Supporting Information

Electrophilic helical peptides that bind covalently, irreversibly and selectively in a protein-protein interaction site

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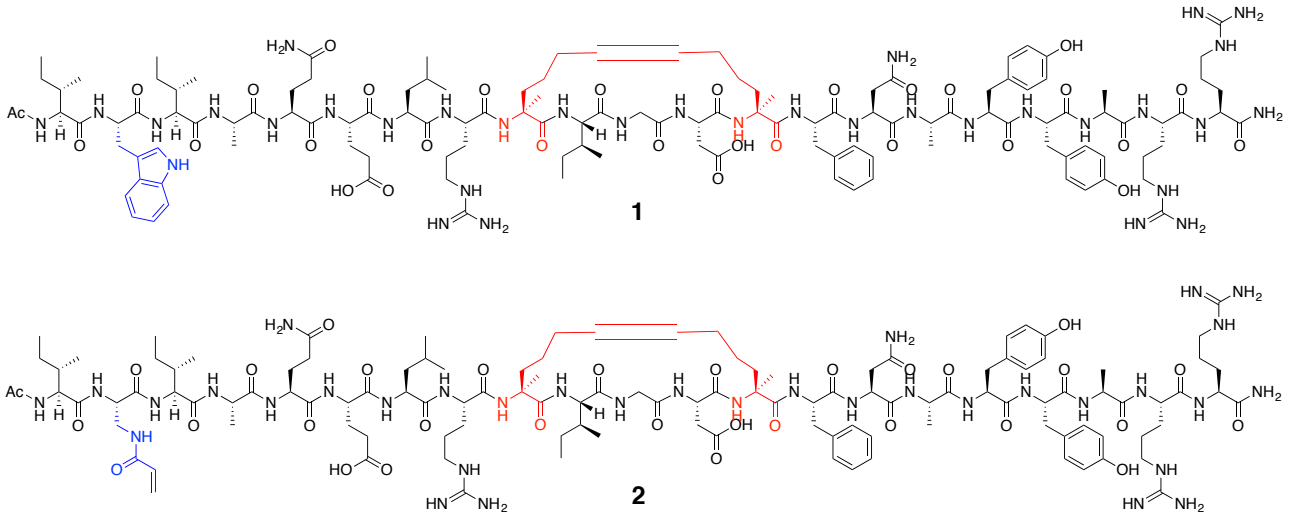

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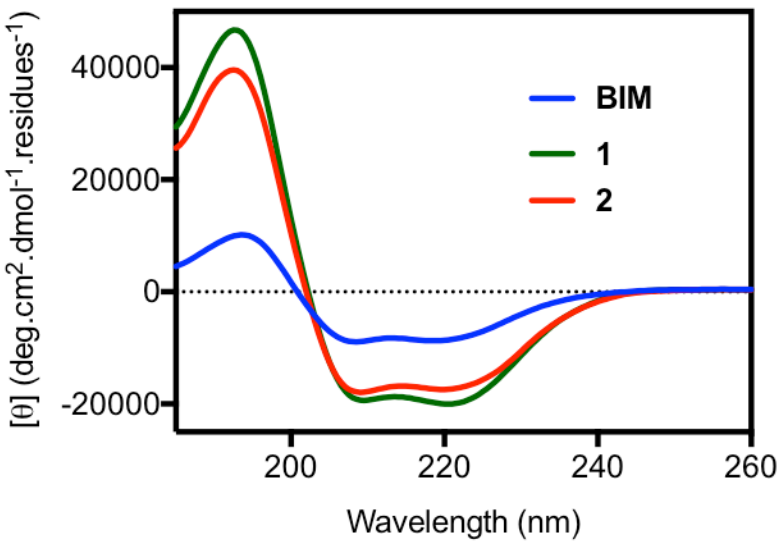


Figure S1. A. Chemical structure of hydrocarbon stapled peptides **1** and **2**. **B**. CD spectra of linear BIM (Ac-IWIAQELRRIGDEFNAYYARR-NH_2, blue), $BIMSAHB_A$ **1** (green) and electrophilic peptide **2** (red) at 50 μM concentration in 20% acetonitrile:80% water. The linear peptide (blue) has some helicity (28% helical), which is significantly increased after insertion of the hydrocarbon sidechain-sidechain linkage between positions 9 and 13 (green, 64% helical), while also replacing Trp2 with Dap(acrylamide) has little further effect (red, 56% helical).

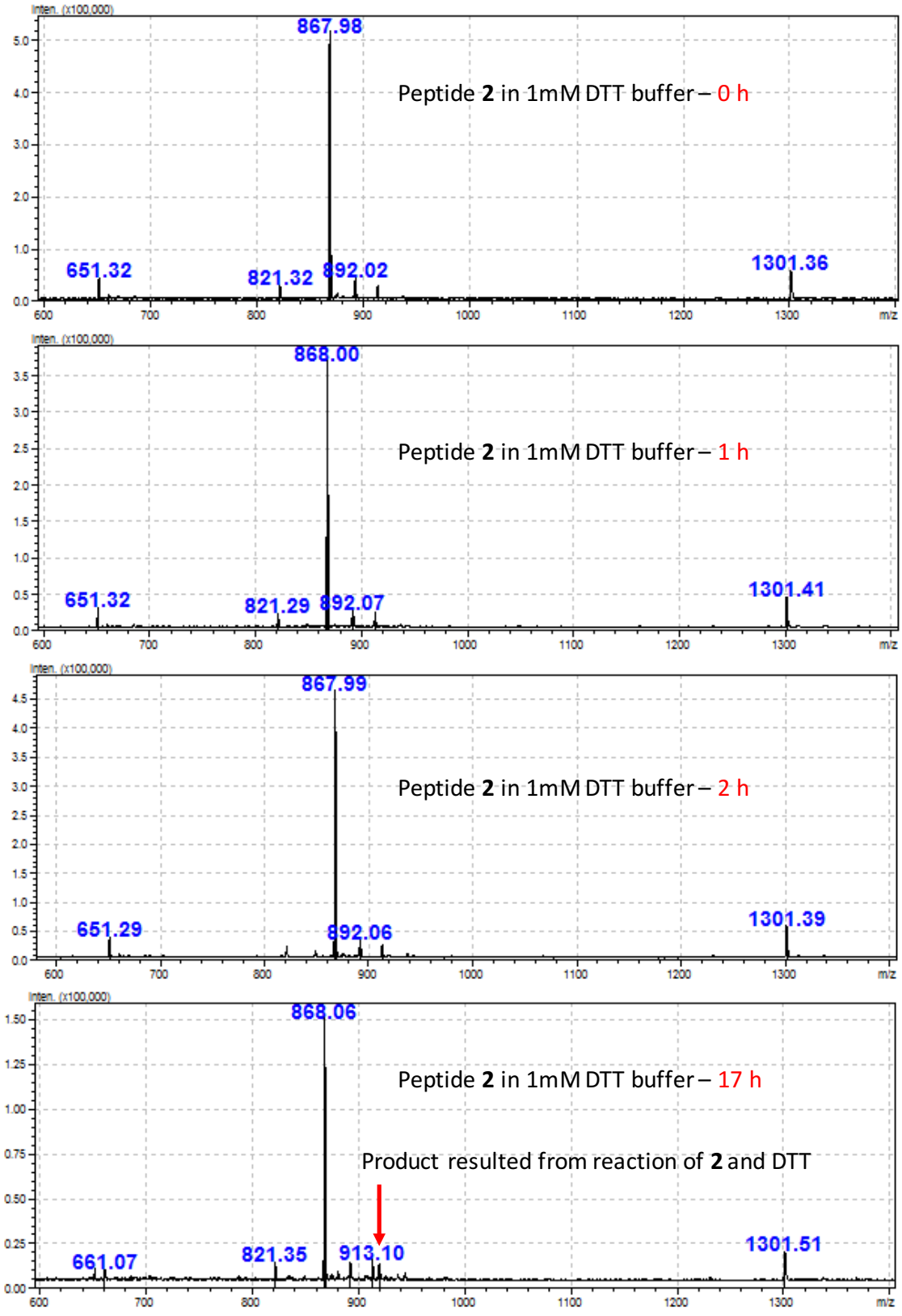


Figure S2. Mass spectra analysis of the integrity of peptide **2** (50 μM) in buffer containing 1 mM DTT, 50 mM Tris, 150mM NaCl, 1 mM EDTA, pH 7.2.

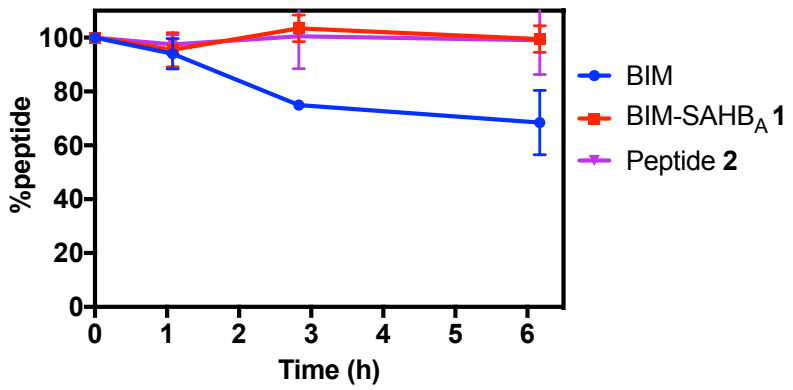


Figure S3. Stability of linear Bim (Ac-IWIAQELRRIGDEFNAYYARR) and hydrocarbon-stapled peptides BimSAHB$_A$ **1** and acrylamide-containing **2**. Conditions: peptides (25 μM) incubated with human serum (190 μL, Sigma Aldrich) at 37^{0}C.

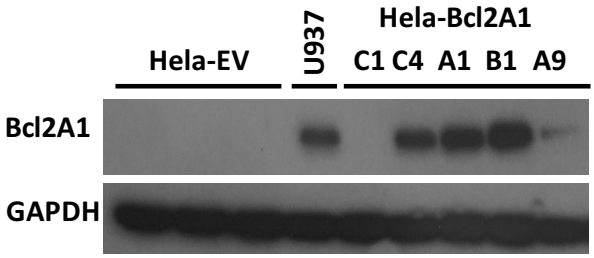


Figure S4. Western blot showing expression of Bcl2A1 in different cell lines. U937 cells endogenously express Bcl2A1, but HeLa cells (HeLa-EV, Empty Vector) do not. A range of different clones were tested to transfect Bcl2A1 into HeLa cells, with the B1 clone showing the highest level of Bcl2A1 expression. Expression of Bcl2A1 was detected using anti-Bcl2A1 antibody (1:2500) and GAPDH was used as a loading control.

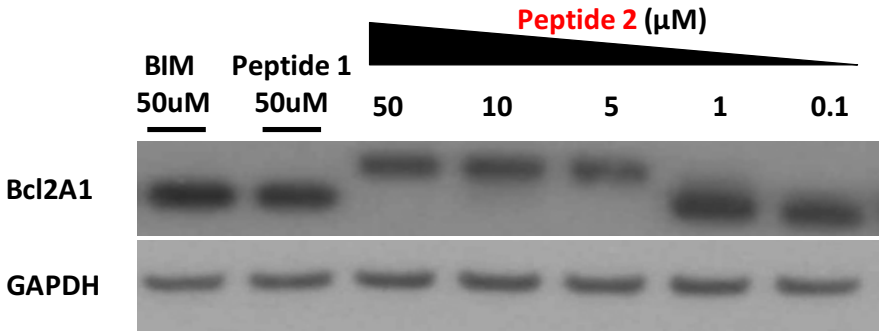


Figure S5. Covalent binding of peptide **2** (at 5-50 μM, but not at 0.1-1μM) to Bcl2A1 after 24h incubation at 37^{0}C with lysates from transfected HeLa-Bcl2A1 cells (B1 clone).

Experimental procedures

Abbreviations

Ac, acetyl; Ahx, aminohexanoic acid; Dap, diaminopropionic acid; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; DCE, dichloroethane; DCM, dichloromethane; DIC, N,N′-diisopropylcarbodiimide ; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DTT, dithioltreitol; Fmoc, 9-fluorenylmethyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid ; FITC, fluorescein isothiocyanate; HATU, 2-(7-Aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(1*H*-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-Hydroxy-7-azabenzotriazole; HR-MS, High-resolution mass spectroscopy; MBHA, 4-methyl-benzylhydrylamine; Mtt, 4-methyltrityl; RCM, ring-closing metathesis; RP-HPLC, reserved-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TCEP, Tris(2-carboxyethyl)phosphine; TFE, 2,2,2-trifluoroethanol; TIS, triisopropylsilane.

1. Peptide synthesis and characterization

Materials

All solvents and reagents used during peptide chain assembly were peptide synthesis grade and purchased from commercial suppliers.

Automated solid phase peptide synthesis

Peptides were assembled using Fmoc-based chemistry on a peptide synthesizer (Symphony, Protein Technologies) utilizing a low loading Rink Amide MBHA resin and standard Fmoc-protected amino acids. Exceptionally, the unusual amino acid Fmoc-(S)-2-(4'-pentenyl)alanine (Fmoc-S_5-OH) was used for the incorporation of the staple handles at positions 9 and 13, and the Mtt-protected building block Fmoc-N^{β}-4-methyltrityl-L-2,3-diaminopropionic acid (Fmoc-Dap(Mtt)-OH) was coupled at the position 2 (or 3) as an attachment site for the warhead moiety. Usually, 4 equiv. of Fmoc-protected amino acid, 4 equiv. of HCTU and 4 equiv. of DIPEA were used in 2 x 30 min coupling cycles. Fmoc deprotection was achieved by treatment with 1:3 piperidine:DMF for 2 × 3 min. For *N*-terminal acetylated peptides, the *N*-terminus was acetylated with Ac_2O:DIPEA (2:1) in DMF for 2 x 5 min followed by on-resin RCM as described below. For peptides containing FITC at the *N*-terminus, the peptide assembly was completed until the incorporation of the *N*-terminal linker Fmoc-β-Ala (for FBID and **10**) or Fmoc-Ahx (for **9**) and the resulting protected peptide was then submitted to RCM as described below. After that, the Fmoc-protecting group was removed and the fluorescent marker was incorporated to the *N*-terminus by treating the free amine resin with FITC (2 equiv) and DIPEA (4 equiv) in DMF overnight.

On-resin ring closing metathesis (RCM)[1]

Prior to RCM, the resin was washed with DCM and dried under high vacuum. The dry resin was then placed in the synthesizer apparatus and swollen in dry DCE under N_2 stream for 10 min and drained. The RCM reaction was performed by treating the resin with a 10 mM solution of Grubbs catalyst 1st generation in dry DCE (2 mL per 50 μmol resin) under N_2 bubbling for 2h. The catalyst solution was drained and a fresh 10 mM Grubbs catalyst solution was added to the resin and reacted for 2h. After that, the resin was washed with DCE, DMF and DCM.

Incorporation of the warhead group (for peptides 2, 5, 6, 7 and 9)

After final assembly of the linear or stapled peptide, the resin was washed with DCM and treated repeatedly with a solution of 3% TFA in DCM (10 x 2 min). After washing with DCM and DMF, a solution of the warhead reagent (acrylic acid, chloroacetic acid or propiolic acid; 4 equiv), HATU (4 equiv) and DIPEA (4 equiv) in DMF was added to the resin and the reaction was agitated for 60

min. After that, the resin was washed with DMF and DCM, dried under vacuum and submitted to cleavage.

Peptide 8

β-chloroalanine containing peptide **8** was assembly as described above, but the building block Fmoc-β-chloroalanine was used to incorporated the chloro-warhead at position 2 instead. Coupling conditions: Fmoc-β-chloroalanine (5eq), DIC (5eq), HOAt (5 eq) in DMF:DCM (1:1) for 2h. Subsequent Fmoc-deprotection after attachment of the β-chloroalanine residue was performed with 1% DBU in DMF for 2 x 1 min.[2]

Cleavage from solid support and peptide purification

Peptides were cleaved from the resin by treatment with TFA:TIS:H_2O (95:2.5:2.5) for 2 h. The crude peptides were precipitated and washed with cold Et_2O, redissolved in 50% acetonitrile/0.05% TFA in water and lyophilized. Peptides were purified by RP-HPLC using a Phenomenex Luna C18 column eluting at a flow rate of 20 mL/min and a gradient of 0 to 60% buffer B (90% CH_3CN/10% H_2O/0.1% TFA in buffer A, 0.1% TFA in water) over 35 minutes.

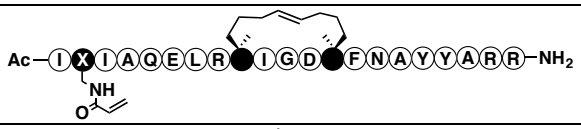
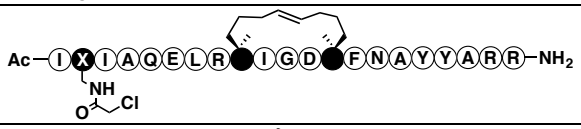
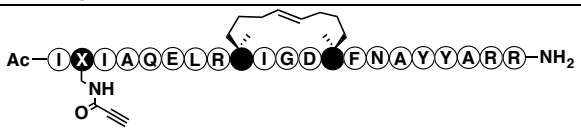
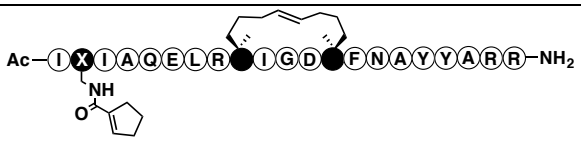
Peptide concentration and storage

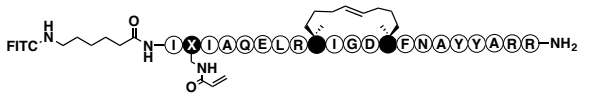
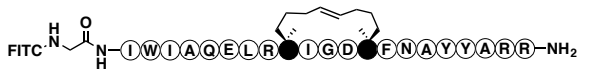
The concentration of pure peptide samples was determined by NMR using Pulcon method.[3] DMSO stocks solution of 10 mM concentration were prepared and stored at -20^{0}C.

Analytical methods

Analytical RP-HPLC was performed on an Agilent system, using a Phenomenex Luna C18 5 um (250 x 4.60 mm) column eluting at a flow rate of 1 mL/min and a gradient of 0 to 60 % buffer B (90% CH_3CN/10% H_2O/0.1% TFA in buffer A, 0.1% TFA in water) over 20 minutes. High-resolution mass spectroscopy was carried out on an Applied Biosystems QSTAR Elite time-of-flight mass spectrometer. The acquired spectrum mass range (m/z ions from +2 to +4) was deconvoluted to determine the observed mass using the program Analyst BioTools.

Table S1. Structure and HR-MS of synthetic peptides.

Compound	Sequence	Expected mass	Observed mass
1		2646.426	2646.040
2		2601.015	2601.426
5		2622.367	2622.409
6		2598.390	2597.978
7		2634.378	2634.020
8		2641.080	2640.475

9		3061.519	3060.526
10		3064.489	3064.004
FBID		2839.336	2839.032

2. Covalent binding by SDS-PAGE electrophoresis and MS-MS spectroscopy

Preparation of Bcl2A1 protein sample

The His6-tag human Bcl2A1 protein (1 to 152 aa) was purchased from Novoprotein (C103). The commercial sample was diluted 4-fold to a concentration of 10 μM Bcl2A1 in buffer containing 50 mM Tris, 150mM NaCl, 1 mM EDTA, 50 mM TCEP, pH 7.2 and incubated in this TCEP buffer for 60 min on an ice bed prior to the binding assays.

Reaction of Bcl2A1 and peptides

Bcl2A1 and peptide were combined in buffer 50 mM Tris, 150mM NaCl, 1 mM EDTA, 2 mM TCEP, 0.005% Tween-20, pH 7.2 to a final concentration of 2 μM Bcl2A1 and 8 μM peptide in a total of 20 μL reaction volume. After incubation at room temperature for 2h, the reaction was immediately submitted to SDS-PAGE analysis.

SDS-PAGE electrophoresis

One-dimensional SDS-PAGE was used to resolve proteins in the reaction mixture using precast 4-12% Bis-Tris pre-cast Novex®Bolt gels in a Bolt® Mini Gel Tank system in Bolt® MES Running Buffer (Life Technologies) following the manufacturer's instructions, which included preparation of the samples by heating at 70^0C with Bolt® reducing agent and LDS sample buffer for 10 min before loading. Gel was stained with Coomassie Blue.

In-gel trypsin digestion

The protein was digested by trypsin using standard in-gel trypsin digestion protocols (ref). Briefly, the individual protein gel bands obtained after electrophoresis were excised, dehydrated and de-stained by treating the gel pieces with a solution of NH_4HCO_3:acetonitrile, followed by digestion with trypsin in NH_4HCO_3 buffer. Digested protein fragments were further extracted from gel with 0.5% formic acid/50% acetonitrile, combined with trypsin digestion supernatant and dried by speed vac.

MS-MS analysis of digested protein

Digest product was redissolved in 0.5% formic acid/50% acetonitrile and were analyzed by LC-MS on a Shimadzu Nexera uHPLC (Japan) coupled to a Triple TOF 5600 mass spectrometer (ABSCIEX, Canada) equipped with a duo electrospray ion source. 10 μl of each extract was injected onto a 2.1mm x 100mm Zorbax C18 1.8um column (Agilent) at 400 μl/min. Linear gradients of 1-80% solvent B over 20 min at 200 μL/minute flow rate, followed by a steeper gradient from 80% to 96% solvent B in 1 min were used for protein elution. Solvent B was held at 96% for 5 min for washing the column and returned to 1% solvent B for equilibration prior to the next sample injection. Solvent A consisted of 0.1% formic acid (aq) and solvent B contained 90/10 acetonitrile/ 0.1% formic acid (aq). The ionspray voltage was set to 5300V, declustering potential (DP) 100V, curtain gas flow 25, nebuliser gas 1 (GS1) 25, GS2 to 35, interface heater at 150^oC and the turbo heater to 450^oC. The mass spectrometer acquired 250ms full scan TOF-MS data over the

mass range 350-2200. The data was acquired and processed using Analyst TF 1.6 software (ABSCIEX, Canada). Digested Bcl2A1 was identified by database searching using ProteinPilot v4 (ABSCIEX) against the UniProt_Sprot_20110925 database (> 90% confidence identity to known Bcl2A1 sequence). MS-MS spectrum of digested protein was scanned for the inserted peptide sequence IAQELR sequence (also for FNAYYAR). A sole protein fragment was found and further characterized as fragment **4** (Figure 3A).

3. Fluorescence polarization (FP) binding assays

Preparation of Bcl2A1 protein sample
The untagged human Bcl2A1 protein (1 to 152 aa) was purchased from Abcam (ab151841). The commercial sample was diluted 4-fold to a concentration of 10 μM Bcl2A1 in buffer containing 50 mM Tris, 150mM NaCl, 1 mM EDTA, 50 mM TCEP, pH 7.2 and incubated in this TCEP buffer for 60 min on an ice bed prior to the binding assays.

FP assay conditions
FP measurements were performed at room temperature using a PHERAstar FS plate reader equipped with fluorescein excitation (490 nm) and emission (520 nm) filters. Samples were placed on a 384-well black plate (Corning) with a total volume in each well of 20 μL with a running buffer containing 50 mM Tris, 150mM NaCl, 1 mM EDTA, 2 mM TCEP, 0.005% Tween-20, pH 7.2.

Direct binding FBID to Bcl2A1
FBID (5 nM) was incubated with different concentrations of Bcl2A1 (two-fold dilutions ranging from 1 to 1000 nM) in running buffer. FP was measured after 1, 2 and 3h incubation and K_d and IC_{80} values for Bcl2A1 binding to FBID were determined by nonlinear regression analysis using Prism software 7.0 (GraphPad). The experiment was repeated three times.

Direct binding FBID to Bcl2A1 in the presence of unlabelled peptide
Bcl2A1 (500 nM) was incubated with unlabelled peptide (125, 250, 500 or 1000 nM) in running buffer at room temperature for 1h. Subsequently, each Bcl2A1/peptide sample was diluted in a series of two-fold dilutions and combined with FBID to a final concentration of 5nM FBID and 250-0.25 nM Bcl2A1 in running buffer. After 1h incubation, FP was measured and the binding curves were obtained using the Prism software. As a control, the same experiment was performed in the absence of unlabelled peptide. Fluorescence polarization was normalized, so that 0% mP refers to FP signal of unbound FBID ligand and 100% mP refers to maximal mP value achieved when ligand FBID is saturated with protein. The experiment was repeated three times.

Competition assays: disrupting Bcl2A1/FBID interaction by titration with unlabelled peptide
The IC_{80} value calculated from the direct binding assay was used to determine the Bcl2A1 concentration to be applied in the competition assays. Bcl2A1 (15 nM) was preincubated with FBID (5 nM) for 1h in running buffer and combined with a serial dilution of the peptide (ranging from 4 μM to 2 nM). After 2h incubation, fluorescence polarization was measured and IC_{50} values were determined by nonlinear regression analysis using Prism software 7.0. Fluorescence polarization was normalized, so that 0% mP refers to FP signal of unbound FBID ligand and 100% mP refers to the FP signal observed for the Bcl2A1/FBID complex in absence of competing peptide. Apparent K_i values were calculated from the observed competitive IC_{50} values after 2h incubation, K_d of FBID/Bcl2A1 interaction and known concentrations of labelled peptide and protein using a formula described in reference 4. The experiment was repeated three times.

Real-time kinetic curves for disruption of Bcl2A1/FBID interaction by unlabelled peptides
Bcl2A1 (500 nM) was incubated with FBID (10 nM) in running buffer for 30 min at room temperature and added to a solution of unlabelled peptide to a final concentration of 250 nM

Bcl2A1 250 nM, FBID 5 nM and peptide 500 or 1000 nM. After addition of the peptide to the Bcl2A1/FBID complex solution, the plate was immediately placed inside the plate reader and FP was recorded every minute over 3.5 hours. The time-course change in FP values was computed into the Prism software to determine the kinetic curve for the covalent binding of the peptide to the protein. As a control, the same experiment was performed in the absence of unlabelled peptide. The experiment was repeated three times.

Mass spectroscopy of intact Bcl2A1 conjugates
His6-tagged human Bcl2A1 protein (Novoprotein, C103) was diluted to a concentration of 10 µM Bcl2A1 in buffer containing 50 mM Tris, 150mM NaCl, 1 mM EDTA, 10 mM TCEP, pH 7.2 and incubated in this buffer for 30 min on an ice bed. Peptides were combined to a final concentration of 5 µM Bcl2A1 and 50 µM peptide in a total of 20 µL reaction volume. After 5h, the covalent reaction was analyzed by LC-MS on a Shimadzu Nexera uHPLC system coupled to a Triple TOF 5600 mass spectrometer (ABSCIEX) using Zorbax C18 column (Agilent).

4. Cell-based assays

Cell culture
U937 cells were cultured in RPMI supplemented with 105 FBS, 10 U/mL penicillin, 10 U/mL streptomycin, 2 mM L-glutamine, 2 mM NEAA and 1 mM HEPES.

Confocal live cell microscopy
U937 cells were treated with 1µM of **9** for 2h at 37°C followed by 30 min incubation with 50 nM MitoTracker Deep Red FM (Invitrogen) and 5µg/ml Hoechst (Invitrogen). Cells were washed and plated onto glass bottom poly-D-lysine coated dishes (MatTek Corporation). Confocal images were acquired using a Ziess LSM 710 FCS x63 objective lens.

Flow cytometry analysis of U937 uptake of peptides
U937 cells were treated with 10µM of fluorescein-labelled peptides for 4h at 37°C followed by washing and quenching by trypan blue to remove non-specific binding. Cells were then analysed by flow cytometry on a Gallios Flow Cytometer (Becton Dickson) and fluorescein-positive cells were quantified using FlowJo (Tree Star Inc).

U937 live cells and cell lysates covalent binding assay
U937 cells were plated at a density of 5 x 10^5 cells and treated with peptides in the presence of 5% FBS for 24h. After incubation, cells were washed and lysed with Cell Lysis Buffer supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). For cell lysate assays, U937 cells were lysed and peptides were added only to lysate at indicated concentration and incubated overnight at room temperature. Samples were reduced at 70°C for 5 min followed by western blot analysis using anti-Bcl2A1 (Cell Signaling Tech) and anti-GAPDH (Sigma Aldrich).

References
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(2) de Araujo, A. D.; Mobli, M.; King, G. F.; Alewood, P. F. *Angew. Chem. Int. Ed.* **2012**, *51*, 10298-10302.
(3) Wider, G.; Dreier, L. *J. Am. Chem. Soc.* **2006***, 128,* 2571 -2576.
(4) Nikolovska-Coleska, Z., Wang, R., Fang, X., Pan, H., Tomita, Y., Li, P., et al. *Anal. Biochem.* **2004**, *332*, 261-273. An online webtool is available to calculate Ki: http://sw16.im.med.umich.edu/software/calc_ki/.