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Electronic Supplementary Information

Targeting anti-apoptotic Bcl2 proteins with scyllatoxin-based BH3 domain mimetics

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Reagents and chemicals. All Fmoc-protected amino acids, Fmoc-PAL-AM resin and coupling reagents used for peptide synthesis were purchased from Novabiochem (Billerica, MA). *N,N*-diisopropylethylamine (DIEA), *N*-methyl-2-pyrrolidone (NMP), piperidine, oxidized and reduced glutathione, isopropyl β-D-1-thiogalactopyranoside (IPTG), trypsin and ammonium persulfate were obtained from Sigma-Aldrich (St. Louis, MO). Tris (base), bisacrylamide, sodium dodecyl sulfate, tetramethylethylenediamine (TEMED), methanol, glacial acetic acid, phenol, imidazole, LB agar, sodium chloride, ampicillin, bacterial protein extraction reagent (B-PER) and protease inhibitors were purchased from Thermo Fisher Scientific (Waltham, MA). Protein marker was obtained from New England Biolabs (Ipswitch, MA). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Morris Plains, NJ). 5-carboxyfluorescein (5-CF) was obtained from Santa Cruz Biotechnology (Dallas, TX). Acetonitrile (ACN) and D-(+)-glucose were purchased from Alfa Aesar (Ward Hill, MA). Ni-NTA agarose resin was purchased from Molecular Cloning Laboratories (San Francisco, CA). LB Medium was obtained from MP Biomedicals (Santa Ana, CA). Unless otherwise stated, all other reagents were obtained from commercial sources and used without further purification.

Solid phase peptide synthesis. All peptides described herein (Table S1) were synthesized on Fmoc-PAL-AM resin (25 μmol scale) using standard Fmoc-based

synthesis protocols.[1, 2] All amino acid couplings and deprotection reactions were performed in a microwave-accelerated reaction system (CEM, Matthews, NC) using software programs written in-house. The resin was washed thoroughly with NMP following each coupling and deprotection step. Amide bond formation was achieved by treating the resin with 5 equivalents of amino acid, 5 equivalents of PyClock and 10 equivalents of DIEA in NMP. Equivalents were based on the resin loading level. Terminal Fmoc groups were removed by treating the resin with 25% (v/v) piperidine in NMP containing 0.1 M HOBt to minimize aspartamide formation.[3] Iterative cycles of amino acid coupling and deprotection were performed until peptides of desired length were achieved.

Peptide labeling with 5-carboxyfluorescein. Following synthesis, resin-bound peptides were labeled at the N-terminus with 5-CF.[4, 5] To facilitate labeling, Fmoc-Gly-OH was coupled onto the N-terminus of resin-bound peptides using solid phase peptide synthesis methods described above. Fmoc deprotection of the N-terminal glycine was achieved by incubating the resin with 25% (v/v) piperidine in NMP containing 0.1 M HOBt. Following deprotection and washing, the resin-bound peptide was transferred immediately to a solution of NMP containing 3 equivalents of 5-CF, 3 equivalents of HCTU (Peptides International, Louisville, KY) and 7.5 equivalents of DIEA. Equivalents were based on the loading level of the resin. This reaction was allowed to stir in the dark at room temperature for 24 hours. Following labeling, the resin was washed with NMP and dichloromethane and dried under vacuum.

Cleavage and purification of fluorescently labeled peptides. Resin-bound peptides were globally deprotected and cleaved from the resin using a reagent composed of 88% TFA, 5% water, 5% phenol and 2% triisopropylsilane (Sigma-Aldrich). This reaction mixture was added to the resin and allowed to incubate at 38 °C for 30 minutes in a microwave reactor (CEM). Following cleavage, the peptide was precipitated in cold diethyl ether, pelleted by centrifugation and re-suspended in 15% (v/v) ACN in water. This mixture was then frozen and lyophilized to dryness. Following lyophilization, crude peptide powders were re-suspended in 15% (v/v) ACN in water and purified across a reversed-phase C18 column (Grace, 10 μm, 250 x 10 mm) using an Agilent ProStar HPLC system. Peptides were eluted over 35 minutes with a linear gradient of 15-50% solvent B (0.1% TFA in ACN) over solvent A (0.1% TFA in water). Absorbance was monitored at 214 and 450 nm to distinguish between labeled and unlabeled peptide products. Product peaks were combined, frozen and lyophilized twice. Purified peptides were reconstituted in water and stored at 4 °C protected from light. The concentrations of stock peptide solutions were quantified using an extinction coefficient for 5-CF of 83,000 M^{-1} cm^{-1} at 450 nm in water (Setareh Biotech, Eugene, OR).

General characterization of peptides by LC/MS. The identities of all peptides described herein were confirmed using LC/MS (Table S1). Peptide masses were determined using a Waters Acquity liquid chromatography system coupled to a Waters Xevo QTof mass spectrometer set to positive ion mode. Peptides were resolved across a reversed-phase C18 column (Waters) and eluted over 7 minutes with a linear gradient

of 5-95% solvent B (0.1% formic acid in ACN) over solvent A (0.1% formic acid in water). All mass data were processed using MassLynx Software version 4.1 (Waters).

General characterization of peptides by analytical HPLC. Peptide purities were evaluated by analytical RP-HPLC using an Agilent ProStar HPLC system. Peptides (2.5 μM in water) were analyzed across a reversed-phase C18 column (Grace, 5 μm, 50 x 2.1 mm) and eluted over 20 minutes with a linear gradient of 5-95% solvent B (0.1% TFA in ACN) over solvent A (0.1% TFA in water). All peptides were purified to >95% as determined by product peak integration of analytical HPLC chromatograms (Figure S1). Analytical HPLC data were processed using OpenLab CDS ChemStation Software (Agilent) version 1.06 and KaleidaGraph version 4.5 (Synergy Software).

Oxidation of reduced ScTx peptides. All reduced ^{Flu}ScTx-based peptides described herein were synthesized, cleaved, purified and characterized as described above. Oxidation (folding) reactions were performed as described previously.[6] Briefly, reduced peptides (25 mg) were dissolved in 25 mL folding buffer (100 mM Tris, 100 mM NaCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, pH 8.0) and were allowed to stir for 3 h at room temperature in the dark. Following the reaction, the solution was acidified with 2.5 mL 50% TFA in water, loaded directly onto a C18 reverse-phase HPLC column (Grace, 10 μm, 250 x 10 mm) and purified as described above (Figure S3). Fully oxidized ScTx-based proteins were confirmed by observing product retention time shifts in HPLC traces and loss in mass corresponding to six hydrogen atoms[6] (Table S1).

Disulfide bridge assignment. To confirm correct positioning of the disulfide bonds, the fully oxidized peptide ScTx-Bax (100 μg) was mixed with trypsin (10% w/w) in 100 μL digestion buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.8) and allowed to incubate at 37 ºC for 3 hours.[6] Following the reaction, 100 μL of 50% (v/v) TFA in digestion buffer was added to stop the proteolysis reaction. The full reaction volume was then loaded onto a C18 RP-HPLC column (Grace, 5 μm, 50 x 2.1 mm) and eluted over 50 minutes with a linear gradient of 0-50% solvent B (0.1% TFA in ACN) over solvent A (0.1% TFA in water). All major peaks resolved by HPLC were collected and analyzed by mass spectrometry (Figure S4). A similar protease solution without peptide was also analyzed as a negative control and showed no autolytic fragments following 3 h of incubation at 37 ºC (data not shown).

Circular dichroism. Stock peptides were diluted to a final concentration of 10 μM in binding buffer (50 mM Tris, 100 mM NaCl, pH 8.0) with or without 30% (v/v) 2,2,2-trifluoroethanol. All peptide solutions were allowed to incubate at 20 ºC for 10 minutes before being analyzed by circular dichroism spectroscopy. Wavelength scans were performed on a Jasco J-715 spectropolarimeter at 20 ºC. Each spectrum represents a background subtracted (buffer only) average of four scans. Data were processed with J-700 Software version 1.5 (Jasco) and KaleidaGraph version 4.5 (Synergy Software).

Plasmid purification and transformation. Plasmids coding for human Bcl2-ΔTM were obtained from the DNASU Plasmid Repository (Tempe, AZ) in transformed phage resistant DH5-α *E. coli* bacterial cells (Clone ID: HsCD00286806). Bcl2-ΔTM was

supplied in a pMCSG7 vector, which adds an N-terminal polyhistidine tag (His_6) to Bcl2-ΔTM to facilitate purification.[7] Plasmids were isolated from DH5-α cells using a plasmid purification kit (Qiagen, Valencia, CA) and subsequently transformed into BL21(DE3) competent cells (Agilent, Santa Clara, CA) according to the manufacturer's instructions. Following transformation, the cells were stored at -80 ºC as glycerol stocks.

Protein expression and purification. His-tagged Bcl2-ΔTM protein was expressed from BL21(DE3) competent cells using a modification of methods described previously.[8] Briefly, bacterial cells were grown as 1 L cultures in LB media containing 100 μg/mL ampicillin to an optimized OD_{600} of 0.8 at 37 ºC. Protein expression was then induced for 4 h at 37 ºC with the addition IPTG (1 mM final concentration). Following induction, the bacteria were pelleted and stored at -80 ºC. To extract the protein, bacterial pellets (500 mL fractions) were re-suspended in 10 mL cold bacterial protein extraction reagent (B-PER) (Thermo Fisher Scientific) containing 10 mM imidazole and protease inhibitor (Thermo Fisher Scientific). This suspension was allowed to shake at 4 ºC for 10 minutes before centrifugation at 15,000 x *g* for 15 minutes at 4 ºC. The cleared lysate containing Bcl2-ΔTM was then added across a freshly prepared Ni-NTA agarose (MC Labs, San Francisco, CA) column according to the manufacturer's instructions. The protein was then eluted from the column with elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0) and immediately dialyzed into binding buffer (50 mM Tris, 100 mM NaCl, pH 8.0). To determine overall purity of the eluted protein, samples (20 μL) of the collected fractions were loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Visualization of the proteins was achieved by staining the gel with

Coomassie Blue (Figure S5A). Following dialysis, the protein was concentrated using centrifugal filtration units (Millipore) to a final concentration of 15 μM. Protein concentrations were determined using standard Bradford assays.[9] Concentrated protein solutions were aliquoted, flash frozen and stored -80 ºC.

Circular dichroism of purified Bcl2-ΔTM. A stock solution of Bcl2-ΔTM protein was diluted to a final concentration of 10 μM in binding buffer (50 mM Tris, 100 mM NaCl, pH 8.0). The solution was allowed to equilibrate at 20 ºC for 10 minutes before being analyzed by circular dichroism spectroscopy. Following equilibration, wavelength scans were performed on a Jasco J-715 spectropolarimeter from 200 to 250 nm at 20 ºC (Figure S5B). To determine the melting temperature (Tm) of Bcl2-ΔTM, a solution of Bcl2-ΔTM (10 μM in binding buffer) was analyzed from 5 to 95 ºC at 222 nm using a Jasco J-715 spectropolarimeter (Figure S5C). Taking the first derivative of melt curve resulted in a Tm of 58.0 ºC for Bcl2-ΔTM. All data were processed using J-700 Software version 1.5 (Jasco) and KaleidaGraph version 4.5 (Synergy Software).

Fluorescence polarization binding assays. Fluorescence polarization experiments were performed in #3575 384-well plates (Corning, Corning, NY) using a SpectraMax M5e multi-mode plate reader (Molecular Devices, Sunnyvale, CA). Fluorescence polarization was measured using an excitation wavelength of 498 nm and an emission wavelength of 525 set to an automatic cutoff of 515 nm. An average of 100 reads was recorded for each well. Serial dilutions of Bcl2-ΔTM were prepared in binding buffer (50 mM Tris, 100 mM NaCl, pH 8.0) and an aliquot of fluorescently-labeled peptide was

added to each dilution at a final concentration of 25 nM. Binding reactions were then allowed to incubate in the dark at 25 ºC for 1 hour. This incubation time was sufficient for binding reactions to reach equilibrium, as judged by an absence of change in the observed polarization value of the sample with the lowest protein concentration over 4 h. Polarization data were processed using SoftmaxPro version 6.4 (Molecular Devices) and binding curves were fit using KaleidaGraph version 4.5 to a single-site binding model described by the equation outlined below.[10]

$$FP_{obs} = FP_0 + (FP_{max} - FP_0) \times \frac{1}{2L} \times \left\{ (Kd + P + L) - \sqrt{(Kd + P + L)^2 - (8 \times P \times L)} \right\}$$

FP_{obs} is the fluorescence polarization at protein concentration P; FP_0 is the fluorescence polarization of ligand in the absence of protein (P = 0); FP_{max} is the maximum fluorescence polarization at saturation of protein with ligand. P and L represent the total respective concentrations of protein and ligand, and Kd is the dissociation constant.

Peptide	Sequence	Reduced		Oxidized	
		Calc. (+m/z)	Obs. (+m/z)	Calc. (+m/z)	Obs. (+m/z)
^{Flu}Bax-BH3	^{Flu}GSTKKLSECLKRIGDELDSNM-NH_2	2680.45	2681.36	-	-
^{Flu}Bak-BH3	^{Flu}GQVGRQLAIIGDDINR-NH_2	2081.26	2081.91	-	-
^{Flu}Bak-BH3$^{\Delta\Delta PP}$	^{Flu}GQVPRQLAIIPDDINR-NH_2	2161.32	2161.84	-	-
^{Flu}wtScTx	^{Flu}GAFCNLRMCQLSCRSLGLLGKCIGDKCECVKH-NH_2	3841.97	3842.60	3835.92	3836.45
^{Flu}wtScTx$^{\Delta\Delta\Delta}$	^{Flu}GAFBNLRMBQLSBRSLGLLGKBIGDKBEBVKH-NH_2	3734.52	3734.56	-	-
^{Flu}ScTx-Bak	^{Flu}GAFCGQVGCQLACIGDGLLGKCIGDKCECVKH-NH_2	3581.79	3582.51	3575.54	3576.27
^{Flu}ScTx-Bak$^{\Delta\Delta\Delta}$	^{Flu}GAFBGQVGBQLABIGDGLLGKBIGDKBEBVKH-NH_2	3474.43	3474.63	-	-
^{Flu}ScTx-Bax	^{Flu}GSCKKLSCBLKCIGDGLLGKCIGDKCECVKH-NH_2	3579.96	3580.67	3573.91	3574.74
^{Flu}ScTx-Bax$^{\Delta\Delta\Delta}$	^{Flu}GSBKKLSBBLKBIGDGLLGKBIGDKBEBVKH-NH_2	3472.51	3472.67	-	-

Table S1. Sequences and mass data of peptides used in this work. Functional BH3 epitope is shown in red; conserved BH3 domain aspartic acid is cyan.

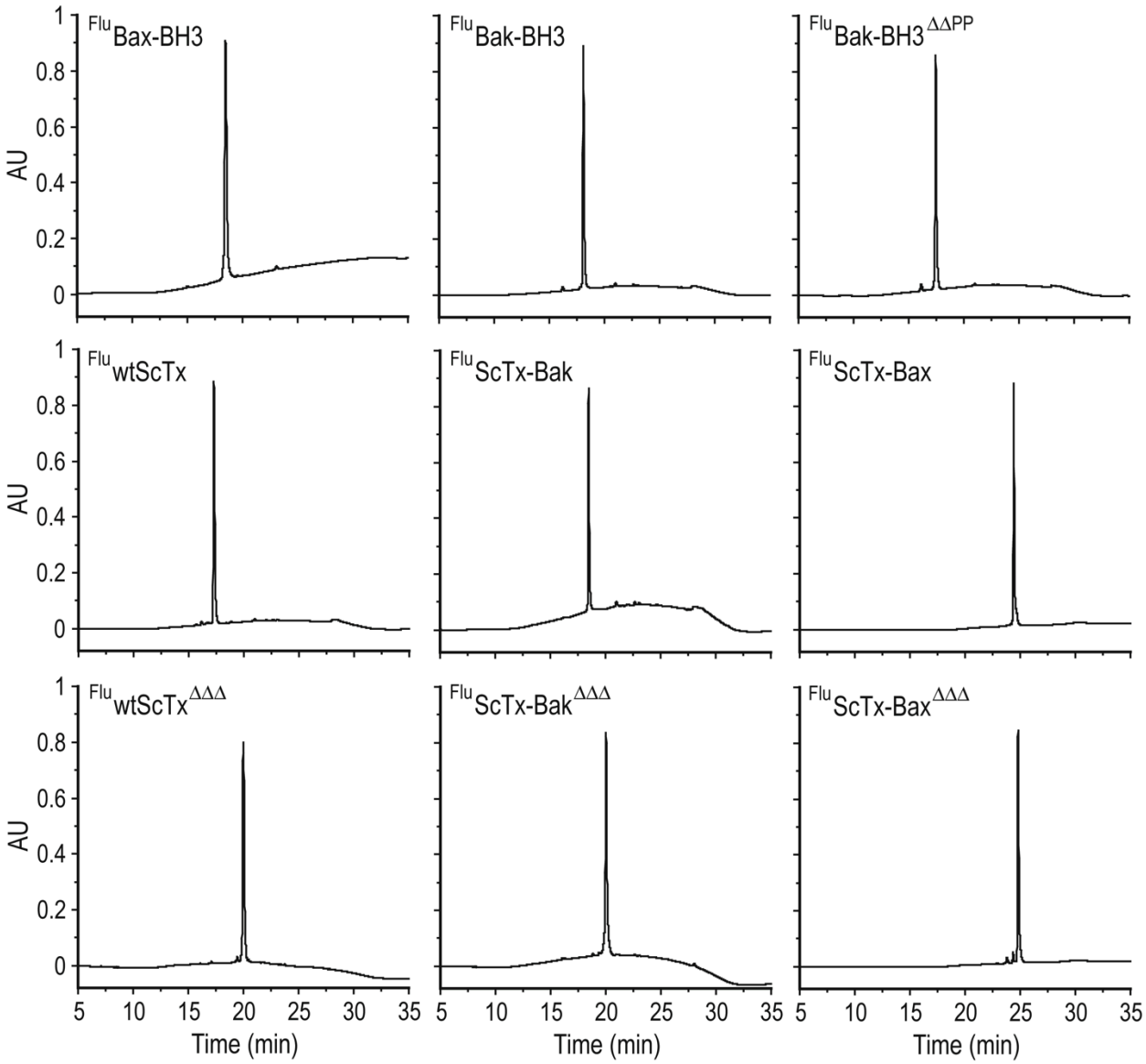

Figure S1. Analytical HPLC chromatograms of peptides used in this work. All spectra were monitored at 214 nm. AU, normalized absorbance units. See ESI text for details.

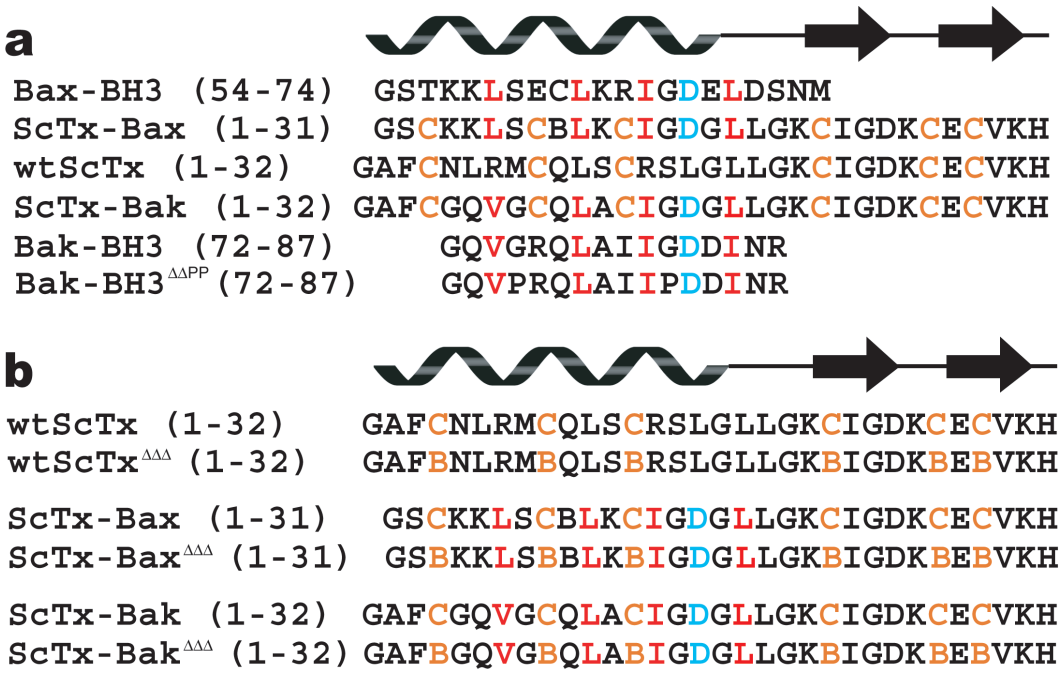

Figure S2. A. Sequence alignment of BH3-domain peptides and ScTx-based BH3 domian mimetics. Residue numbers are shown in parentheses. Key α-helical residues required for Bcl2-ΔTM recognition are colored red; cysteines that contribute to ScTx folding are orange; conserved BH3 domain aspartic acid residue is cyan. B. Sequence alignment of ordered and intrinsically disordered ScTx-based BH3 domian mimetics. Key α-helical residues required for Bcl2-ΔTM recognition are colored red; aminobutyric acids and cysteines that contribute to ScTx folding are orange; conserved BH3 domain aspartic acid residue is cyan.

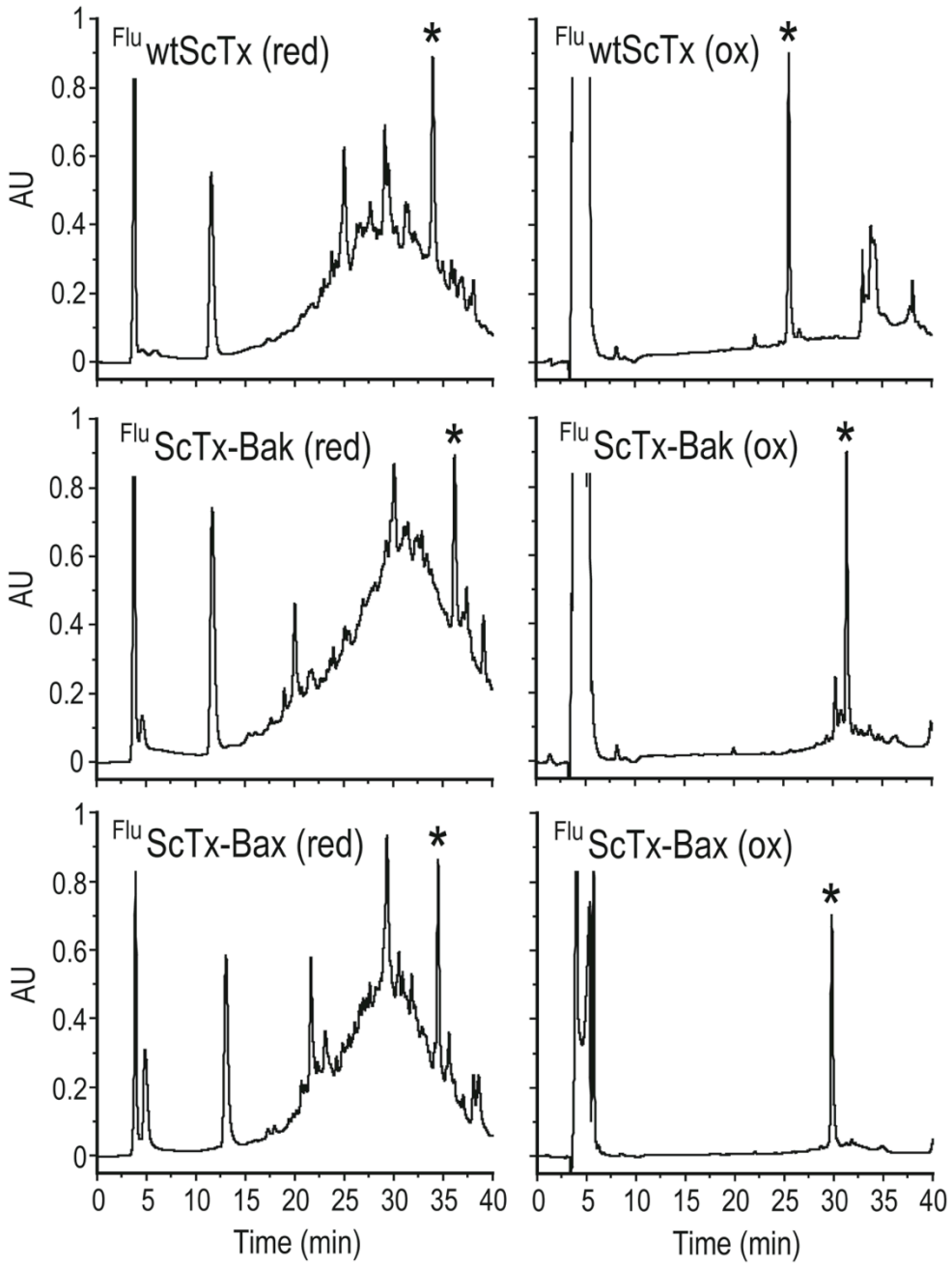

Figure S3. Preparatory RP-HPLC chromatograms of reduced (red) and oxidized (ox) ScTx-based peptides. Spectra on the left represent crude HPLC chromatograms of reduced peptides following synthesis and cleavage from the resin. Peaks marked with an astrisk (*) are those of fluorescently labeled, reduced product. Spectra on the right represent HPLC chromatograms of fully oxidized peptides following the folding reaction. Peaks marked with an astrisk (*) are those of fluorescently labeled, oxidized product. All spectra were monitored at 214 nm. AU, normalized absorbance units. See ESI text for details.

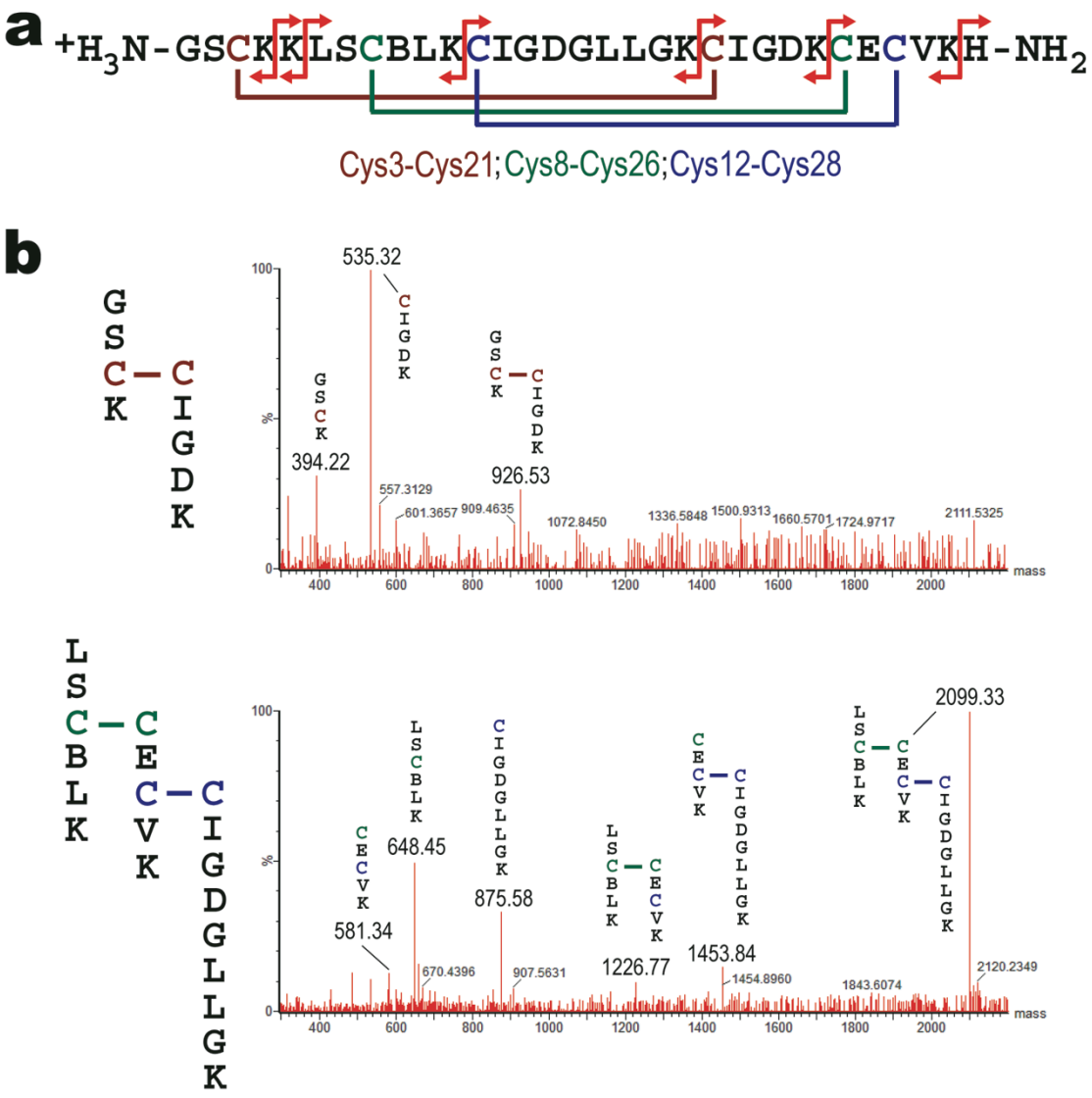

Figure S4. A. Primary sequence of fully oxidized ScTx-Bax peptide. Oxidized dicysteine linkages are shown below the primary sequence; trypsin cut sites are indicated by red arrows. B. Mass spectra of peptide fragments isolated from trypsin digest. Dicysteine-linked peptide sequences are shown adjacent to corresponding mass peaks. See text for experimental details.

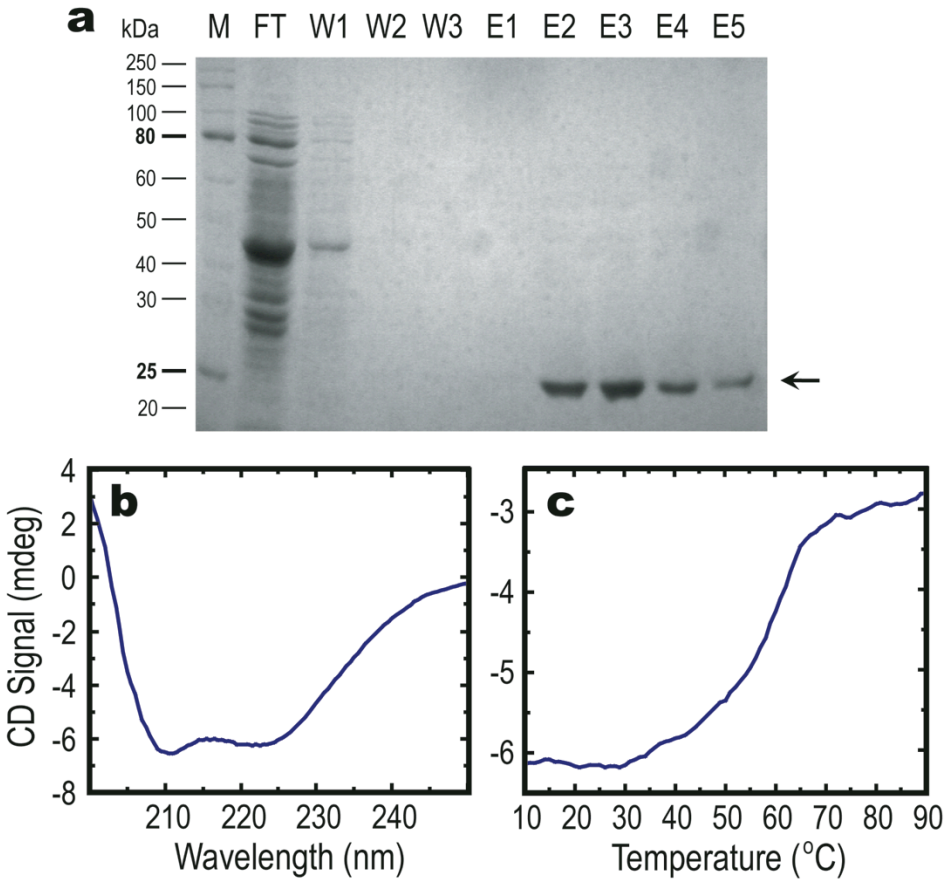

Figure S5. A. SDS-PAGE from Bcl2-ΔTM purification by column chromatography. See ESI text for details. M, size marker; FT, flow through; W1-3, washes; E1-5, elutions. Arrow indicates purified Bcl2-ΔTM at ~25 kDa. B. Circular dichroism wavelength scan of Bcl2-ΔTM in binding buffer at 20 ºC. Spectra represent a background subtracted (buffer only) average of four scans. C. Circular dichroism thermal scan of Bcl2-ΔTM in binding buffer at 222 nm.

SUPPLEMENTAL REFERENCES.

1. C. D. Chang and J. Meienhofer, *Int J Pept Protein Res*, 1978, **11**, 246-249.

2. G. B. Fields and R. L. Noble, *Int J Pept Protein Res*, 1990, **35**, 161-214.

3. S. A. Palasek, Z. J. Cox and J. M. Collins, *J Pept Sci*, 2007, **13**, 143-148.

4. I. M. Kaplan, J. S. Wadia and S. F. Dowdy, *J Control Release*, 2005, **102**, 247-253.

5. K. Nogami, K. Takahama, A. Okushima, T. Oyoshi, K. Fujimoto and M. Inouye, *Chembiochem*, 2014, **15**, 2571-2576.

6. Q. Zhu, S. Liang, L. Martin, S. Gasparini, A. Menez and C. Vita, *Biochemistry*, 2002, **41**, 11488-11494.

7. L. Stols, M. Gu, L. Dieckman, R. Raffen, F. R. Collart and M. I. Donnelly, *Protein Expr Purif*, 2002, **25**, 8-15.

8. K. M. Kim, C. D. Giedt, G. Basanez, J. W. O'Neill, J. J. Hill, Y. H. Han, S. P. Tzung, J. Zimmerberg, D. M. Hockenbery and K. Y. Zhang, *Biochemistry*, 2001, **40**, 4911-4922.

9. M. M. Bradford, *Anal Biochem*, 1976, **72**, 248-254.

10. D. Golemi-Kotra, R. Mahaffy, M. J. Footer, J. H. Holtzman, T. D. Pollard, J. A. Theriot and A. Schepartz, *J Am Chem Soc*, 2004, **126**, 4-5.