Supporting Information for

Rational Design of Proteolytically Stable, Cell-Permeable Peptide-Based Selective Mcl-1 Inhibitors

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General Information

4, 4'-Bisbromomethyl-biphenyl (Bph) was purchased from TCI America and used directly in the cross-linking reactions. All other chemicals and solvents were obtained from commercial sources and used without further purification. ^{1}H NMR spectra were recorded with Inova-500 MHz NMR spectrometer. Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system. Peptides were purified using a Gilson semi-preparative reverse-phase HPLC system equipped with a Phenomenex C_{18} column with a flow rate of 5 mL/min and a gradient of 10-100% ACN/H_2O containing 0.1% formic acid while monitoring at 220 nm and 254 nm. Analytical HPLC were performed using Phenomenex Luna C_{18} or Kinetex C_{18} column (250 × 4.6 mm) with flow rate set at 1.0 mL/min and UV detection set at 220 and 254 nm. Live cell microscopy was performed on a Zeiss LSM-510 meta-NLO system equipped with a Coherent Chameleon Ultra II Ti/Sapphire laser and an external non-descanned detectors. HRMS was performed with an in-house ESI-FTICR mass spectrometer.

Expression and Purification of mMcl-1

BL21DE3 cells bearing pGEX-6P1-GST-mMcl-1-(152-308) plasmid were allowed to grow in 2 L LB medium containing 100 μg/mL ampicillin at 37 °C to OD = 0.8, at which point 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce GST-mMcl-1 expression. The protein expression was allowed to proceed for additional 6 hours before the cells were harvested by centrifugation. The cell pellets were re-suspended in lysis buffer (10 mL/g of cells) containing

1× PBS, 300 mM NaCl, 2 mM DTT, 10 mM $MgCl_2$, 0.5 mM EDTA and 0.5% Tween-20. Then lysozyme (1 mg/mL cells) and DNAse (5-10 U) were added to the re-suspended cell pellets, and the mixture was incubated on ice for 30 min. The cell suspension was passed through a French press before centrifugation at 16,000 rpm for 30 min. The supernatant was collected and loaded to a GST column equilibrated with 10 CV of equilibration buffer containing 1× PBS, 150 mM NaCl and 2 mM DTT. The column was washed with 5-10 CV of equilibration buffer followed by 5 CV of elution buffer containing 50 mM Tris, 150 mM NaCl, and 15 mM reduced gluthathion, pH 8.0. The pure fractions containing GST-mMcl-1 were pooled and desalted through dialysis in a desalting buffer containing 20 mM Tris, 1 mM EDTA, and 2 mM DTT. The protein concentration was determined by Bradford assay to be 6.6 mg/mL, which gave a soluble yield of 5.8 mg/L 1 OD culture. The protein concentration was adjusted to 1 mg/mL with desalting buffer before addition of PreScission protease (10 units of protease per 1 mg of GST fusion protein), and the cleavage reaction was allowed to proceed at 4 ºC overnight. The mixture was then applied ÄKTA FPLC (GE Biosciences) equipped with 1 mL MonoQ column and applied a linear gradient of 0-100% buffer B (buffer A: 20 mM Tris; buffer B: 20 mM Tris + 1 M NaCl) over 60 min with a flow rate of 1 mL/min. The desired mMcl-1 was recovered in the flow-through fractions and was found to be very pure (see Figure S1). The protein was concentrated to 8.1 mg/mL and used in crystallization trial setup.

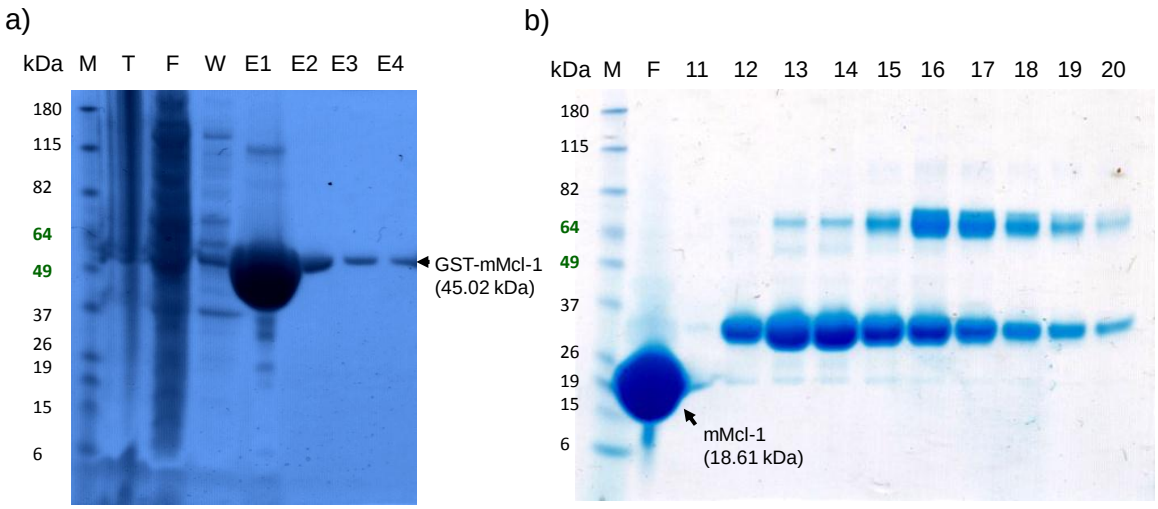


Figure S1. Expression and purification mMcl-1. (a) SDS-PAGE gel of GST-mMcl-1 after GST affinity chromatography: T, total cell lysate; F, flow through; W, washing fraction; E1-E4;

elution fractions. (b) SDS-PAGE gel after FPLC: F, flow through; 11-20 fractions. The desired GST-cleaved mMcl-1 protein band is indicated.

Crystallization and Structure Determination

The mMcl-1 protein was co-crystallized with the cross-linked Noxa-BH3 peptide **2** using conditions optimized from an in-house sparse matrix screen.[S1] The ligand was incubated with the protein at 1.5× molar excess for 45 minutes on ice prior to crystallization setup. Crystals were grown at 20 °C by hanging drop diffusion using a 1:1 mixture of protein and precipitant containing 10-20% PEG 8000, 2% ethylene glycol, 150-350 mM $CaCl_2$, and 50 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS) (pH 8.5). Crystals were mounted in nylon loops and cryoprotected by transferring through three solutions of the precipitant solution supplemented with increasing concentrations of MPD (8%, 16%, 24%) for approximately 30 seconds each and stored in liquid nitrogen. The final cryoprotectant solution contained 24% MPD, 20% PEG 8000, 350 mM $CaCl_2$, 50 mM HEPPS (pH 8.5), and 0.5 mM cross-linked Noxa-BH3 peptide **2**.

Data were collected remotely with BLU-ICE software[S2] at SSRL Beamline 9-2. The mMcl-1/ Noxa-BH3 peptide **2** dataset was solved with MOLREP[S3] using the crystal structure 2NLA as a search model. The model was subjected to iterative rounds of model building (COOT[S4]) and refinement (REFMAC5[S5]) until completion. Diffraction and refinement statistics are presented in Table S2.

Table S1. ESI-MS characterization of the chemically modified Noxa BH3 peptides.

Peptide	Sequence	Mass calculated m/z, $[M+2H]^{2+}$	Mass found m/z $[M+2H]^{2+}$
Noxa	Ac-AAQLRRIGDKVNLRQKLLN	1124.6262	1124.6882
1	Ac-AAC'LRRIGDC'VNLRQKLLN[a]	1188.6033	1188.6592
2	Ac-AAc'LRRIGDC'VNLRQKLLN[b]	1188.6133	1188.6636
3	Ac-AAc'LRAIGDC'VNLRQKLLN	1145.8219	1145.8763
4	Ac-AAc'LRAIGDC'VNLAQKLLN	1103.5545	1103.5944
5	Ac-AAc'LRAIGDC'VNLAQALLN	1074.5068	1074.5574
6	Moc-A_mAc'LRRIGDC'VNLRQKLLN[c]	1204.1069	1204.1682
7	Moc-A_mA_mc'LRRIGDC'VNLRQKLLN	1211.1126	1211.1748
8	Moc-A_mA_mc'LRAIGDC'VNLAQALLN	1097.5161	1097.5705
Fluo-Noxa	Fluorescein-Ahx-AAQLRRIGDKVNLRQKLLN[d]	1354.7014	1354.7487
Fluo-**1**	Fluorescein-Ahx-AAC'LRRIGDC'VNLRQKLLN	1419.1985	1419.2171
Fluo-**2**	Fluorescein-Ahx-AAc'LRRIGDC'VNLRQKLLN	1419.1985	1419.2198
Fluo-**5**	Fluorescein-Ahx-AAc'LRAIGDC'VNLAQALLN	1305.2084	1305.2479
Fluo-**8**	Moc-A_mA_mc'LRAIGDC'VNLAQALLNK(Fluorescein)[e]	1355.6134	1355.6466

[a] C' denotes Bph-linked L-cysteine; Ac = acetyl; all peptides were amidated at C-termini. [b] c' denotes Bph-linked D-cysteine. [c] Moc = methoxycarbonyl; A_m = N-methyl-Ala. [d] Ahx = 6-aminohexanoic acid. [e] Fluorescein was attached to the ε-amino group of lysine.

Table S2. Statistics for mMcl-1 in complex with cross-linked Noxa-BH3 peptide **2**

Wavelength (Å)	0.97945
Resolution (Å)	2.00
Space Group	$P2_12_12_1$
Unit Cell parameters (Å)	
a	42.04
b	47.97
c	68.60
R_{merge}[a](%)	8.5 (28.7)
Completeness[a]	99.5 (99.4)
I/σ[a]	13.7(6.1)
No. observations	67192
No. reflections	9295
Refinement resolution (Å)	39.3-2.0
R-factor[a] (%)	19.1 (20.1)
R-free[a] (%)	23.6 (27.9)
Wilson *B*-value($Å^2$)	27.3
Average *B*-factor	
Overall ($Å^2$)	27.6
Solvent ($Å^2$)	33.4
Ligand ($Å^2$)	26.8
RMS deviation from ideal	
Bond lengths (Å)	0.022
Bond angles (^{o})	1.84

[a] The value in parentheses represents the statistics within the highest resolution shell

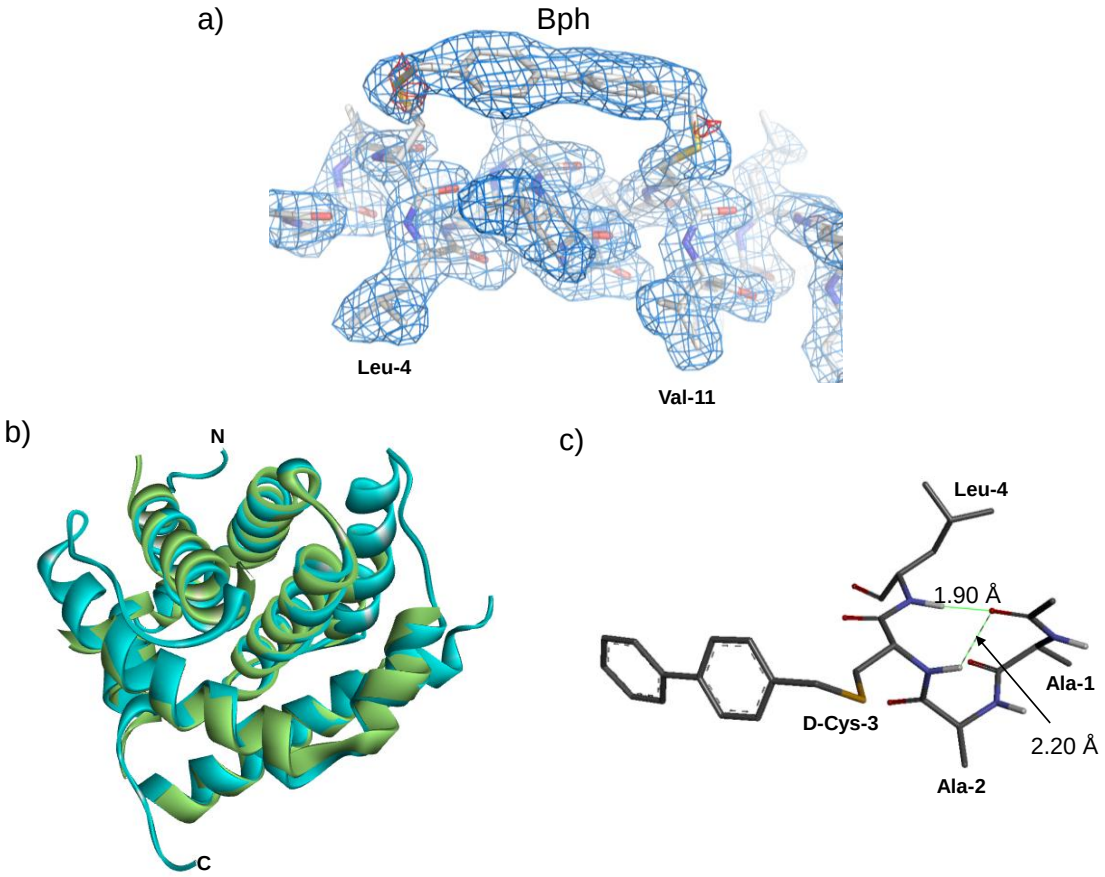
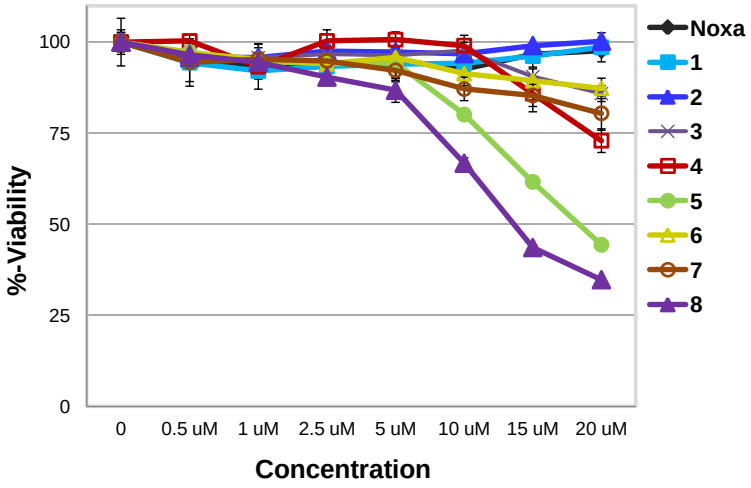


Figure S2. (a) Refined electron density surrounding the peptide and the cross-linker. 2Fo-Fc electron density is shown in blue, contoured at 1σ and Fo-Fc density is shown in red contoured at 3σ.The side chain of Arg6 was removed for clarity. (b) The ribbon model of mMcl-1 structure of determined in this study (colored in green) was superimposed with a previously determined mMcl-1 NMR structure (2JM6, colored in cyan). (c) The N-terminus of Bph-cross-linked Noxa peptide **2** showing a pair of intramolecular hydrogen bonds between acetyl C=O and the N-H's of D-Cys-3 and Leu-4. The N-H groups of Ala-1 and Ala-2 are solvent-exposed.

Table S3. Determination of K_d values by direct FP assay using fluorescein-labeled peptides. The peptides were dissolved in PBS buffer, pH 7.5, containing 5% DMSO and 0.005% Tween-20.

Peptide	GST-Mcl-1 K_d (nM)	GST-Bcl-xL K_d (nM)
Bim	12.4 ± 0.2	8.6 ± 1.6
Noxa	6.7 ± 1.0	> 1,000
Fluo-1	4.9 ± 1.5	> 1,000
Fluo-2	3.4 ± 0.2	> 1,000



Peptide	*%-Viability at 20 μM*	*EC_{50} (N = 3)*
Noxa	97.6 ± 0.9	> 20 μM
1	98.6 ± 4.0	> 20 μM
2	100.3 ± 0.2	> 20 μM
3	85.9 ± 2.2	> 20 μM
4	72.9 ± 3.2	> 20 μM
5	44.3 ± 0.2	18.2 μM
6	87.3 ± 2.8	> 20 μM
7	80.5 ± 4.7	> 20 μM
8	34.8 ± 0.5	13.4 μM

Figure S3. Determination of cell-killing activities of the various Noxa peptides using ATP assay: The Mcl-1-overexpressing U937 cells cultured in DMEM medium supplemented with 5% FBS were treated with the indicated peptide over a concentration range of 0-20 μM for 48 hours, and the cell viability was determined using the CellTiter-Glo reagent (Promega). The calculated EC_{50} values were collected in the Table.

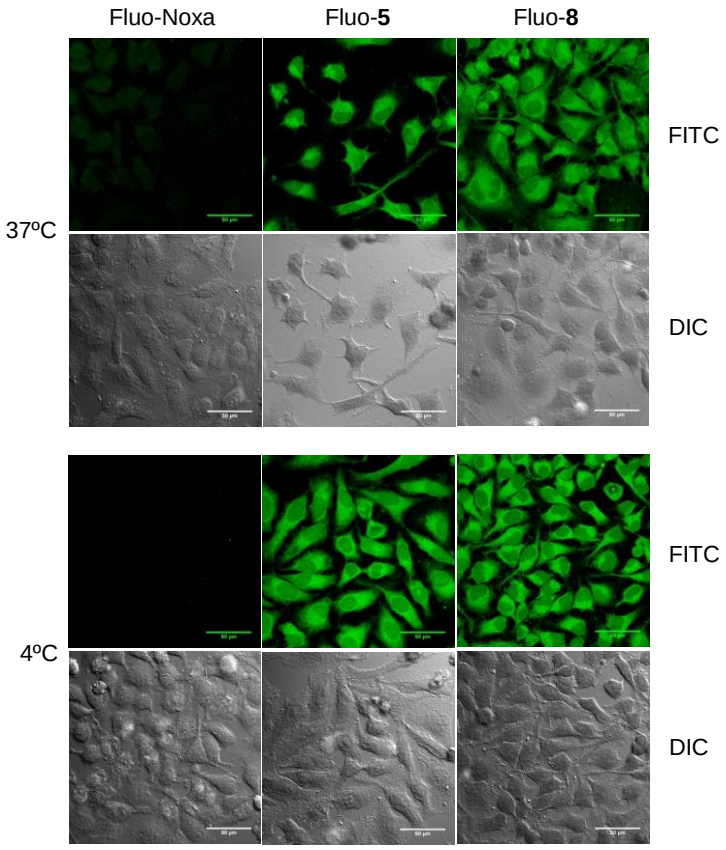


Figure S4. Confocal micrographs of HeLa cells after treatment with 10 μM of fluorescein-labeled peptides: Fluo-Noxa, Fluo-**5**, and Fluo-**8**, for 2 hours. Scale bar = 50 μm.

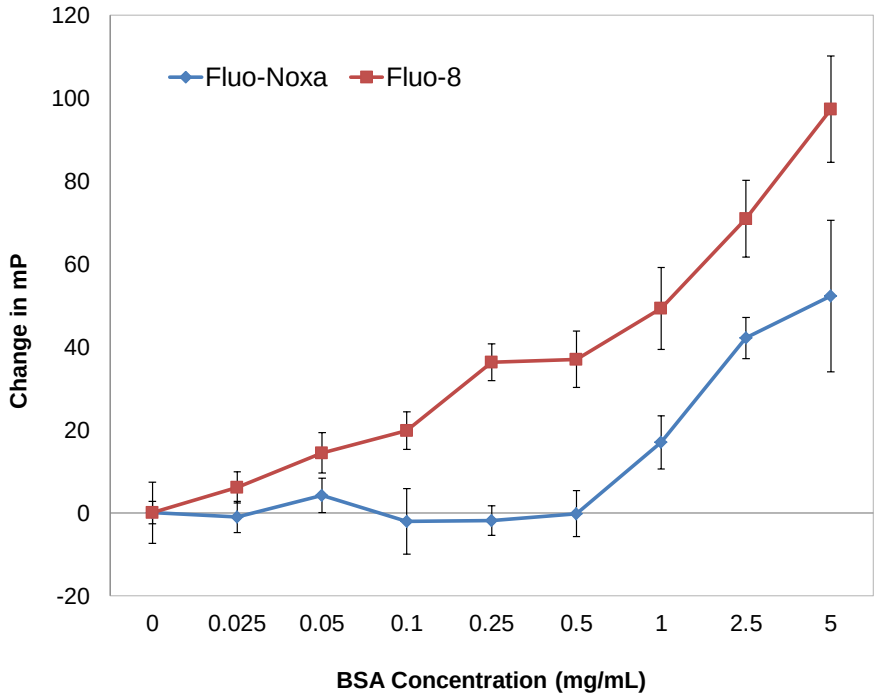
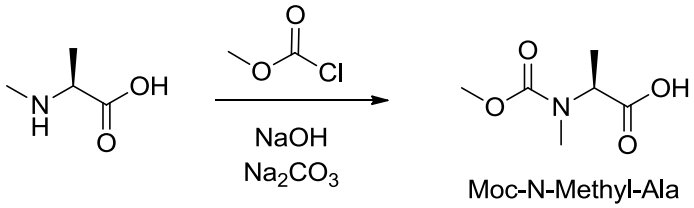


Figure S5. Direct FP assay to measure the fluorescence polarization change (ΔmP) for the fluorescein-labeled Noxa and peptide **8** after addition of various concentrations of BSA (Fraction V, MW = 66 KDa, Fisher Scientific). The fluorescein-labeled peptides (5 nM for Fluo-Noxa; 10 nM for Fluo-**8**) were incubated with BSA in PBS buffer in a total volume of 50 µL in 96-well FP assay plates at 37 °C for 4 hours before signal acquisitions.

Synthesis of 2-(Methoxycarbonyl-methyl-amino)-propionic Acid (Moc-N-Methyl-Ala)[S6]



L-alanine (1.0 g, 10 mmol) was added to a solution containing 20 mL of 1 M sodium hydroxide and sodium carbonate (0.55 g, 5.2 mmol). Methyl chloroformate (0.85 mL, 11 mmol, 1.1 eq) was added at 0 °C over 15 minutes and the stirring was continued for 1 hour. The reaction mixture was stirred at room temperature for another 6 hours before washing with diethyl ether. The aqueous layer was acidified to pH 1-2 and extracted with dichloromethane. The organic phase

was concentrated to give the desired product as a yellow oil (1.11 g, 77% yield): ^{1}H NMR (500 MHz, C_6D_6) δ 9.87 (s, 1H), 4.66-4.86 (m, 1H), 3.7 (s, 3H), 2.83 (s, 3H), 1.43 (d, *J* = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, D2O) δ 176.47, 157.56, 54.12, 53.10, 30.33, 14.52; MS (ESI) calculated for $C_6H_{11}NO_4$ 162.1 $[M+H]^+$, found 162.1.

General Procedure of Solid Phase Synthesis of the Modified Noxa Peptides

The linear peptides were synthesized by following standard Fmoc peptide synthesis protocol with Rink amide resin (substitution = 0.66 mmol/g) on a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ). For each coupling reaction, 5 equiv of Fmoc-amino acid, 5 equiv of HBTU, and 10 equiv of *N*-methylmorpholine (NMP) were used. The coupling reaction was allowed to proceed for 45 min. For coupling of D- and L-Cys, 1 M of trimethylpyridine in DCM/DMF (1:1) was employed together with a reduced pre-activation time (0.5 min) in order to minimize racemization. The Fmoc-deprotection was accomplished by treating peptide-bound resin with 20% piperidine/DMF (3×, 8 min each). After peptides were assembled, *N*-terminal amine was acylated using 10 equiv of acetic anhydride and 10 equiv of DIEA. Peptides were then cleaved by treating resin with a cleavage cocktail containing 95% TFA, 2.5% 1,2-ethanedithiol, 1.5% triisopropylsilane and 1% water for 1.5 hours (extended to 2.5 hours for peptides containing Pbf protecting groups). Peptides were precipitated in diethyl ether, collected by centrifugation, and washed with diethyl ether prior to drying in high vacuum. The crude peptides were purified by Gilson reverse-phase HPLC equipped with a semi-prep Phenomenex C_{18} column running at a flow rate of 5 mL/min and a gradient of 10-90% acetonitrile/H_2O containing 0.1% formic acid. The fractions showing the desired mass and greater than 90% purity by LC-MS were pooled and lyophilized to give the final linear peptides. For synthesis of the fluorescein-labeled peptides, chain elongation was allowed to continue with Fmoc-Ahx-OH. After removal of Fmoc protecting group, the *N*-terminus was conjugated with fluorescein by incubating the peptide with 1.5 equiv of FITC overnight in presence of 2 equiv of DIEA.

Synthesis of N-Methyl-L-Alanine-Substituted Peptides 6, 7, and 8

The standard solid phase synthesis protocol was followed in the assembly of the peptides until before coupling with N-methyl-alanine. For the 1st N-methyl-alanine, we used one of the reported coupling procedure,[S7] in which 4 equiv Fmoc-N-methyl-alanine, 4 equiv PyBOP, 12

equiv HOAt, and 12 equiv DIEA in DMF were added to the resin and the mixture was stirred for 2 hours. For the 2nd N-methyl-alanine as in peptides **7** and **8**, the same procedure was employed except the above synthesized Moc-N-Methyl-Ala. Moc capping of N-methyl-alanine was preferred over acetyl capping because of the known deletion side reactions[S8] when acetyl anhydride was used in N-terminal capping.

Synthesis of Fluorescein Conjugated Peptide 8

Our initial attempts to conjugate fluorescein at the N-terminus after N-methyl-Ala have led to deletion of N-Methyl-Ala. We then decided to conjugate fluorescein at the C-terminus of peptide **8**. To this end, we added an additional lysine residue at the C-terminus so that we can conjugate fluorescein to lysine ε-amino group. The linear peptide was assembled on the solid-support and cleaved, and the cross-linking with Bph was performed as described previously. Afterwards, the dried cross-linked peptide was re-dissolved in appropriate amount of DMF to obtain a peptide solution of 30 mg/mL, to which 1.5 equiv of FITC and 10 equiv of DIEA were added. The mixture was stirred for 2 hours, and the fluorescein-conjugated peptide was precipitated out by addition of cold diethyl ether. The residue was washed with ether to remove excess amount of FITC, and the dried residue was re-dissolved in acetonitrile/water and purified by reverse-phase HPLC.

Peptide Side Chain Cross-Linking by Bph

The cross-linking reactions were carried out by incubating the purified dicysteine-containing peptides with 1.5 equiv of 4,4’-bis(bromomethyl)-1,1'-bipheny (Bph) in a mixed solvent of acetonitrile/30 mM NH_4HCO_3 buffer (1:4 to 2:3 depending on solubility), pH 8.5, to obtain a final peptide concentration of 1 mM. The mixture was stirred at room temperature for 1.5-2 hours. Afterwards, the solvents were evaporated and excess amount of Bph cross-linker was removed by washing the residue with diethyl ether. The residue was then purified by preparative reverse-phase HPLC to give the purified Bph-cross-linked peptide.

Fluorescence Polarization (FP) Assay

FITC-labeled peptides derived from the Bim BH3 domain (DMRPEIWIAQELRRIG DEFNAYYAR) or the Bak BH3 domain (GQVGRQLAIIGDDINR) were used to determine the ability of the tested compounds to interfere with the binding of Bim to Mcl-1 or Bak to Bcl-xL,

respectively. In brief, 20 μL of 20 nM GST-tagged mouse Mcl-1-(152-309) or 37.5 nM GST-tagged human Bcl-x_L-(1-209) fusion protein in phosphate-buffered saline (PBS, pH7.4) containing 0.005% Tween 20 was mixed with 5 μL of the test peptides at varying concentrations in PBS containing 25% DMSO and 0.005% Tween 20 in each well of 96-well half area black polystyrene plates (Corning #3993). Then, 25 μL of 10 nM FITC-labeled Bim BH3 or Bak BH3 peptide (for Mcl-1 and Bcl-x_L, respectively) in PBS containing 5% DMSO and 0.005% Tween 20 was added to each well and thoroughly mixed at 1450 rpm for 3 min with BioShake IQ ThermoMixer (QUANTIFOIL Instruments GmbH) at room temperature. The polarization values in millipolarization units (mP) were measured for 0.2 sec at excitation/emission wavelengths of 480/535 nm using a PerkinElmer 2030 multilabel plate reader. IC_{50} was determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model (SigmaPlot 10.0.1, Systat Software, Inc.). K_i values were then calculated using the equation: $K_i = [I]_{50} / ([L]_{50}/K_d + P_0/K_d + 1)$,[S9] where I_{50} and L_{50} are the free concentrations of the inhibitor and ligand, respectively, at 50% inhibition, P_o is the free concentration of protein in the absence of inhibitor, and K_d is the disassociation constant of the Bim-GST-Mcl-1 complex and has a value of 12.4 nM (Table S3)

Cell Viability Assay

Human histiocytic lymphoma U937 cells, which overexpress Mcl-1 and are insensitive to ABT-737, were seeded at 25,000 cells per well on 96-well plates (BD Falcon, #353296) in 100 μL of RPMI1640 medium containing 5% FBS and treated in triplicate with increasing concentrations of the tested peptides. Cell viability was determined at 48 h after treatment by measuring the intracellular ATP level using the CellTiter-Glo Luminescenct Cell Viability Assay kit (Promega G7571).

Circular Dichroism Measurement

Circular dichroism spectra were recorded with JASCO J-715 CD spectrometer at room temperature using a 0.1-cm path length cuvette. The spectra were recorded in the wavelength range of 185-250 nm and averaged over 3 scans with a resolution of 0.5 nm, a bandwidth of 1.0 nm and a response time of 4 s. The sensitivity and scan rate of the spectrometer were set to 100 mdeg and 50 nm/min, respectively. All peptides were dissolved in acetonitrile/H_2O (1:1) to reach

the final concentrations of 0.2 mg/mL. The mean residue ellipticity was plotted to wavelength, and the percent helicity of each peptide was calculated based on $[\theta]_{222}/[\theta]_{max}$. $[\theta]_{max}$ was calculated according to the formula: $[\theta]_{max} = -39500 \times (1-3/n)$ where n is the number of amide bonds.[S10]

Fluorescence Activated Cell Sorting

HeLa Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in two 24-well plates in a 37 ºC, 5% CO_2 incubator to reach confluency of around 70%. Cells were then washed twice with PBS before switching to serum-free DMEM medium. One of the plates was kept in incubator at 37ºC while other plate was kept at 4 ºC for a period of 30 minutes, then appropriate amounts of fluorescein conjugated peptides (1 mM in DMSO) were added to obtain a final peptide concentration of 10 µM. The culture plates were then incubated for additional 2 hours at these two different temperature settings. Then medium was removed and cells were washed with PBS (3×) followed by treatment with trypsin to detach the cells. After a brief low-speed centrifugation, HeLa cells were collected and re-suspended in PBS for the FACS analysis.

Live Cell Confocal Microscopy

HeLa cells were cultured in 10 glass-bottom microwell dishes of 35-mm size to reach the confluency of nearly 80%. After switching to serum free medium, 5 of the dishes were incubated for 30 min at 37 ºC and the remaining 5 were kept at 4 ºC. Each of the plates was treated with 10 µM of a specified fluorescein-labeled peptide for 2 hours. Afterwards, cells were washed with PBS and the medium was switched to Earle's balanced salt solution supplemented with MEM essential and non-essential amino acids. The fluorescent images were acquired with a Zeiss LSM-510 meta-NLO system equipped with a Coherent Chameleon Ultra II Ti/Sapphire laser and an external non-descanned detector.

Proteolytic Stability Studies

Stability against chymotrypsin: To a 60 µL ammonium bicarbonate solution were added 100 µL of peptide solution (10 µM, dissolved in ammonium bicarbonate buffer, pH 7.5, containing 10% DMSO) and 40 µL of α-chymotrypsin (5 µg/mL) from bovine pancreas (Sigma, Type II, activity > 40 units/mg protein), and the mixture was incubated at 37 ºC. At the specified time, an aliquot

of the above digestion mixture was withdrawn and quenched by the addition of equal volume of 5% TFA in acetonitrile. Afterwards, 10 µL of 0.2 mM Fmoc-glycine was added to the above sample as an injection control. LC-MS analysis was then performed for each sample. The amount of intact peptide remained in the mixture was quantified by the integration area of the intact peptide ion counts. The digestion at each time points was repeated two or three times to give the average values along with standard deviations.

Stability against trypsin: To an 80 µL ammonium bicarbonate solution (pH 7.5) were added 100 µL of peptide solution (10 µM, dissolved in ammonium bicarbonate buffer, pH 7.5, containing 10% DMSO) and 20 µL of trypsin (5 µg/mL) from porcine pancreas (Sigma, 13,000-20,000 BAEE units/mg protein), and the mixture was incubated at 37 ºC. At the specified time, an aliquot of the above digestion mixture was withdrawn and quenched by the addition of equal volume of 5% TFA in acetonitrile. The LC-MS analysis was carried out in a similar manner as described previously.

Stability in mouse serum: To 200 µL of fresh non-sterile mouse serum (Equitech-Bio, Kerrville TX) was added 25 µl of peptide solution (45 µM, dissolved in ammonium bicarbonate buffer, pH 7.5, containing 10% DMSO), and the mixture was incubated at 37 ºC. At the specified time, an aliquot of incubation mixture was withdrawn and quenched by addition of equal volume of 15% trichloroacetic acid in acetonitrile to precipitate out serum proteins over ice for 30 min. The mixture was then centrifuged at 13,500 rpm for 10 min, and the supernatant was collected and analyzed by LC-MS in a similar manner as described previously.

Complete Reference for 2c, 6 and 12:

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