Supporting Information

Design of Bcl-2 and Bcl-xL Inhibitors with Subnanomolar Binding Affinities Based Upon a New Scaffold

Haibin Zhou[+^], Jianfang Chen[+^], Jennifer L. Meagher[#^], Chao-Yie Yang[+^], Angelo Aguilar,[+^] Liu Liu[+], Longchuan Bai[+], Xin Cong[+], Qian Cai[+], Xueliang Fang[+], Jeanne A. Stuckey[#], and Shaomeng Wang[+*]

[+]Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, and [#]Life Sciences Institute, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0934, USA

* To whom correspondence should be addressed

Tel: 734-615-0362

Fax: 734-647-9647

E-mail: shaomeng@umich.edu

^These authors contribute equally

Table of Contents:

I. Predicted modeling of **6** and **7** (S2)

II. Fluorescence polarization competitive binding assays (S2-S4)

III. Crystallographic studies (S4-S5)
IV. References (S6)

I. Molecular modeling

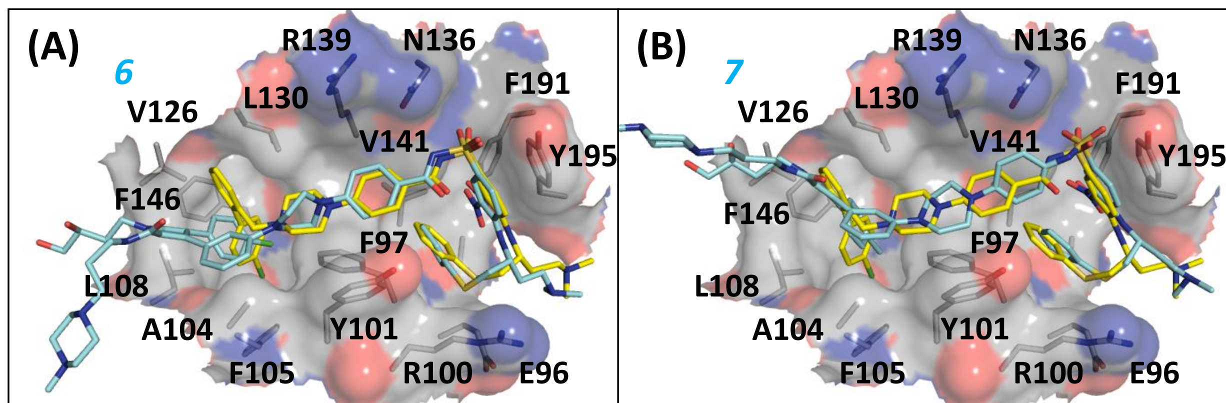

Figure S1 (A), (B) The predicted binding models of **6** and **7** superimposed with crystal structure of **1**.

II. Fluorescence polarization based binding assays

Protein expression and purification

Human Bcl-2 protein. The isoform 2 construct of the human Bcl-2 with an N-terminal 6xHis tag was used. Bcl-2 protein was produced in *E. coli* BL21(DE3) cells. Cells were grown at 37°C in 2xYT containing antibiotics to an OD600 of 0.6. Protein expression was induced by 0.4 mM IPTG at 20°C for 20 h. Cells were lysed in 50mM Tris pH 8.0 buffer containing 500 mM NaCl, 0.1% bME and 40 µl of Leupectin/Aprotin. The protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The protein was further purified on a Superdex75 column (Amersham Biosciences) in 25 mM Tris pH 8.0 buffer containing 150 mM NaCl and 2 mM DTT.

Human Bcl-xL protein. Human Bcl-xL protein, which has an internal deletion for the 45-85 amino acid residues and a C-terminal truncation for the amino acid residues 212-233, was cloned into the pHis-TEV vector (a modified pET vector). Protein was produced in *E. coli* BL21 (DE3) cells with an N-terminal 8xHis tag. Cells were grown at 37°C in 2xYT containing antibiotics to an OD600 of 0.6. Protein expression was induced by 0.4 mM IPTG at 25°C for 16 h. Cells were lysed in 50mM Tris, pH7.5 buffer containing 200mM NaCl, 0.1%bME and Leupectin/Aprotin.

His-TEV-Bcl-xL protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The His-TEV-Bcl-xL protein was further purified on a Superdex75 column (Amersham Biosciences) in 20mM Tris pH7.5 buffer containing 150mM NaCl and 5mM DTT.

Human Mcl-1 protein. The Mcl-1 fragment consisting of 171-327 amino acid residues was cloned into the pHis-TEV vector (a modified pET vector) through BamHI and EcoRI sites. Mcl-1 protein with an N-terminal 8xHis tag was produced in E. coli BL21 (DE3) cells. Cells were grown at 37°C in 2xYT containing antibiotics to an OD600 density of 0.6. Protein expression was induced by 0.4 mM IPTG at 37°C for 4 hours. Cells were lysed in 50 mM Tris pH 8.0 buffer containing 500 mM NaCl, 0.1% bME and 40 µl of Leupectin/Aprotin. Mcl-1 protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN), following the manufacturer's instructions. The protein was further purified on a Source Q15 column (resin and column are from Amersham Biosciences) in 25 mM Tris pH 8.0 buffer, with NaCl gradient.

Development and optimization of quantitative fluorescence polarization (FP)-based binding assays for Bcl-2, Bcl-xL and Mcl-1 proteins

Sensitive and quantitative fluorescence polarization (FP)-based binding assays were developed and optimized to determine the binding affinities of Bcl-2 family protein inhibitors to the recombinant Bcl-2, Bcl-xL, and Mcl-1 proteins.

Determine K_d values of fluorescent probes to proteins

Fluorescein tagged BIM (81-106), BAK (72-87), and BID (79-99) BH3 (Bcl-2 Homology 3) peptides, which were named Flu-BIM, Flu-BAK, and Flu-BID, were synthesized in our laboratory using a peptide synthesizer. Their Kd values were determined to Bcl-2, Bcl-xL and Mcl-1 proteins with a fixed concentration of Flu-BIM, Flu-BAK, or Flu-BID, respectively, in saturation experiments. Based upon our analysis of the dynamic ranges for the signals and their K_d values, Flu-BIM was selected as the tracer in the Bcl-2 binding assay, Flu-BAK was selected as the tracer for the Bcl-xL binding assay and Flu-BID was selected as the tracer for the Mcl-1 binding assay, respectively. The K_d value of Flu-BIM to Bcl-2 was 0.55 ± 0.15 nM, the K_d value

of Flu-BAK to Bcl-xL was 4.4 ± 0.8 nM, the K_d value of Flu-BID to Mcl-1 was 6.9 ± 1.0 nM, respectively, in our saturation experiments.

In the saturation experiments, fluorescence polarization values were measured using the Infinite M-1000 plate reader (Tecan U.S., Research Triangle Park, NC) in Microfluor 2 96-well, black, round-bottom plates (Thermo Scientific). To each well, 1nM of Flu-BIM or 2nM of Flu-BAK or 2nM of Flu-BID and different concentrations of Bcl-2 or Bcl-xL or Mcl-1 were added to a final volume of 125 μl in the assay buffer (100mM potassium phosphate, pH 7.5, 100 μg/ml bovine γ-globulin, 0.02% sodium azide, Invitrogen, with 0.01% Triton X-100 and 4% DMSO). Plates were mixed and incubated at room temperature for 2 hours with gentle shaking. The polarization values in millipolarization units (mP) were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Equilibrium dissociation constants (K_d) were then calculated by fitting the sigmoidal dose-dependent FP increases as a function of protein concentrations using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA).

III. Bcl-xL Crystallographic studies

Expression and Purification of Bcl-xLΔTM ΔLP

Bcl-xLΔLPΔTM (ΔLP is a deletion of residues 45-84 and ΔTM is a deletion of residues 212-233) was cloned into an expression vector with a TEV protease cleavable 8-His tag. The protein was expressed in C41 cells overnight at 20 °C by induction with IPTG. The protein was purified from the soluble fraction of the cell lysate by affinity chromatography using Ni-NTA resin in a buffer containing 50 mM TrisHCl pH 7.5, 200 mM NaCl, and 0.1% βME. The amino terminal His tag was cleaved by incubation with TEV protease and the protein was further purified by anion exchange (Source Q) and gel filtration chromatography (Superdex 75) in buffer containing 20mM TrisHCl pH 7.5, 150 mM NaCl and 0.1% βME.

Table S1: Data Collection and Refinement Statistics

Data Collection	**Bcl-xL-4**
SpaceGroup	$P4_22_12$
Unit Cell a, b, c (Å)	103.748, 103.748, 35.320
Wavelength (Å)	0.97872
Resolution (Å)[1]	1.7 (1.73 – 1.7)
Rsym (%)[2]	5.2 (40.8)
<I/sI>[3]	>20 (5)
Completeness (%)[4]	99.9 (100.0)
Redundancy	14.3 (14.4)
Refinement	
Resolution (Å)	1.7
R-Factor (%)[5]	0.1854
Rfree (%)[6]	0.2036
Protein atoms	1170
Water Molecules	110
Unique Reflections	21781
R.m.s.d.[7]	
Bonds	0.009
Angles	0.88
MolProbity Score[8]	6.11
Clash Score[8]	1.34
RSR of compound[9]	0.288
RSCC of compound[9]	0.827
PDB ID	3SPF

[1]Statistics for highest resolution bin of reflections in parentheses.
[2]$R_{sym} = \Sigma_h \Sigma_j | I_{hj} - \langle I_h \rangle | / \Sigma_h \Sigma_j I_{hj}$, where I_{hj} is the intensity of observation j of reflection h and $\langle I_h \rangle$ is the mean intensity for multiple recorded reflections.
[3]Intensity signal-to-noise ratio.
[4]Completeness of the unique diffraction data.
[5]$R\text{-factor} = \Sigma_h | |F_o| - |F_c| | / \Sigma_h |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes for reflection h.
[6]R_{free} is calculated against a 10% random sampling of the reflections that were removed before structure refinement.
[7]Root mean square deviation of bond lengths and bond angles.
[8]Molprobity Server.[1]
[9]Predeposition Electron Density Server.[2]

References:

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2. Kleywegt, G. J.; Harris, M. R.; Zou, J. Y.; Taylor, T. C.; Wahlby, A.; Jones, T. A., The Uppsala Electron-Density Server. *Acta Crystallogr D Biol Crystallogr* **2004,** *60* (Pt 12 Pt 1), 2240-9.