Supplementary Material for:

Rapid Optimization of Mcl-1 Inhibitors using Stapled Peptide Libraries Including Non-Natural Side Chains

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1. Supplementary Methods

1.1 Synthesis of stapled peptide libraries

We used Tentagel Macrobead resin (200 μm, ~0.25 mmol g^{-1}, Rapp Polymere) with HMBA linker, which enables independent side chain de-protection and cleavage steps (under strongly acidic and basic conditions, respectively). Synthetic libraries were synthesized manually on a 0.1 mmol scale using standard Fmoc chemistry. For standard couplings, a four-fold excess of amino acids and coupling reagents (N,N'-diisopropylcarbodiimide (DIEA) / hydroxybenzotriazole (HOBT) 1:1) relative to resin loading was used. All couplings were performed as double couplings, with reactions run until completion as indicated by a negative Kaiser test. All α-4-pentenyl alanine residues as well as natural and unnatural side chains at randomized positions were activated by means of DIC / 1-hHydroxy-7-azabenzotriazole (HOAT) (ten-minute pre-activation). The molar excess of amino acid and coupling reagents was reduced for olefin bearing amino acids (S_5) to 1.5-fold for the first and 0.8-fold for the second coupling. Prior to deprotection, possibly non-acylated N-termini were capped by adding a mixture of acetic anhydride/ DIEA / dimethylformamide (DMF) (1:0.1:5) (3 x 10 min). Piperidine (20%) in DMF was used for Fmoc deprotection (4 × 10 min). After deprotection, the resin was washed with DMF (6 x 5 min) and, if necessary, the resin was split into separate pools for coupling different amino acids. The resin was then washed twice with DMF (6 x 5 min) and re-pooled if the resin had been split prior to coupling. The elongation cycle was repeated until the sequence was complete. Finally, all peptides were N-acetylated using a mixture of acetic anhydride/DIEA/DMF (1:0.1:5). The ring-closing metathesis reaction was carried out on resin-bound, fully protected peptides in 1,2-dichloroethane (DCE) at room temperature (20–25 °C) using Grubbs' first-generation catalyst.[1,2] The resin was washed with 1 ml of dichloromethane (DCM) (3 × 1 min) and with 1 ml of DCE (3 × 1 min) and then treated with 2-3 ml of a 6 mM solution of Grubbs' first-generation catalyst in DCE. After the first round of the 2 h metathesis, we repeated the same procedure for a second round of catalyst treatment with fresh catalyst solution. The resin was then washed with 1 ml of DCE (3 × 1 min) and then with 1 ml of DCM (3 × 1 min). The residual ruthenium catalyst interferes with fluorescence-based on-bead screening, therefore, it is removed from beads prior to the fluorescence based on-bead screening by complexing with water-soluble tris(hydroxymethyl)phosphine (THP)[3] and extracting the catalyst-THP complex with water. 5 mL of 1 M THP in isopropanol was added to 0.5 g of resin after metathesis reaction and stirred vigorously under nitrogen. After 12 h, the color of beads changed from purple brown to faint yellow. The beads were extensively washed with isopropanol and water. Before side chain deprotection, 3 mL of DCM was added and beads were vigorously stirred for 10 minutes. Finally, the beads were treated for 120 min with a side-chain deprotection cocktail (2.5:2.5:95) of water, tri-isopropylsilane and trifluoroacetic acid.

1.2 Screening of stapled peptide libraries

We optimized the bead-binding screen by trying various washing conditions and blocking reagents (BSA, gelatin, cell lysate). As reported also by Kodadek et al., we found the mixture of proteins present in an E. coli lysate to be a particularly effective blocking reagent.[4] Conditions that proved optimal, based on control experiments, were 20 mM Tris-Cl buffer (pH 8.0), 150 mM NaCl, 0.1% Tween-20, and 10 mg/ml cell lysate (prepared as described below); this buffer is called blocking buffer (BB) in the sections below. Screening for binders was performed as described below.

The resin was swollen overnight in DMF and then transitioned from DMF to aqueous buffer (20 mM Tris-Cl buffer (pH 8.0), 150 mM NaCl) gradually over the course of 2 hours. Then the entire amount of resin was split into 24-well low-binding plates. Beads were washed with BB by adding/removing buffer 3 times using flat-tip gel-loading pipette tips, which were found not to permit passage of swollen beads. Then, fresh BB was added, and beads were blocked in buffer for one hour. After removing buffer, a mixture of biotinylated target protein in the presence of excess myc-tagged competitor protein (pre-incubated in BB for 10 minutes) was added and beads were incubated for 2 hours. The c-myc-tagged Bcl-2 receptors without the C-terminal transmembrane region and without the N-terminal domain of Mcl-1 were expressed and purified as in Dutta et al.[5] The biotinylated Bcl-2 receptors were expressed and purified as in Foight et al.[6] Beads were then washed (5 x 2 min) with fresh buffer. Streptavidin-coated quantum dots (5 nM Qdots-SA605), also pre-incubated for 10 minutes in buffer, were added in 25 μL total volume and incubated for 30 minutes. Beads were then washed (5 x 2 min) with fresh buffer. Only beads bound to biotinylated protein can interact with Qdots-SA605, generating hits. Beads were visualized and collected manually with a pipet tip using a fluorescence microscope using the standard blue/green filter set (emission=515 nm, excitation=460-490 nm). Brightly glowing beads were picked manually with a pipet tip. Stapled peptides were cleaved from the resin using 1.0 M sodium hydroxide (10 min) and desalted using C18-packed 10 μL micropipette tips. MALDI mass spectra were obtained using a Brucker MALDI-TOF mass spectrometer.

1.3 Pre-screening with large-particle flow cytometer

For library LB, the library was sorted first with Union Biometrica BioSorter, a large-particle flow cytometer prior to fluorescence microscopy. Beads were poured into the sample cup at a density of about 50 beads/mL. Gating and sorting regions for bead sorter were defined using time-of-flight (TOF) to identify uniform sized beads and red fluorescence intensity (RED) to monitor quantum dot binding. The top 5% of bright beads of the expected size were collected. The selected beads were dispensed into 96-well plates. We used accompanying FlowPilot™ Software for data analysis. About 300 of the collected beads were then analyzed under a fluorescence microscope.

1.4 Preparation of cell lysate for screening experiments

E. coli BL21 cells were grown overnight and harvested by centrifugation at 4000 rpm. The cell pellet was suspended in sonication buffer (50 mM Tris buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and then 100 μl of phenylmethanesulfonyl fluoride (1 mg ml^{-1}) was added. The cells were centrifuged again and finally re-suspended in 10 ml of the same buffer. The cells were lysed by sonication and the soluble and insolubale fractions separated by centrifugation at 20000 rpm to remove cell debris. The clear cell lysate was dialyzed against 20 mM Tris buffer, pH 7.4, 150 mM NaCl, 0.1% Tween 20. The concentration of the lysate was estimated with Coomassie Plus protein assay reagent. BSA was used to plot a standard curve.

1.5 Synthesis of individual peptides

Peptides were synthesized on Rink amide resin LL (0.24 mmol g^{-1}) in a manner similar to that employed for the library construction except that split and pool steps were not necessary. Peptide cyclization was carried out in the same manner as used for library construction. Stapled peptides were all N-acetylated using a mixture of acetic anhydride/DIEA/DMF (1:0.1:5) after final Fmoc-deprotection. The peptides were cleaved

off the resin and deprotected by a cocktail including 95% Trifluoroacetic acid (TFA), 2.5% water and 2.5% tri-isopropylsilane. The crude peptides were purified by reversed-phase HPLC on a C18 column with a linear water:acetonitrile gradient and the identity of the peptides was confirmed by MALDI-TOF mass spectrometric analysis. For biotinylation, a spacer is desired, therefore a short PEG linker (Fmoc-NH-PEG5-CH2COOH) was added at the N terminus. The biotinylation reaction employs a 2 ml solution of 0.1 M biotin-OSu/0.4 M DIEA, stirring overnight. For derivatization of Bim peptide with fluorescein isothiocyanate (FITC), a β-alanine was added at the N terminus as a short linker. Then, capping with FITC was accomplished by stirring the resin overnight in 2 ml of 0.1 M fluorescein isothiocyanate (isomer I)/0.2 M DIEA.

1.6 CD measurements

CD spectra were recorded on an AVIV model 420 CD spectrometer equipped with a temperature controller using 1 mm pathlength cells and a scan speed of 5 nm/min. The spectra were averaged over 5 scans with the baseline subtracted based on buffer controls. The samples were prepared in 20 mM Tris buffer pH 7.4, with the final peptide concentration 20 μM. The concentrations of unfolded peptides were determined by the UV absorption, in 6.0 M guanidine hydrochloride aqueous solution. Ellipticity was normalized to concentration (c [mol L^{-1}]), number of residues and path length using:

$$[\theta] = [\theta]_{obs} / (10000 \times l \times c \times n)$$

where $[\theta]_{obs}$ is the measured ellipticity in millidegrees and $[\theta]$ the normalized ellipticity in 10^3 deg cm^2 $dmol^{-1}$ $residue^{-1}$. Each sample was prepared and measured three times and baseline corrected. Fractional helical content was calculated from the mean residue molar ellipticity at 222 nm and the number of backbone amides using the equation:

percent helical content= $[\theta]_{222}/(-44000 + 250T)(1-3/n) \times 100$, where n is the number of amino-acid residues in the peptide and T is the temperature in degrees Celsius.[7]

1.7 Fluorescence Polarization Assay

Competition fluorescence polarization assays were performed by titrating 23-mer unlabeled peptides over a concentration range of 0-10 μM. A 21-mer variant of N-terminally fluoresceinated Bim peptide (F-IWIAQELRRIGDEFNAYYARR) was used as the competitor peptide. In the competition assay, receptor concentrations were 50 nM, and fluoresceinated Bim was at 25 nM, in a final volume of 100 μl. Plates were mixed and incubated at 25 °C for 2 hours. Experiments were done in quadruplicate. Competition fluorescence anisotropy data were fit to a complete competitive binding model (equation 17 in Roehrl et al., 2004) using Prism.[8,6]

1.8 Bio-layer Interferometry

All experiments were performed using an Octet Red 96 System (ForteBio Inc.) Super Streptavidin (SSA) sensors were pre-wetted in PBS containing 1% [w/v] BSA, 0.05% [v/v] Tween-20 and 2% (v/v) DMSO, pH 7.5 for 10 min prior to use. Biotinylated stapled peptides (see above) at a concentration of 0.1 mg mL^{-1} were loaded on biosensors. Mcl-1 at 3 concentrations ranging from 40 to 400 nM was bound to Mcl-1 for 10 min and then allowed to dissociate in phosphate buffer over 15 min. Negative control runs were performed as above except that no biotinylated peptide was loaded onto the sensors. The capture levels for controls were less than 5% of the signal from experimental

samples using concentrations of Mcl-1 ranging from 40 to 400 nM. Binding parameters were fit using the accompanying Octet Software version 8.2 assuming 1:1 binding.

1.9 Protease degradation assay

The proteolytic stability of peptides toward α-chymotrypsin (from bovine pancreas, 40.0 units mg^{-1}) was analyzed by analytical HPLC. The amount of intact peptide was quantitated by serial injection over time. Protease reaction samples contained 20 μM peptide in 10 mM phosphate buffer pH 7.4 plus chymotrypsin (0.5 ng μL^{-1}). Aliquots of 5 μL were removed at fixed time points and quenched with 95 μL acetonitrile containing 0.1% TFA. All samples were immediately subjected to analytical HPLC.

1.10 Cellular BH3 profiling assay

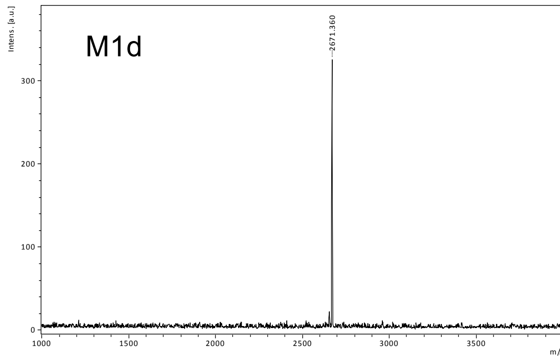
Peptides were serially diluted into plates from 200 μM to 0.2 nM using 10-fold dilutions in DTEB (Derived from Trehalose Experimental Buffer: 135 mM trehalose, 50 mM KCl, 20 μM EDTA, 20 μM EGTA, 0.1% BSA, 5mM succinate, 10 mM HEPES-KOH pH 7.5) containing 0.005% w/v digitonin, 10 mM 2-mercaptoethanol, 2 μM JC-1, and 20 μg mL oligomycin. Three replicate wells for each peptide, for each cell line, were prepared by adding 15 μL of the peptide dilutions to each well of a black, untreated 384-well plate. Control wells containing no peptide or 20 μM FCCP (carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone, a chemical uncoupler of oxidative phosphorylation) were included for zero and complete depolarization, respectively. Multiple plates were produced from the same stock and frozen at −80°C for later use. Frozen plates were brought to RT prior to use, cells were suspended in DTEB at a density of 1.34×10^6 cells/mL, and 15 μL of cell suspension was added to each well of the dilution series to yield wells ranging from 0.1 nM to 100 μM peptide with 20000 cells/well. Fluorescence of JC-1 aggregates was measured at 590 nm with 545 nm excitation on a Tecan Safire2 at 5 min intervals for 3 h. The area under each signal-vs-time curve was normalized to the untreated and FCCP values to produce the fractional depolarization. Curves were plotted as log [peptide] vs. percent depolarization, and dose−response curves were fit using Graphpad PRISM 6. For curves without an upper baseline, an upper limit on the EC_{50} was estimated by fitting the curve with a complete upper baseline.

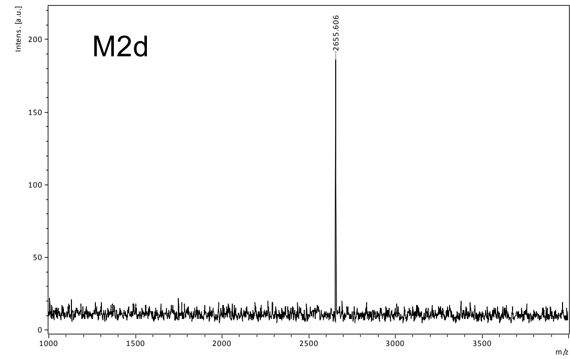
1.11 Sequence decoding by MALDI-TOF mass spectrometry

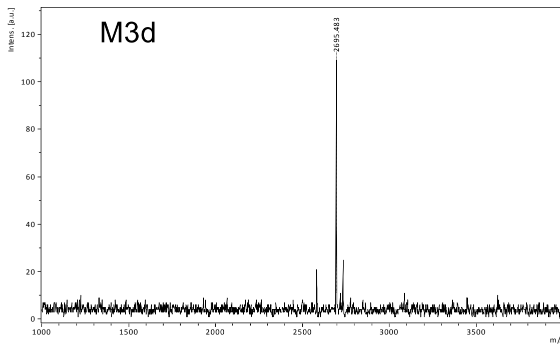
After screening, stapled peptides were cleaved from the bead using 1.0 M sodium hydroxide followed by acidification using formic acid. The peptides were desalted using C18-packed 10 μL micropipette tips (ZipTips, Millipore) according to the manufacturer instructions. Each peptide sequence was determined according to its unique mass. All peptides in the library differed by at least 0.5 Dalton from other library members. MALDI mass spectra were obtained using 1:1 mixtures of the cleaved sample with α-cyano-4-hydroxycinnaminic acid (CHCA) matrix on a Bruker MALDI-TOF mass spectrometer. Below are the mass spectra of the best binders, M1d, M2d, M3d, M4d, M5d and M6d, as determined by MALDI mass spectrometry, along with their predicted theoretical masses.

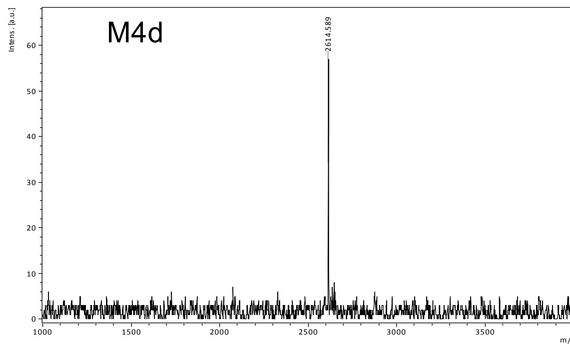
Peptides	Sequences	Theoretical $[M+H]^+$
M1d	Ac-IWBTQGLRRLGDEIxAYYxRR	2671.4051
M2d	Ac-IWBJQGLRRLGDEIxAYYxRR	2655.4669
M3d	Ac-IWBJQGZRRLGDEIxAYYxRR	2695.6123
M4d	Ac-IWBJQGLDRLGDEIxAYYxRR	2614.3928
M5d	Ac-IWBJQGLQRLGDEIxAYYxRR	2627.4244
M6d	Ac-IWBTQGLDRLGDEIxAYYxRR	2630.3310

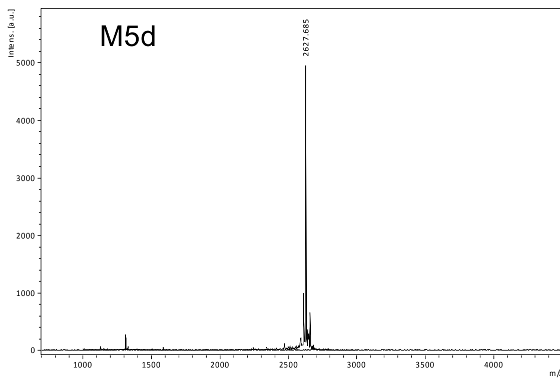
J: Aib, B: Nle, Z: Cha x: α-4-pentenyl alanine

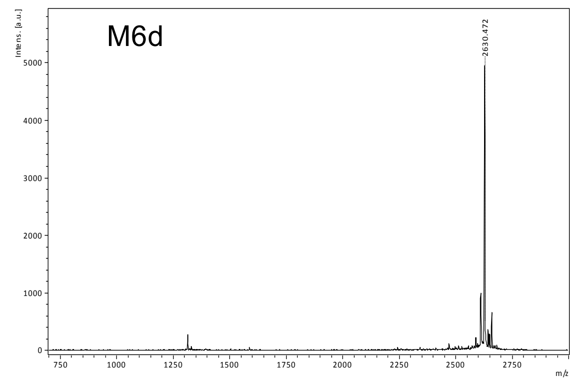










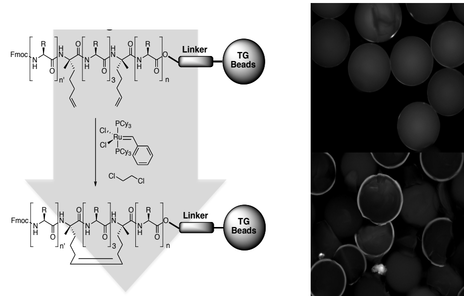


2. Supplementary Results and Analysis

2.1 On-bead screening of control stapled peptides

We determined optimized screening conditions as well as the optimal peptide loading ratio using resin-linked control peptides with known affinities for binding to Mcl-1. We applied biotinylated Mcl-1 to the peptide-displaying beads and then detected bound protein with fluorescent streptavidin-coated quantum dots. Stewart et al. have identified a series of Mcl-1-binding peptides, including Mcl-1 BH3 SAHBa (stapled) that binds to Mcl-1 with K_d = 43 nM, and Mcl-1 BH3 peptide (unstapled) that binds with K_d = 245 nM.[9] Decreasing the concentration of biotinylated Mcl-1 and increasing the concentration of cell lysate as blocking reagent led to a decrease in overall signal of beads carrying stapled peptides, which helps resolve differences between resin-linked peptides with different binding, despite the narrow range for their K_d values. (See inset figure: Mcl-1 BH3 (top) and Mcl-1 SAHBa (bottom) micrographs).

Mcl-1 BH3 KALETLRRVGDGQRNETAF
Mcl-1 SAHBa KALETLRS_5VGDS_5QRNETAF
S_5= α-4-pentenylalanine



2.2 First generation library LA

The first-generation combinatorial library, library LA, was generated to establish the feasibility of our strategy and optimize our protocols. LA served as a proof of principle prior to making and screening of the library B (LB).

Library LA was based on a stapled variant of the BH3 sequence of Bim.[10] We replaced the 3c and 3g positions with crosslinked α-4-pentenylalanine groups, and sampled residues near the stapled positions. The library included point mutations, and combinations of mutations, that have been shown to either be supportive or disruptive of binding to Mcl-1 or Bcl-x_L (see below). Library LA included 2 amino-acid choices varying the residue size and/or charge at 6 positions near the staple, leading to 128 peptides for screening. LA was synthesized at 0.1 mmol scale, using 333 mg of resin including ~53,333 beads. Assuming conditions of random mixing and equally efficient synthesis, there were approximately 416 copies of each stapled peptide in the library.

Both the stapling chemistry and subsequent tests for binding were carried out while peptides were bound to the resin, with one unique peptide per bead. Beads were incubated with Mcl-1 and/or Bcl-x_L (receptors differently labeled), and beads with high fluorescence under the microscope were collected by pipette, cleaved from the bead using 1.0 M sodium hydroxide (10 min), desalted using C18-packed 10 μL micropipette tips and finally subjected to sequence decoding by MALDI-TOF. The libraries were designed such that each peptide had a unique mass with a difference of at least 0.5 Dalton from other library member, to allow for facile and accurate identification of sequences.

Sequences of peptides identified as binders (found more than once in the hit pool) from LA were in excellent agreement with the expected impact of known mutations (see sequence logos 1 and 2 below). Successful isolation of tight binders was further confirmed by fluorescence polarization binding assays.

Randomized positions

2d$^{I/F}$, 2e$^{A/T}$, 2g$^{E/R}$, 3b$^{R/D}$, 3e$^{G/E}$, 4a$^{V/F}$, 4e$^{Y/K}$

1. Screening LA for binding to biotinylated Mcl-1 in the presence of excess amount of unlabeled Bcl-x_L.

Screening condition: 0.2 μM ^{Bio}Mcl1 and 2.5 μM myc Bcl-x_L

Peptide	# Beads	Sequence																					
				2d	2e		2g		3b			3e			4a				4e				
A1	22	I	W	I	T	Q	E	L	R	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A
A2	16	I	W	I	T	Q	E	L	D	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A
A3	13	I	W	I	A	Q	E	L	D	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A
A4	11	I	W	F	A	Q	E	L	D	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A
A5	9	I	W	I	A	Q	R	L	R	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A
A6	9	I	W	I	T	Q	R	L	R	X	I	G	D	X	V	N	A	Y	K	A	R	R	A
A7	6	I	W	F	T	Q	E	L	R	X	I	G	D	X	V	N	A	Y	K	A	R	R	A
A8	5	I	W	I	T	Q	E	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
A9	5	I	W	F	T	Q	E	L	D	X	I	G	D	X	F	N	A	Y	K	A	R	R	A

2. Screening LA for binding to biotinylated Bcl-x_L in the presence of excess amount of unlabeled Mcl-1.

Screening condition: 0.2 μM ^{Bio}Bcl-x_L and 2.5 μM myc Mcl-1

Peptide	# Beads	Sequence																					
				2d	2e		2g		3b			3e			4a				4e				
B1	16	I	W	F	A	Q	E	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
B2	13	I	W	I	A	Q	E	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
B3	12	I	W	F	A	Q	R	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
B4	10	I	W	I	T	Q	R	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
B5	8	I	W	I	A	Q	E	L	R	X	I	G	D	X	F	N	A	Y	Y	A	R	R	A
B6	7	I	W	F	A	Q	R	L	R	X	I	G	D	X	F	N	A	Y	Y	A	R	R	A
B7	7	I	W	F	T	Q	R	L	R	X	I	G	D	X	F	N	A	Y	K	A	R	R	A
B8	3	I	W	F	A	Q	E	L	D	X	I	G	D	X	V	N	A	Y	Y	A	R	R	A

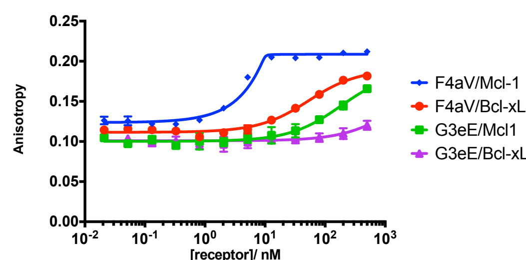
We included mutations in library LA at positions where prior studies provided information about what we could expect, and compared the results of our screen to prior observations:[11,6,5,10]

1. 2e: at position 2e, Mcl-1 accommodates a range of amino acids, including Val, Pro and Thr. In contrast, Bcl-x_L has a strong preference for Gly and Ala.[6,7] We mutated this position with Ala and Thr and found, as expected, that Ala was selected more frequently than Thr when screening for selective Bcl-x_L binding, with the reverse preference observed when screening for Mcl-1 selective binding.
2. 2g: mutation of Glu to Arg at 2g disfavors Mcl-1 binding but not Bcl-x_L binding, as assessed using peptide SPOT arrays.[5] Our screening results confirm a preference for Glu over Arg at 2g for stapled peptides binding to Mcl-1 that is not seen for binding to Bcl-x_L.
3. 3b: Glu or Asp at position 3b are less destabilizing for binding to Mcl-1 than binding to Bcl-x_L.[11,5,10] We mutated this position with Arg and Asp. In screening,

3b was conserved as Arg in peptides that bound to Bcl-x_L, but we found both Asp and Arg in selective Mcl-1 binders.

4. 3e: position 3e, which is typically occupied by small amino acids in native BH3 sequences, does not tolerate substitution with residues larger than Gly, Ala or Ser, for either Mcl-1 or Bcl-x_L binding.[5] We mutated this position to Gly or Glu. All peptides identified as binding to both Mcl-1 or Bcl-x_L had Gly at position 3e.
5. 4a: Bcl-x_L exhibits a preference for large hydrophobic residues at 4a, whereas Mcl-1 binding tolerates many other subsitutions.[5] Given a choice between Val and Phe, position 4a was restricted to Phe in stapled peptides binding to Bcl-x_L. Selective binders of Mcl-1 showed a strong preference for Val, consistent with prior work.[5]
6. 4e: Mutation of Tyr to Lys at 4e is known to favor Bcl-x_L binding over Mcl-1 binding.[11] We also observed this preference for stapled peptides identified as binding selectively to Bcl-x_L (sequence logo 2).

These results were further confirmed by affinity measurement of selected variants in solution. Two examples are described here.



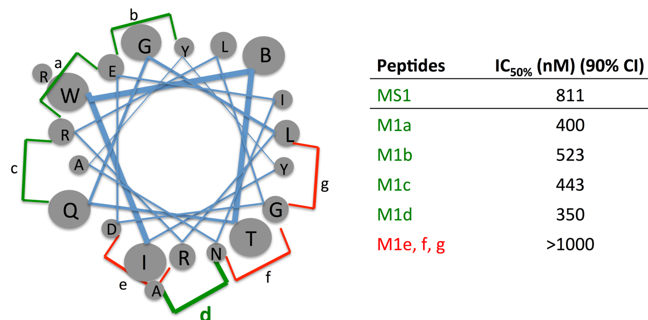
As indicated in sequence logo 1 (screening with Mcl-1), we observed a significant preference for Val over Phe at position 4a and an absolute selection for Gly over Glu at position 3e. Therefore, we synthesized BimSAHBa-G3eE and BimSAHBa-F4aV and tested their binding by fluorescence polarization. BimSAHBa-F4aV binds tightly to Mcl-1 compared to Bcl-x_L. As expected, G3eE is a disruptive mutation for binding of stapled peptides to both Mcl-1 and Bcl-x_L. These preliminary library-screening results demonstrated the selection of side chains that are known to be supportive for Mcl-1 binding and confirmed the efficacy of modified libraries for affinity-based design of new stapled peptides.

3. Supplementary Figures and Tables

Table S1. Sequences and masses of BH3 peptides used for solution assays

Peptide	Sequence	Mass exp.
FITC-Bim 21mer	FITC-βA-IWIAQELRRIGDEFNAYYARR-NH_2	3098.7
MS1	Ac-RPEIWMTQGLRRLGDEINAYYAR-NH_2	2848.6
M1a	Ac-IWBTQGLRS_5LGDS_5INAYYARR-NH_2	2570.2
M1b	Ac-IWBTQGLRRLGDS_5INAS_5YARR-NH_2	2563.9
M1c	Ac-IWBTS_5GLRS_5LGDEINAYYARR-NH_2	2571.5
M1e	Ac-IWBTQGLS_5RLGS_5EIN AYYARR-NH_2	2583.8
M1f	Ac-IWBTQGLRRLS_5DEIS_5AYYARR-NH_2	2683.3
M1g	Ac-IWBTQGS_5RRLS_5DEINAYYARR-NH_2	2684.1
M1d	Ac-IWBTQGLRRLGDEIS_5AYYS_5RR-NH_2	2669.1
M2d	Ac-IWBJQGLRRLGDEIS_5AYYS_5RR-NH_2	2654.5
M3d	Ac-IWBJQGZRRLGDEIS_5AYYS_5RR-NH_2	2693.1
Biotin-PEG-M1d	Biotin-PEG-IWBTQGLRRLGDEIS_5AYYS_5RR-NH_2	3190.6
Biotin-PEG-M2d	Biotin-PEG-IWBJQGLRRLGDEIS_5AYYS_5RR-NH_2	3174.8
Biotin-PEG-M3d	Biotin-PEG-IWBJQGZRRLGDEIS_5AYYS_5RR-NH_2	3214.8
FITC-BimSAHBa-F4aV	FITC-βA-IWIAQELRS_5IGDS_5VNAYYARR-NH_2	3016.4
FITC-BimSAHBa-G3eE	FITC-βA-IWIAQELRS_5IEDS_5FNAYYARR-NH_2	3135.78

S_5= α-4-pentenyl alanine, J= Aminoisobutyric acid (Aib), Z= Cyclohexyl alanine (Cha), B= Norleucine (substituted for methionine to optimize activity of the ruthenium catalyst)



Peptides	$IC_{50\%}$ (nM) (90% CI)
MS1	811
M1a	400
M1b	523
M1c	443
M1d	350
M1e, f, g	>1000

Figure S1. Constructs with varying staple positions. Staples that impaired binding are in red and those that improved the binding to ^{myc}Mcl-1 are in green. The staple in M1d is highlighted in bold.

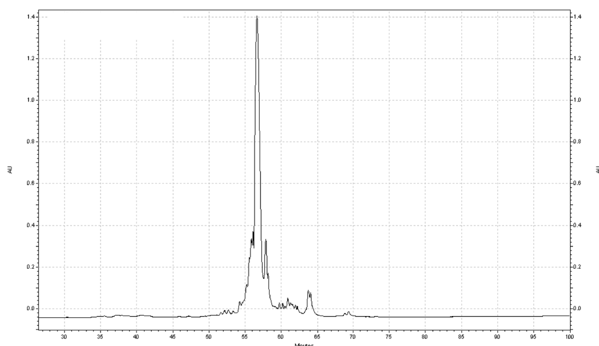


Fig. S2. Representative HPLC chromatogram of the products obtained upon cleaving a pool of 50-100 beads after synthesis using optimized library synthesis protocols.

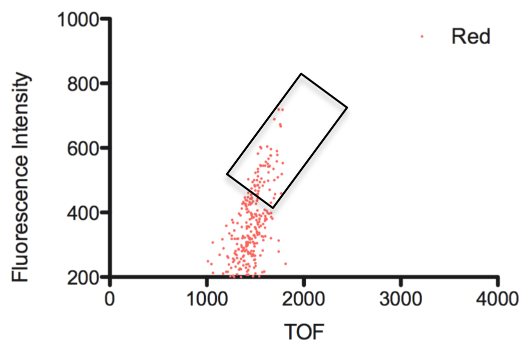


Fig. S3. Sorting beads on bead sorter using time-of-flight (TOF) to sort uniform sized beads and red fluorescence intensity (RED) to detect binding to Mcl-1. The gating and sorting region was chosen to collect beads with the highest 5% fluorescence signal.

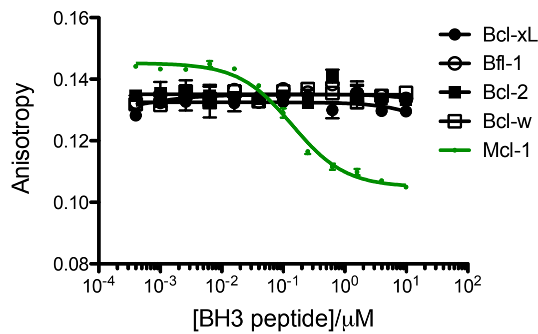


Figure S4. Competition fluorescence anisotropy experiments. Unlabeled M2d was mixed with fluoresceinated Bim BH3 and one of Mcl-1, Bfl-1, Bcl-w, Bcl-x_L or Bcl-2. Error bars show the standard error of four replicates.

Figure S5. Comparisons of modeled structures of M3d, MS1 and stapled peptide Mcl-1-SAHB$_D$ (3MK8) bound to Mcl-1. (Left) View of the p2 pocket of Mcl-1-SAHB$_D$ (pale yellow, PDB 3MK8) and M3d (magenta, model based on 3MK8). Mcl-1:SAHB$_D$ has a leucine at this site and Mcl-1:M3d has Cha. (Right) Area around position 2e in models of MS1 (pale yellow, model based on 3MK8, threonine residue at 2e) and M3d (magenta, also a model based on 3MK8, Aib residue at 2e).

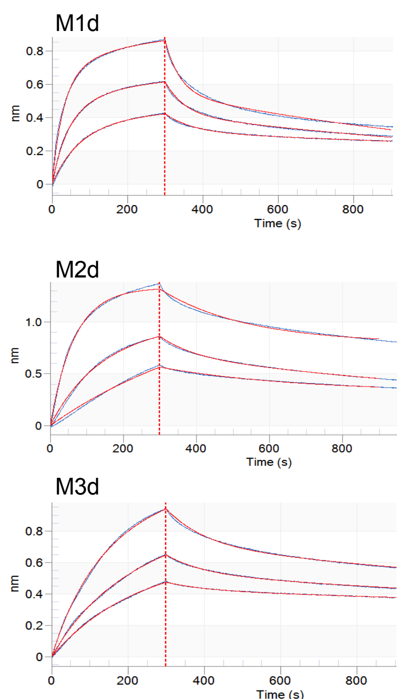


Table S2. Kinetic binding data for stapled peptides.

	k_{on} (M^{-1} S^{-1})× 10^4	k_{dis} (s^{-1}) × 10^{-4}	K_d (nM)
M1d	21.2 ± 0.7	32.4 ± 0.1	15.3 ± 0.1
M2d	6.5 ± 0.02	5.81 ± 0.008	8.9 ± 0.5
M3d	3.5 ± 0.007	1.8 ± 0.002	5.1 ± 0.3

Figure S6. Fitting of the kinetics for 1:1 binding of M1d, M2d and M3d to Mcl-1. Fits were done using Octet Software version 8.2.

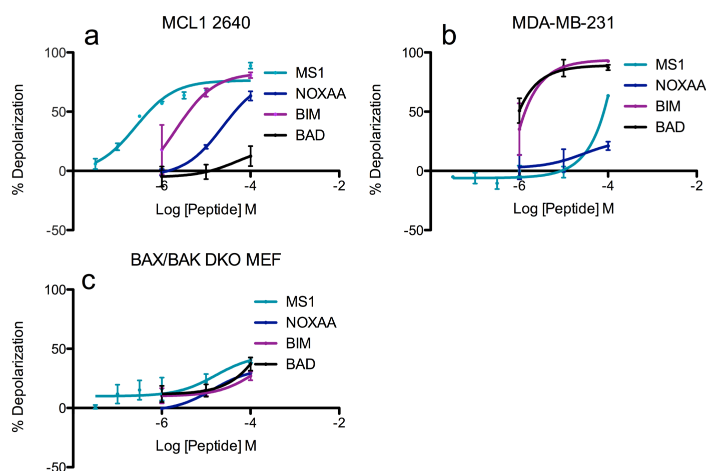


Figure S7. BH3 profiling of cell lines using engineered (MS1) and native BH3 peptides (Bim, NoxaA). Mcl-1 2640 cell line is dependent on Mcl-1 whereas MDA-MB-231 is dependent on Bcl-x_L, as indicated by response to NoxaA and Bad, respectively. BBDL is a Bax/Bak deficient leukocyte. Error bars indicate the standard deviation over 3 or more replicates.

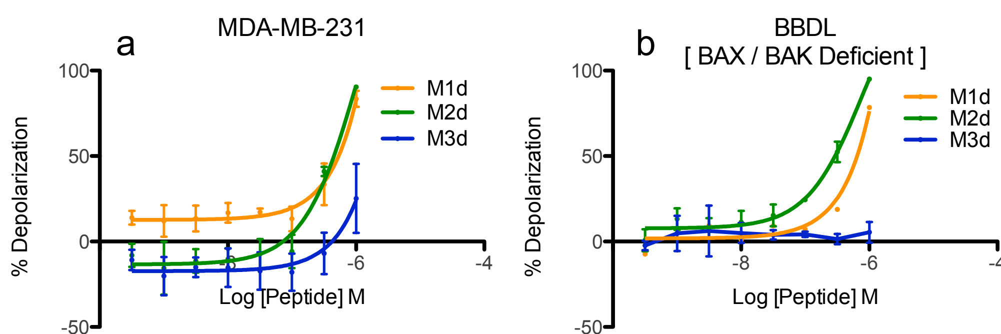


Figure S8. a) BH3 profiling of stapled BH3 peptides M1d, M2d and M3d in MDA-MB-231 cells. The cell line is dependent on Bcl-x_L as determined by BH3 profiling with target-specific BH3-only peptides. b) BH3 profiling of stapled BH3 peptides M1d, M2d and M3d in BBDL cell line, which is a Bax/Bak deficient leukocyte. Error bars indicate the standard error over 3 or more replicates.

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