}$-XL	28 ± 5

3.4 Proteolytic stability

Proteolysis of peptides is altered by addition of a crosslinker. Samples of peptides were incubated in sodium phosphate buffer (100 mM, pH 7) at 20 ºC for 1 hour in the presence of the indicated concentrations of trypsin. Samples were then rapidly frozen in liquid nitrogen then individually thawed immediately before injection into a Dionex Ultimate 3000 analytical with an Acclaim 120 C18 3 μM 120 Å 4.6 x 150 mm column, eluting with 0 to 60% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes.

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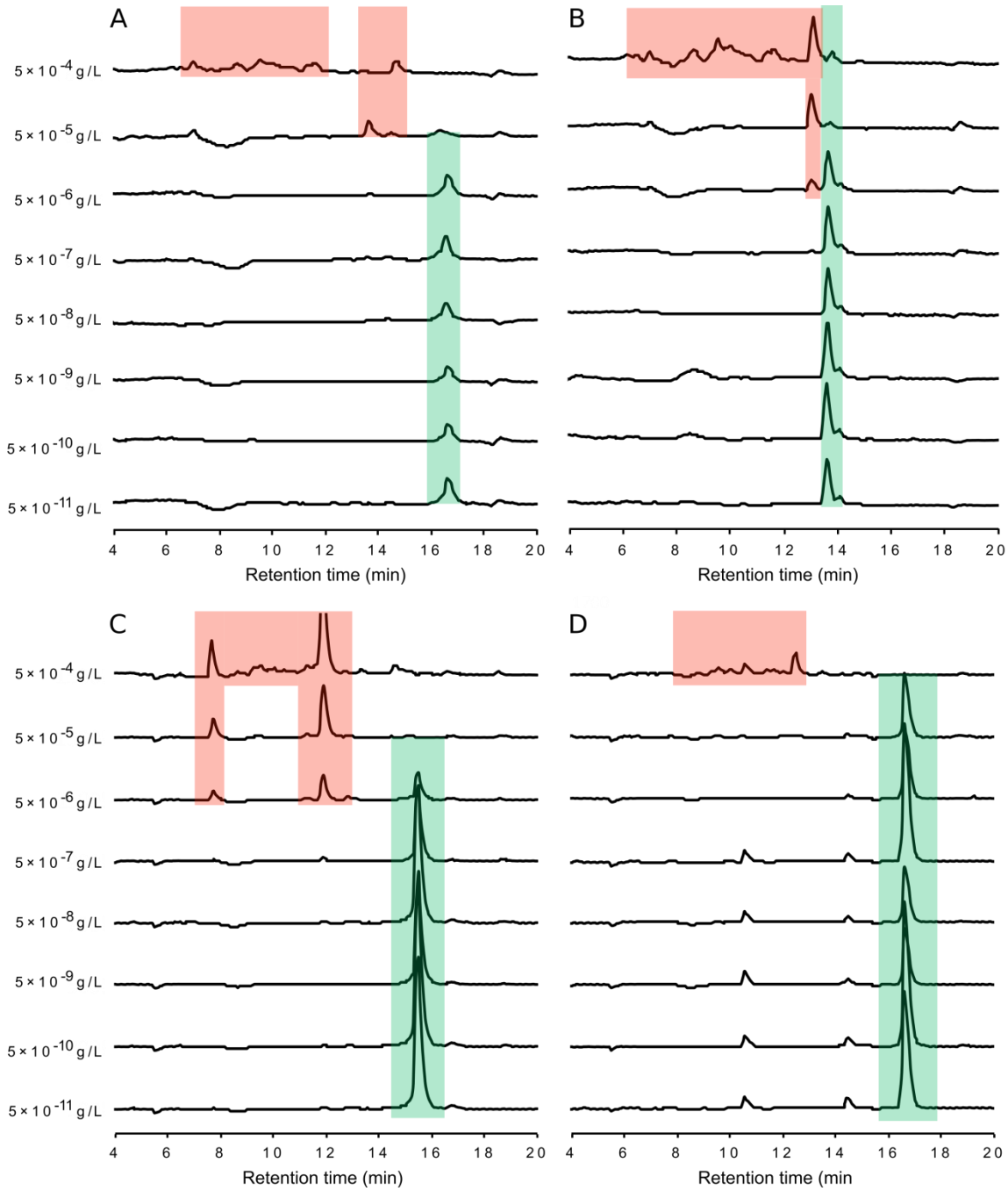

Fig. S5 Crosslinker position affects proteolysis. A) Ac-Bak$_{I81F}^{i,i+7}$ B) Ac-Bak$_{I81F}^{i,i+7}$-XL C) Ac-Bak$_{I81F}^{i,i+11}$ and D) Ac-Bak$_{I81F}^{i,i+11}$-XL.

3.5 Reductive stability

Glutathione concentrations equivalent to intracellular reducing potentials do not cause reduction of azobenzene peptides. Samples (50 μM) were incubated in sodium phosphate buffer (100 mM, pH 7) alone or with or without prior UV irradiation in buffer containing reduced glutathione (GSH, 10 mM) and oxidised glutathione (GSSG, 1 mM) to approximate the reducing potential of the cytosol.[S4] After 2 hours incubation at 37 ºC the samples were rapidly frozen in liquid nitrogen then defrosted and analysed by HPLC using an Acclaim 120 C18 3 μM 120 Å 4.6 x 150 mm column, eluting with 0 to 60% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes.

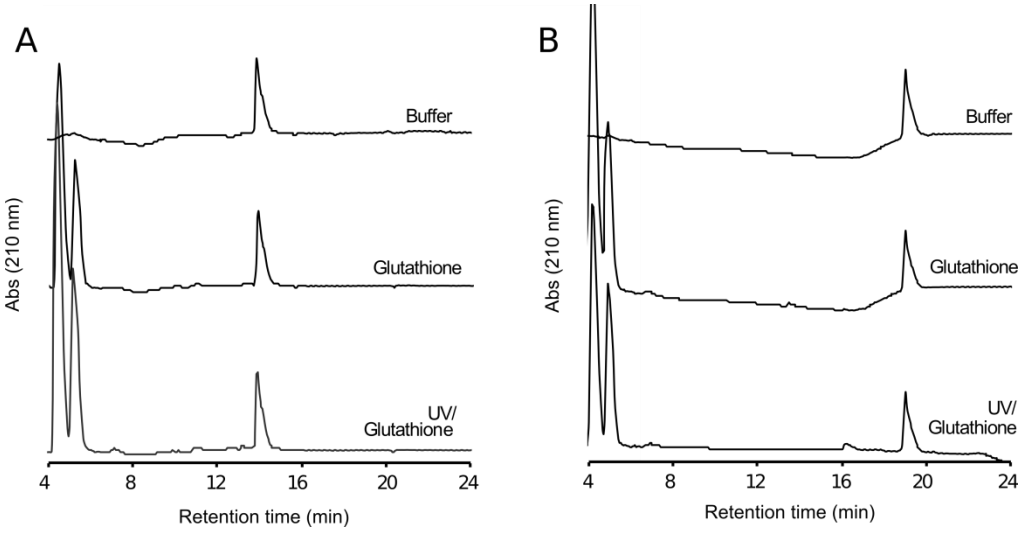

Fig. S6 The azobenzene crosslinker is not effected by gluathione. A) Ac-Bak$_{I81F}^{i,i+7}$-XL and B) FAM-Bak$^{i,i+11}$-XL.

These results mirror other reported results suggesting that in contrast to some other azobenzene molecules,[S5] this crosslinker is not effected by this reducing potential of glutathione.[S4,S6]

4 Assays

4.1 Retention of molecular components of the apoptotic pathway in a permeabilised cell system

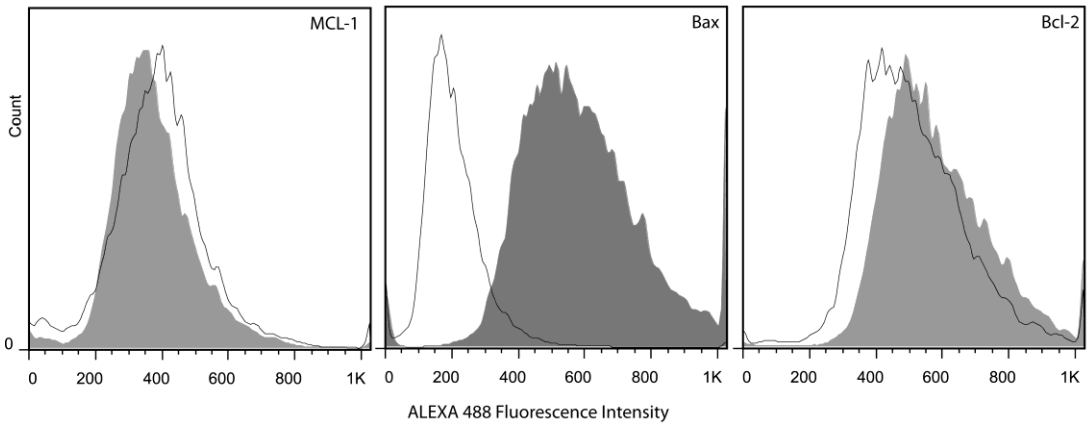

Fig. S7 SU-DHL-4 cells (at 2x10^5/mL) were permeabilised and held according to the protocol described in the main text, then immunostained with fluorescently labelled antibodies. In brief, samples were fixed in 4% paraformaldehyde, followed by 0.2% Triton permeabilisation and blocked in bovine serum albumin. The relevant primary was added [anti-MCL-1 (S-19) from Santa Cruz (sc-819); anti-Bax from BD Biosciences (96109830); anti-Bcl-2 from Sigma (B3170)]. Samples were washed, then a secondary Alexa488-labelled antibody added [Life Technologies, Invitrogen]. Data for 10^4 cells were acquired using a FACS Calibur flow cytometer [FL-1 (530/30 nm wavelength emission; linear scale)]. Permeabilised cells (open histograms) were compared to non-permeabilised control cells maintained in full media prior to fixation (filled histogram).

4.2 Imaging overall mitochondrial health

Cells were exposed to buffer, peptide or ABT-737 for 30 mins then labeled with the membrane potential independent dye MitoTrackerOrange CMTRos (Life Technologies; M-7510) at 0.1 ng/mL for 10 minutes at 37 ^{o}C. The cells were then fixed in respiration buffer containing 4% paraformaldehyde for 20 minutes to obtain high resolution information about mitochondrial morphology and integrity. Fluorescence intensity images at 580/40 nm were acquired by confocal laser scanning microscopy (Radiance, BioRad attached to a Nikon inverted microscope). A 512 x 512 xy optical slice was acquired; using a x60, 1.4 Plan-Apochromat oil immersion lens (providing typically 0.15 μm per pixel resolution). Optical sections were collected at 1 mm steps. A threshold was applied to the mitochondrial fluorescence at a high stringency (at 120 arb units), to create masks identifying the healthy mitochondrial membrane (in membrane potential terms) within cells, any treatment which compromised mitochondria reduced the signal below this threshold. Fluorescence intensity distribution in cells; healthy cells show looped structures usually polarized into a

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cap on one side of the cell. Treatment with active peptide (FAM-Bak$^{i,i+11}$-XL or irradiated Ac-Bak$_{I81F}^{i,i+7}$-XL) shows this pattern both redistributes throughout the cell and the total fluorescence diminishes.

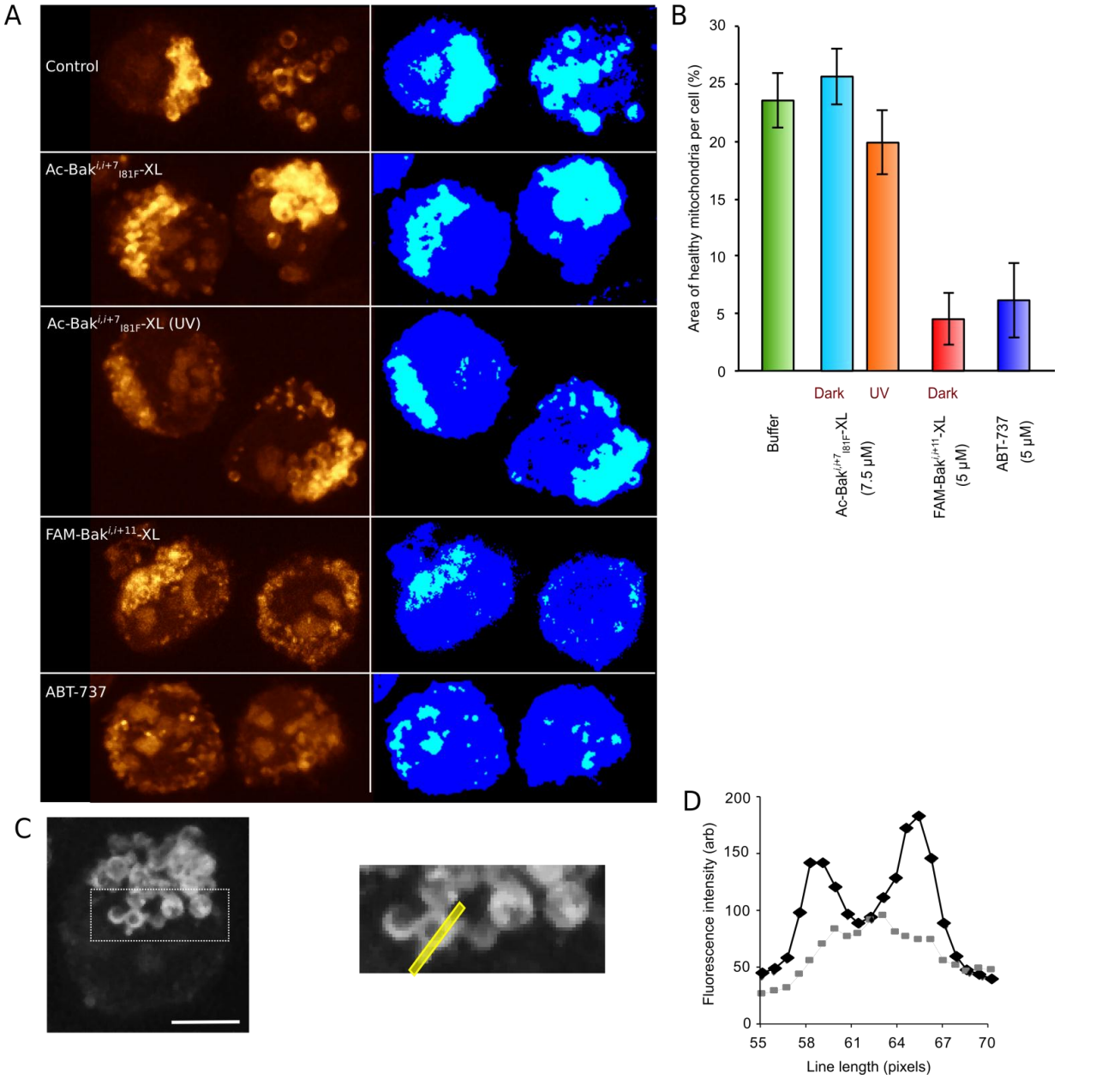

Fig. S8 Peptides case changes in mitochondrial morphology. A) Binarisation at high threshold gives the area of healthy mitochondria (cyan), merged with the binarisation at a low threshold to give the area of the whole cell (blue). B) Comparison of these two areas gives the percentage of healthy mitochondria per cell area for cells exposed to buffer only (n=7); Ac-Bak$_{I81F}^{i,i+7}$-XL (n=23); irradiated Ac-Bak$_{I81F}^{i,i+7}$-XL (n=17); FAM-Bak$^{i,i+11}$-XL (n=3); ABT-737 (n=12). C) Morphology of a healthy mitochondrial network (box-and magnified) in a whole cell (round dotted line). D) Intensity plot (along yellow line) across a mitochondrial loop (diamond line) and across a collapsed network after ABT treatment (square grey line). Provides insight on how the healthy mitochondria classification is derived. Bar 5 μm.

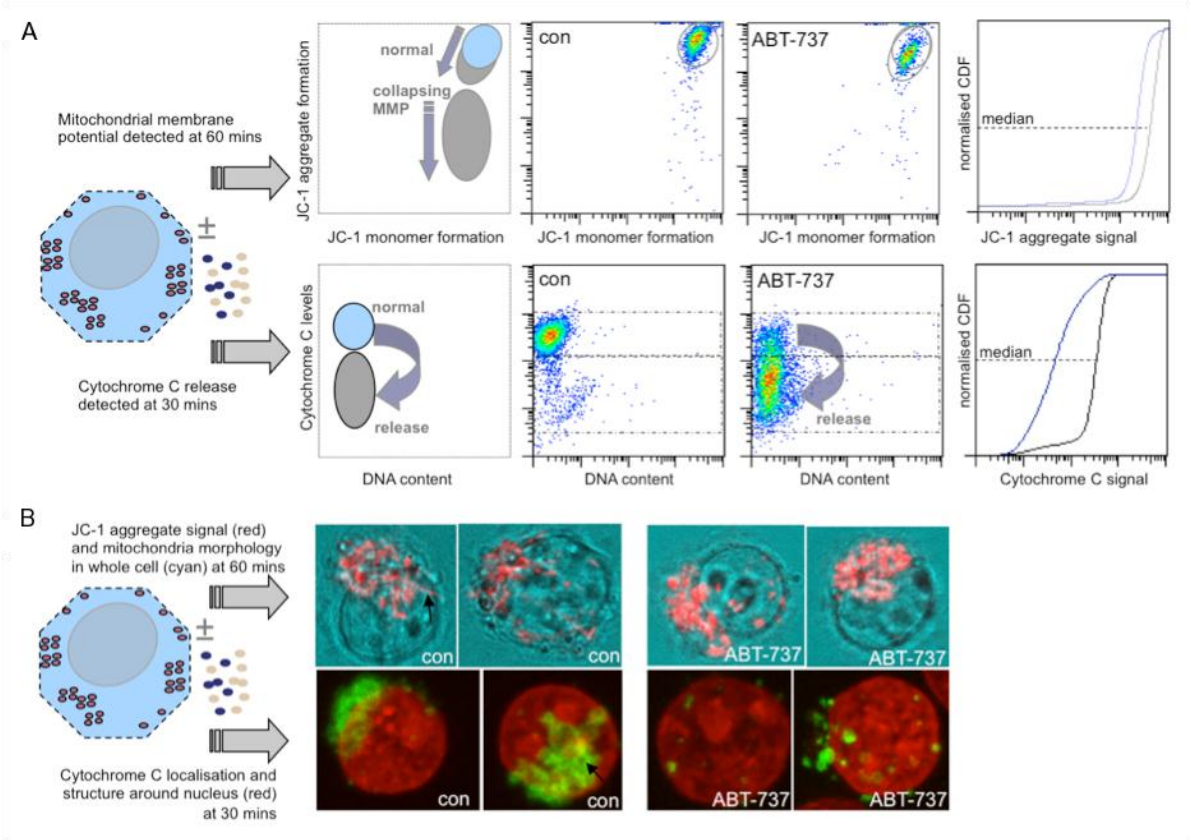

Fig. S9 Schematic to demonstrate in-cell assays for induction of apoptosis-like processes. Cells were permeabilised in respiration buffer and exposed to peptide over 30-60 minutes followed by single cell analysis using flow cytometry collecting 10,000-25,000 events to detect low frequency events and provide a sensitivity range for recoverable and non-recoverable perturbations. A) JC-1 (5,5´,6,6´-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Using flow cytometery to detect the relative spectral shift, movement of cell cohorts down the y-axis indicates loss of aggregate after 60 minutes quantified as shifts in the JC-1 aggregate signal median (upper row), enabling the extraction of dose responses. Immunofluorescence detection of cytochrome c release after 30 minutes is used as an end-point assay conducted in a timeframe that closely matches that of the nanoswitch life times (lower row). Collection of large numbers of events was essential to adequately detect minority population exhibiting cytochrome c release. Median or region analyses, where the extent of release or the frequency of cells undergoing release are determined for a set threshold for cytochrome c fluorescence intensity (typically set at channel 10^2 for a 4 decade range), were used to detect apoptosis induction. Release can be correlated with cell cycle position as measured by DNA content using DRAQ5™. B) Confocal laser scanning microscopy was used to understand mitochondria distribution in SU-DHL-4 cells. Dual channel images were collected of JC-1 labeled cells as described. JC-1 aggregate (red) or cytochrome c (green) signals showed that typically healthy mitochondria in these cells are coalesced and sit polarized in the round cell (cyan). There is very little cytoplasm in these cells hence the JC-1 signal is peri-nuclear (red). Upon treatment with ABT-737 the reticular-looped structures (arrow) disappear, the organelles redistribute and cytochrome C is located in these distinct punctuate structures throughout the cell.

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4.3 Apoptosis-like processes induced by Bak-derived peptides are cell cycle dependant

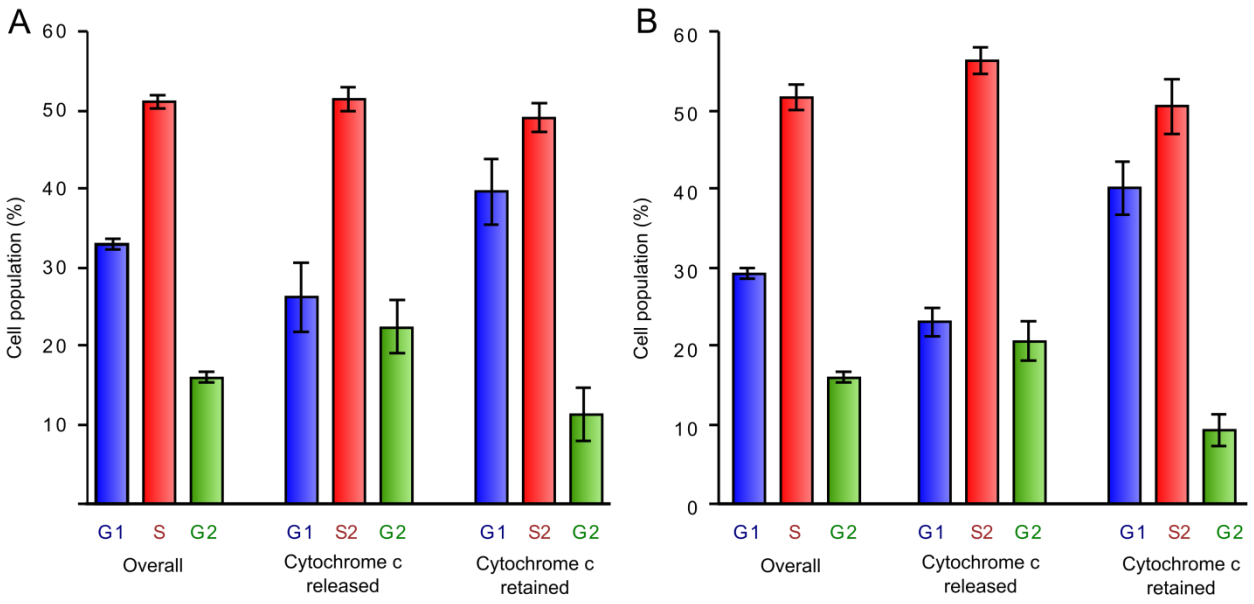

Fig. S10 Cytochrome c release defines two populations that contain cells at different stages of the cell cycle. Both A) FAM-Bak$^{i,i+11}$-XL and B) Ac-Bak$_{I81F}^{i,i+7}$-XL show the same pattern of apoptosis induction over the different phases of the cell cycle. Compared to the overall debris-gated population (left grouping) the fraction of cells that retain cytochrome c (middle grouping) contain relatively less G1 and more G2 cells so that the fraction of cells that have lost cytochrome c (right grouping) have more G1 and fewer G2 cells.

References

1. J. W. Chin and A. Schepartz, *Angew. Chem. Int. Ed.*, 2001, **40**, 3806-3809.
2. P. Wysoczanski, R. J. Mart, E. J. Loveridge, C. Williams, S. B.-M. Whittaker, M. P. Crump and R. K. Allemann, *J. Am. Chem. Soc.*, 2012, **134**, 7644-7647.
3. S. Kneissl, E. J. Loveridge, C. Williams, M. P. Crump and R. K. Allemann, *ChemBioChem*, 2008, **9**, 3046-3054.
4. Y. X. Zhang, F. Erdmann and G. Fischer, *Nat. Chem. Biol.*, 2009, **5**, 724-726.
5. A. A. Beharry, O. Sadovski and G. A. Woolley, *J. Am. Chem. Soc.*, 2011, **133**, 19684-19687.
6. F. Z. Zhang, K. A. Timm, K. M. Arndt and G. A. Woolley, *Angew. Chem. Int. Ed.*, 2010, **49**, 3943-3946.