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The discovery of small molecule chemical probes of Bcl-X_L and Mcl-1

Michael Prakesch [a], Alexey Yu Denisov [c], Marwen Naim [d], Kalle Gehring [c,*], Prabhat Arya [a,b,*]

[a] Ontario Institute for Cancer Research, MaRS Centre, South Tower, 101 College Street, Toronto, Ont., Canada M5G 1L7
[b] Steacie Institute for Molecular Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ont., Canada K1A 0R6
[c] Department of Biochemistry, McGill University, 3655 Promenade Sir William Osler, Montréal, Que., Canada H3G 1Y6
[d] Computational Chemistry & Biology Group, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, Que., Canada H4P 2R2

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ABSTRACT

A tetrahydroaminoquinoline-based library was generated with the goals of finding small molecule modulators of protein–protein interactions. Several library members as well as other related intermediates were tested for their ability to bind to Bcl-X_L and Mcl-1 by in silico and ^{15}N NMR studies. The NMR study led to the identification of the tetrahydroaminoquinoline-based nude scaffold, **7** as a weak binder (K_d = 200 μM for Bcl-X_L and K_d = 300 μM for Mcl-1) to both proteins. Using this scaffold as the starting material, we then synthesized a focused library of only 9 derivatives by applying the principles of a fragment-based approach. All these derivatives were then tested by NMR and this led to the discovery of a novel, small molecule (MIPRALDEN, **17**) as a binder to Mcl-1 and Bcl-X_L (K_D = 25 and 70 μM). This finding is novel because to our knowledge there are not many small molecules known in the literature that bind to Mcl-1.

1. Introduction

The programmed cell death (apoptosis) machinery follows several well-organized cell signaling networks where one of the two major pathways (i.e., the mitochondrial process or intrinsic pathway) involves a highly regulated series of proteins.[1,2] Three subfamilies of the protein known as the Bcl-2 family interact together and decide whether the cell should die or survive. One group is prone to inducing apoptosis (e.g., Bax, Bak, and Bok), the second one is also pro-apoptotic and is called 'BH3-only pro-apoptotic' (Bad, Bik, Bim, Bid, Hrk, Bcl-rambo, Bmf, Bcl-G, PUMA, and Noxa), and the third group is known to be pro-survival (e.g., Bcl-2, Bcl-X_L, Bcl-w, Bcl-B, Mcl-1, and A1).[3–5] It was shown that cancer cells over express Bcl-2 family proteins around mitochondria and contribute to tumor initiation, progression and resistance to therapy.[6,7] Thus, triggering apoptosis and inducing cancer cells to die could be one therapeutic way to fight cancers and an especially attractive one if apoptosis could be induced using small molecules.

In cases where the structural information of the protein is known and if the protein could be subjected to a low-throughput screening study by NMR, the use of the fragment-based approach to identify small molecule ligands is gaining momentum.[8–11] An excellent example of this approach is the work from Abbott Laboratories on the discovery of ABT-737 as a small molecule binder to Bcl-X_L. ABT-737 is a nanomolar (nM) binder to Bcl-X_L and the synthesis of this small molecule was achieved using the structural information of Bak:Bcl-X_L protein–protein (p–p) interaction.[6] Although ABT-737 is a strong and selective binder to Bcl-X_L, it was shown not to target Mcl-1 protein, thereby conferring resistance to the drug. Further, it was shown that the down-regulation of Mcl-1, when ABT-737 is used, removed its lethality effect.[12,13] Hence, finding a small molecule targeting Mcl-1 only, or even both Bcl-X_L and Mcl-1, could serve as a highly desirable chemical probe to investigate its biological functions.

2. Results and discussion

2.1. Library generation of tetrahydroquinoline derivatives with the goals of targeting protein–protein interactions

Targeting protein–protein (p–p) interactions by small molecules remains a challenging undertaking since these

* Corresponding authors. Tel.: +1 613 993 7014; fax: +1 613 952 0068.
E-mail addresses: kalle.gehring@mcgill.ca (K. Gehring), prabhat.arya@nrc.ca (P. Arya).

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interactions cover a relatively large surface and involve multiple hot-spots that may have extensive hydrophobic surfaces.[14] With the goals of finding small molecule probes of p–p interactions,[15] we are developing high-throughput approaches leading to the library generation of tetrahydroquinoline alkaloid-inspired compounds. With this objective, the synthesis of the tetrahydroaminoquinoline scaffold, **1**, was achieved as shown in Scheme 1.[16] To develop the solid-phase synthesis method, compound **2** was obtained following several easy transformations. This included the introduction of a three-carbon spacer and changing the *N*-Teoc to the *N*-Fmoc protecting group giving derivative **2**. These transformations allowed the compound to be compatible with the solid-phase synthesis that utilizes silylation as the mode of immobilization. The loading of compound **2** was performed using the Broad Institute loading-protocol and it was achieved in high yields (76% after cleavage from the support). When carried out on a large scale, the loss of the *N*-Fmoc protecting group was observed, yielding **3** as the free amino alcohol derivative. The loaded compound **3** could then be used in the generation of a 105-membered library by selectively introducing the first diversity using a DIC coupling with acids, R_1CO_2H. Under these conditions, there was no sign of the amide coupling using the free secondary amine. This group appears to be involved in an intra-molecular hydrogen bonding with the carboxyl ester group (see Supporting material for more details). Following this, the second diversity was then introduced using acid chloride (R_2COCl) coupling to give compound **4**. After the *N*-Alloc removal and the coupling of the third diversity member, using R_3COCl, cleavage of the all the library members gave 105 discrete compounds with the general structure **6,** in good yields. No purification was required since the HPLC of each library member showed an average purity greater than 90%.

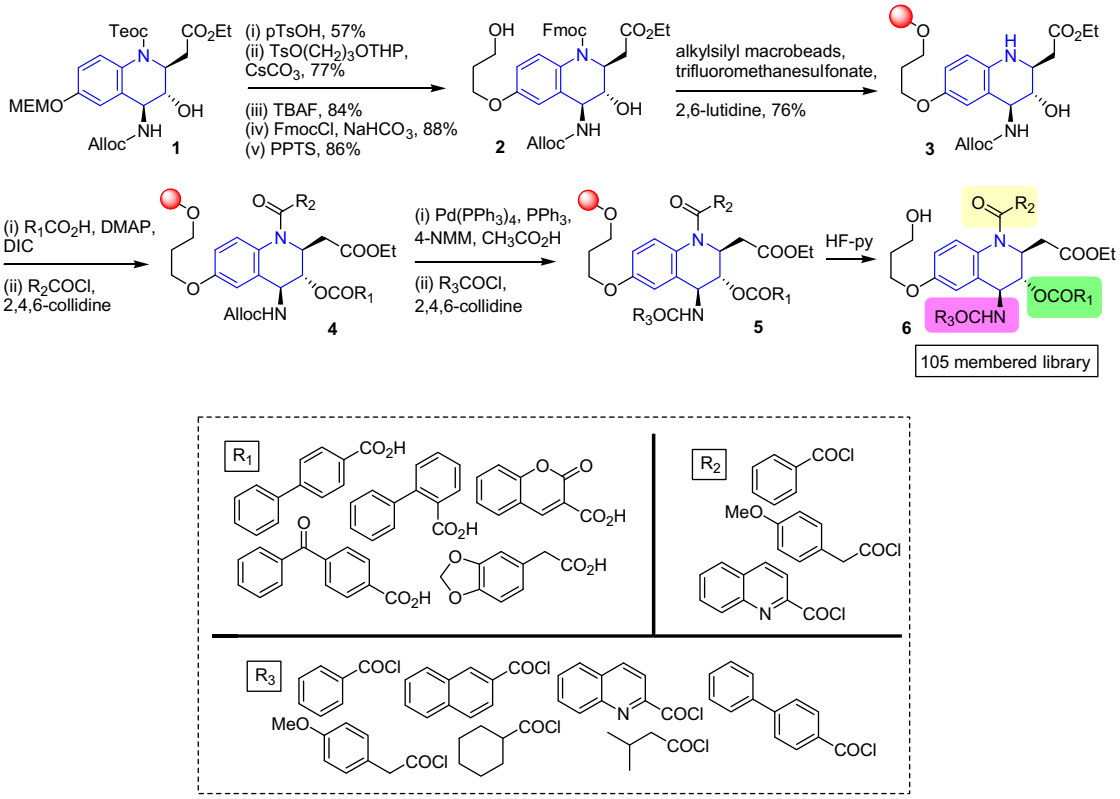
2.2. In silico screening of the generic library

In a search of small molecule binders to Bcl-2 proteins family, the library members were then tested by virtual screening against two anti-apoptotic proteins: Bcl-X_L Protein (1YSI and 1YSN) and Bcl-2 (1YSW). The screening studies resulted in the identification of several compounds from the library showing good scores (see Fig. 1). The in silico identified library members showing good scores were then re-synthesized in solution to be tested further in NMR-binding experiments with Bcl-X_L protein. Due to their poor solubility, all the leads identified by in silico were not compatible with the NMR studies.

2.3. A search for the small molecule scaffold by NMR screening

Shown in Scheme 2 are two small molecule derivatives, **7** and **8**, that were obtained from **1** and **2** with the goal of validating our in silico studies. To enhance their solubility in an aqueous media, the protecting groups around the scaffold were removed to increase the number of hydrogen-bonding donors. Compounds **7** and **8** were then subjected to ^{15}N NMR studies for their ability to interact with Bcl-X_L.

Interestingly, scaffold **8** was found to be a poor binder to the hydrophobic cleft of Bcl-X_L with a K_D of 10 mM. While **7** was still a weak binder, it had a better interaction with the protein: $K_D = 200\ \mu M$. The perturbed residues were localized in the helices α-2, -5, and -7 with the most strongly affected amino acid residues being 96–102, which is a typical site for the earlier known small molecule drugs (ABT-737 and Gossypol). Further, NMR studies combined with computational experiments also confirmed the location of **7** in the upper section of the Bcl-X_L hydrophobic cleft. Scaffold **7** was



Scheme 1. The library generation from the tetrahydroaminoquinoline scaffold.

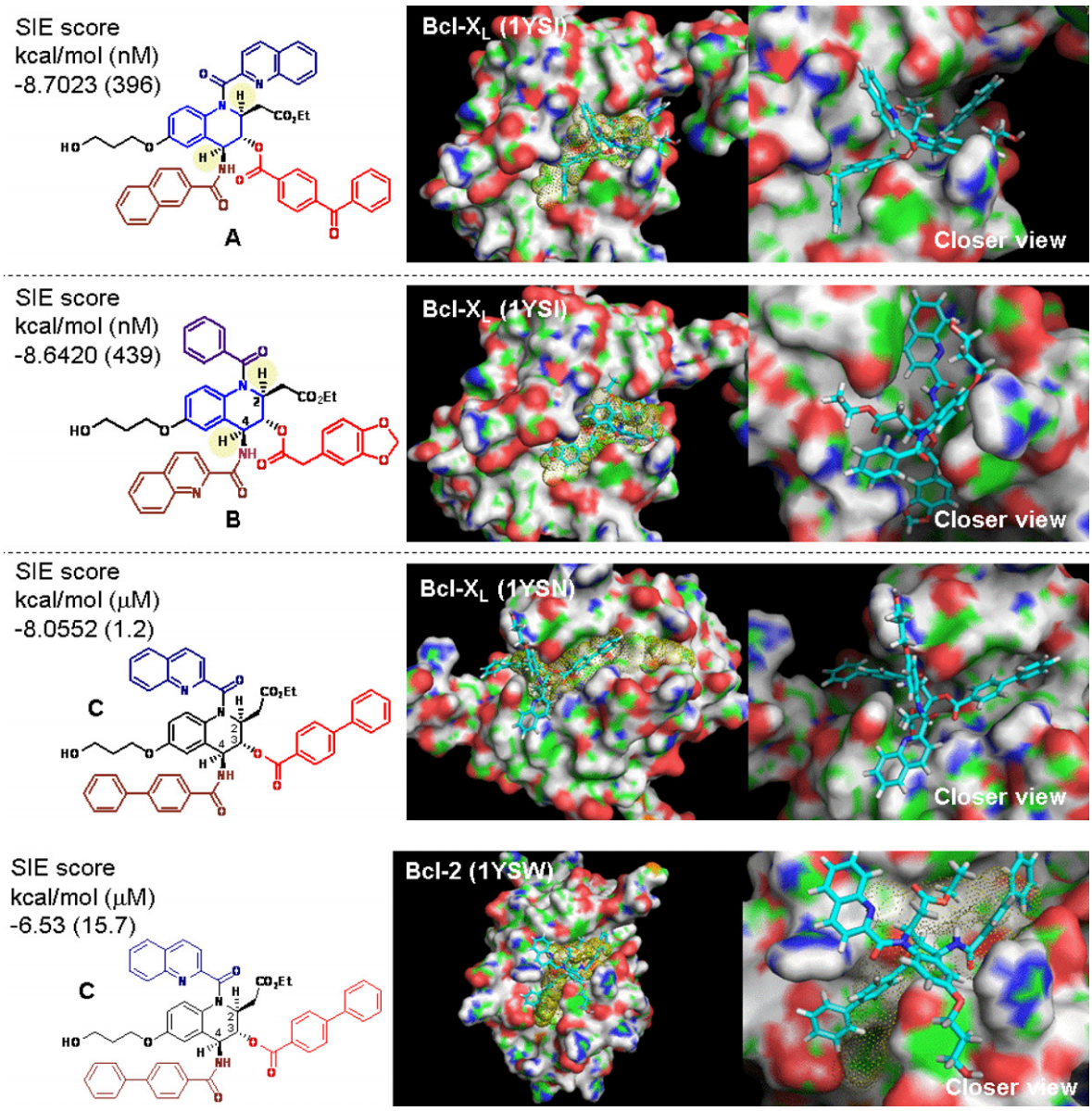
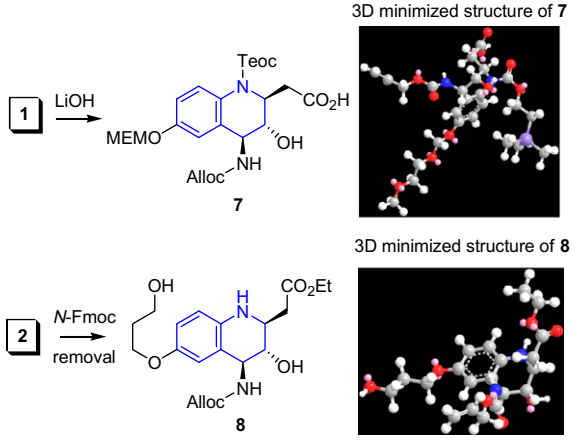


Figure 1. In silico Studies with Bcl-X_L and Bcl-2 using the tetrahydroaminoquinoline library. (A–C) The library members.



Scheme 2. Two derivatives of the tetrahydroaminoquinoline scaffold.

also tested with Mcl-1 protein and it showed similar interactions with the BH3-peptide-binding site formed by helixes α-2, -3, -4, and -5 with a K_D of 300 μM. The in silico docking of compound **7** was then performed using Fred® with both proteins, Bcl-X_L and Mcl-1, and 100 best poses are shown in Figure 2A and B. The best pose is represented in Figure 2C and D, where compound **7** is interacting at the same site of the original Bak-peptide.

Although identified as a weak binder in our NMR experiments, these findings were very attractive and offered an excellent opportunity to derivatize this 'nude' scaffold further using a fragment-based type approach. At this stage, it was decided to synthesize only a small set of compounds comprising the functionalization of both amines of the nude scaffold, **7**. Due to the presence of the hydroxyl and carboxylic acid groups in each derivative, it was anticipated that the solubility in an aqueous media would not be the limiting factor.

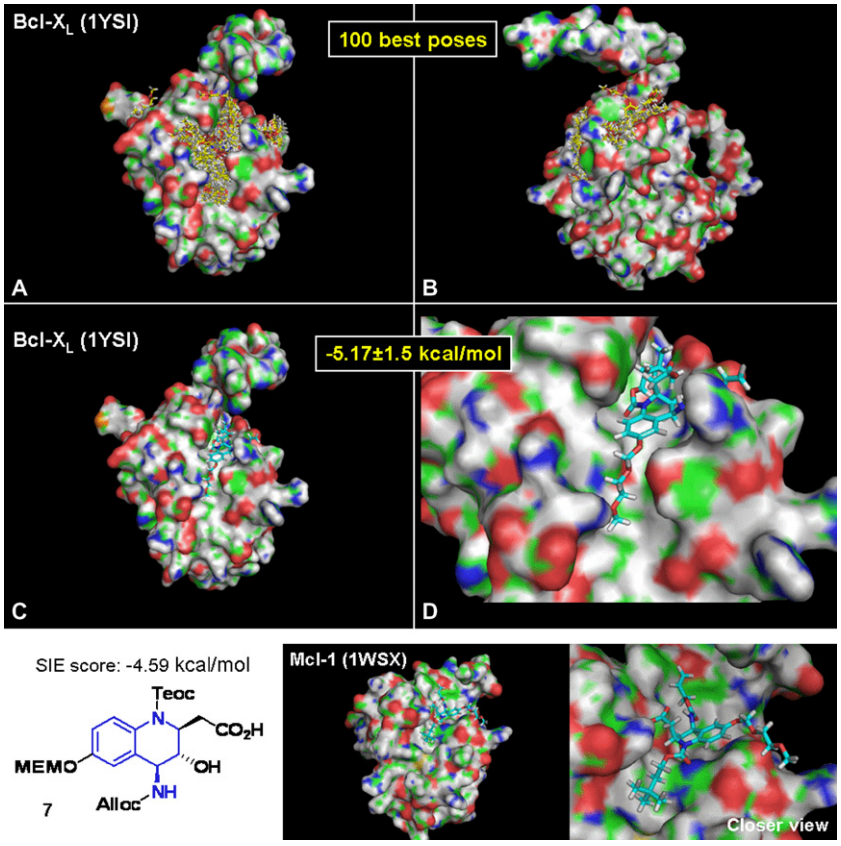
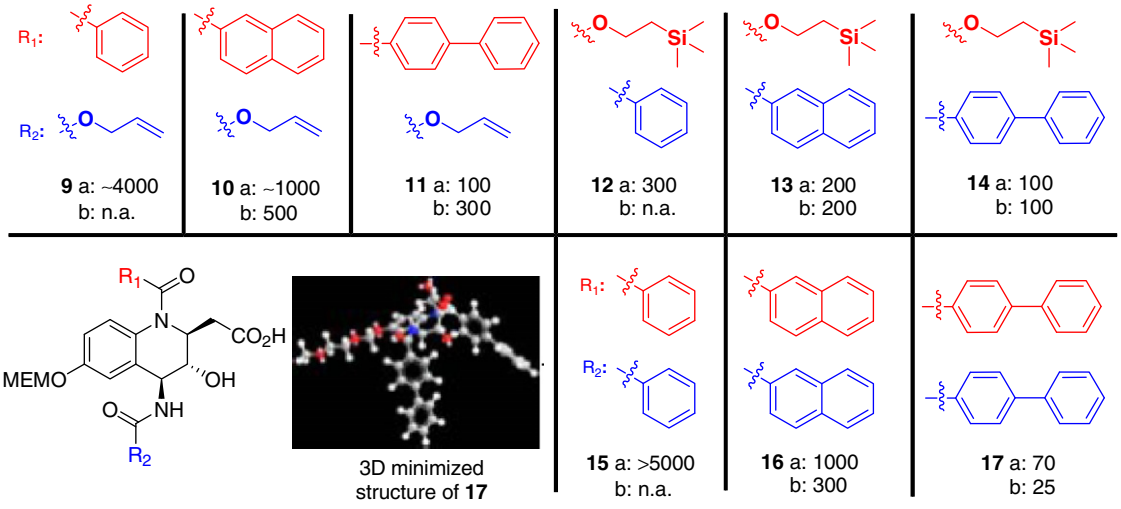


Figure 2. In silico docking of 7 with Bcl-X$_L$ and Mcl-1.



a: Binding with Bcl-X$_L$ protein in μM; b: Binding with Mcl-1 protein in μM; n.a.: not available

Figure 3. A focused, nine-membered library by solution-phase synthesis.

2.4. The use of a fragment-based approach in designing focused nine-membered library

Shown in Figure 3 are nine compounds that were then synthesized in solution. To explore the potential of the chiral scaffold **7** to expand the distal hydrophobic sites, the plan was to systematically introduce several hydrophobic groups (i.e., Ph, Bn and 4-biphenyl) at both amines via an amide bond. In particular, we were very interested in exploring the use of the 4-biphenyl groups at both amines because it has been shown in the past to be an excellent fragment when it comes to protein binding.[17]

2.5. NMR-binding experiments with Bcl-X$_L$ and Mcl-1

Compounds **9**–**17** were tested for their ability to interact with Bcl-X$_L$ and Mcl-1 proteins and the results are shown in Figure 3. To date, as we expected, our best result with Mcl-1 is the MIPRALDEN (compound **17**), which binds with a K_D = 25 μM. Shown in yel-

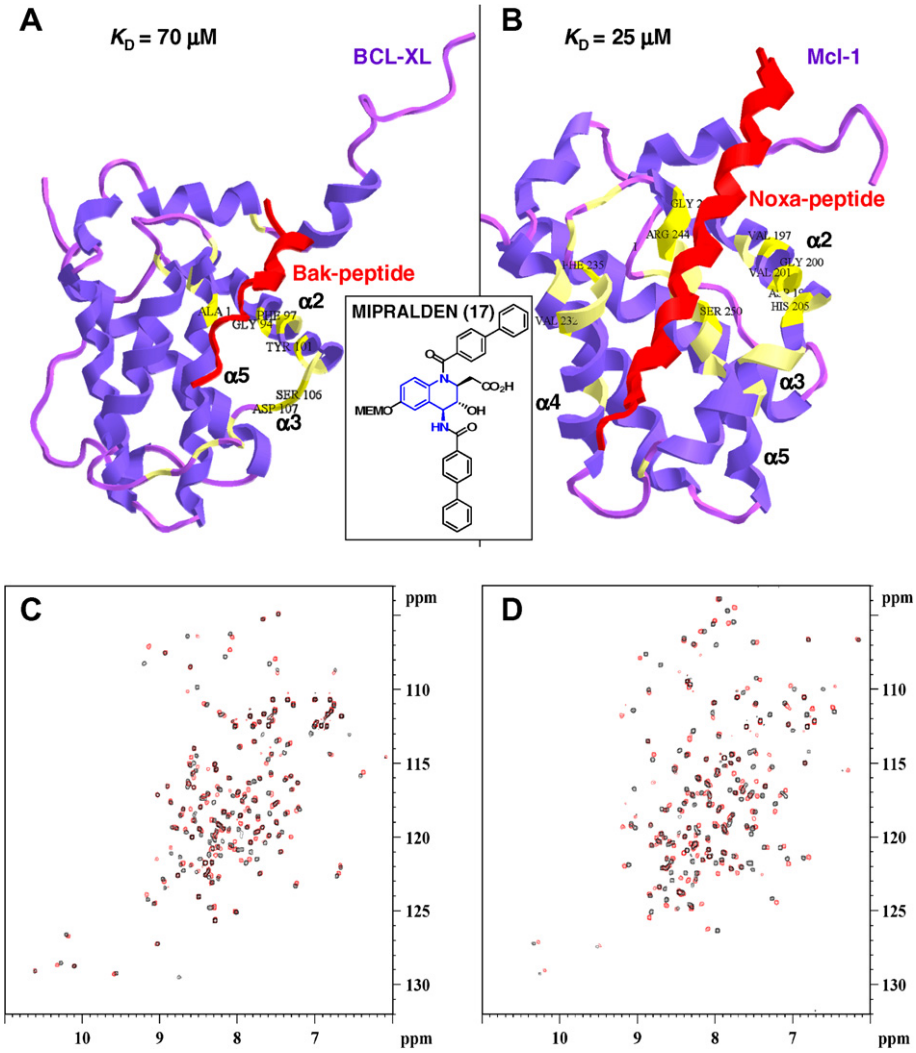


Figure 4. Interactions of **17** with Bcl-X_L and Mcl-1 by NMR. (A and B) Proximity of residues (in yellow) of Bcl-X_L and Mcl-1 with **17** (MIPRALDEN). (C and D) ^{15}N–^{1}H HSQC for Bcl-X_L (C) and Mcl-1. (D) Without (black) and with (red) **17**. (E) Magnitude of the amide chemical shift changes $[(\Delta^1H \text{ shift})^2 + (\Delta^{15}N \text{ shift} \times 0.2)^2]^{1/2}$ in Bcl-X_L upon **17** binding. The positions of α helices as well as BH domains in Bcl-X_L are shown. (F) Magnitude of the amide chemical shift changes $[(\Delta^1H \text{ shift})^2 + (\Delta^{15}N \text{ shift} \times 0.2)^2]^{1/2}$ in Mcl-1 upon **17** binding. The positions of α helices as well as BH domains in Mcl-1 are shown.

low in Figure 4A and B are the amino acid residues from Bcl-X_L and Mcl-1 proteins that were affected by **17** (see also Figure 4C and D for ^{15}N–^{1}H HSQC). Magnitudes of amide chemical shift changes in ^{15}N–^{1}H HSQC spectra are also shown in Figure 4E and F. The in silico docking with compound **17** using Fred® was further studied with Bcl-X_L and Mcl-1 proteins and the best poses are shown in Figure 5. This is an interesting finding because although several small molecule binders to Bcl-X_L appeared in the literature in recent years[18,19,18,20–23] but to our knowledge there are not many examples of their effective binding with Mcl-1.[23,13,20,24,25] The discovery of **17** also opens up tremendous opportunities for improving the binding, leading to the next generation of analogs. Furthermore, work is also ongoing at several fronts to test the scope of **17** in various biological systems and these findings will be reported as they become available.

3. Experimental procedures

3.1. Synthesis

All the information on the experimental procedures, characterization data, and ^{1}H, ^{13}C NMR spectra is provided as the supplement data (see Supporting information).

3.2. In silico procedure and tools

In the present method, an in-house made scoring function, 'Solvated Interaction Energy' SIE, was coupled with the commercial docking program suit (Omega-Fred from OpenEye Inc., Santa Fe, CA, USA) to produce a virtual screening platform. The scoring function combines molecular mechanics force-fields with a continuum treatment of solvation. It was carefully parameterized using a curated experimental-binding set that consists of diverse protein–small molecule or protein–peptide complexes with known binding affinities and X-ray structures.[26] Moreover, SIE-scoring function was subjected to two validation tests. First, its ability to discriminate the native-binding mode or a close conformation from an ensemble of 100 decoy poses was determined. Second, its ability to identify known binders embedded in a random chemical library in a virtual screening context was tested. Overall, SIE scoring function performed extremely well in both tests.

3.3. Protein structure preparation (Bcl-X_L, Bcl-2 and Mcl-1)

In order to explore in silico compound binding onto Bcl-2 protein family, 2 X-ray complexes of Bcl-X_L and Bcl-2 and solution structure of Mcl-1 were considered, that is, (i) BclX_L (pdb entry

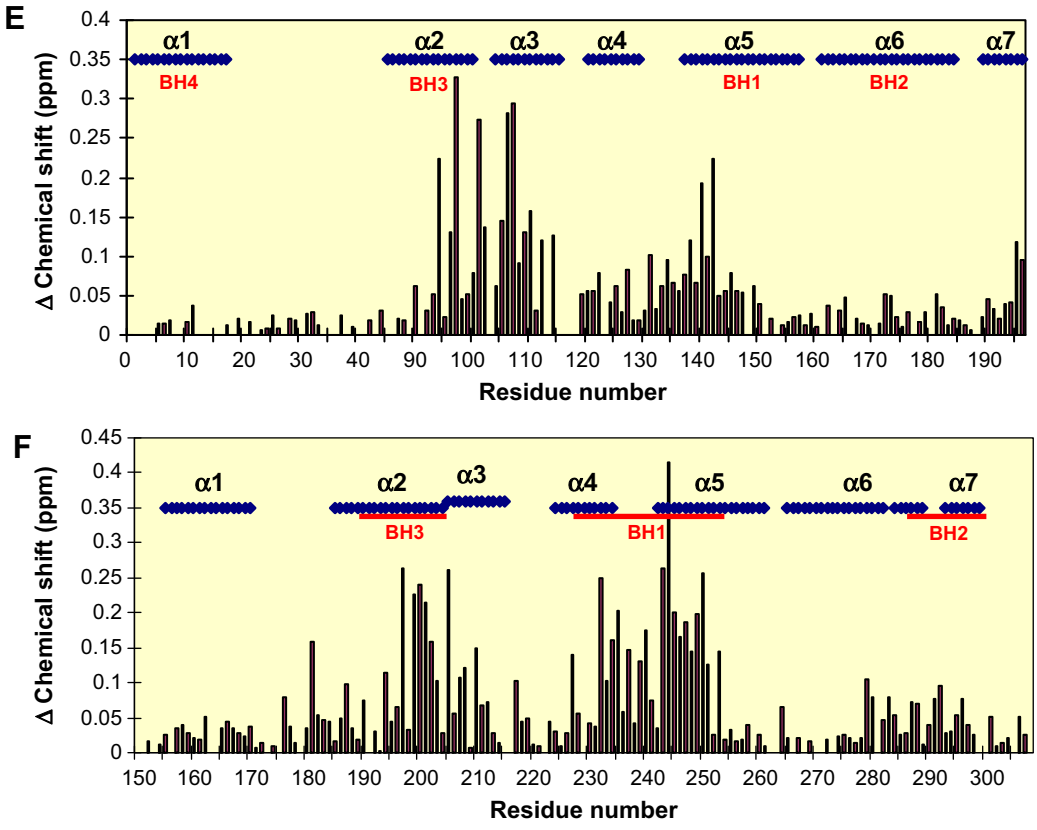


Figure 4. (*continued*)

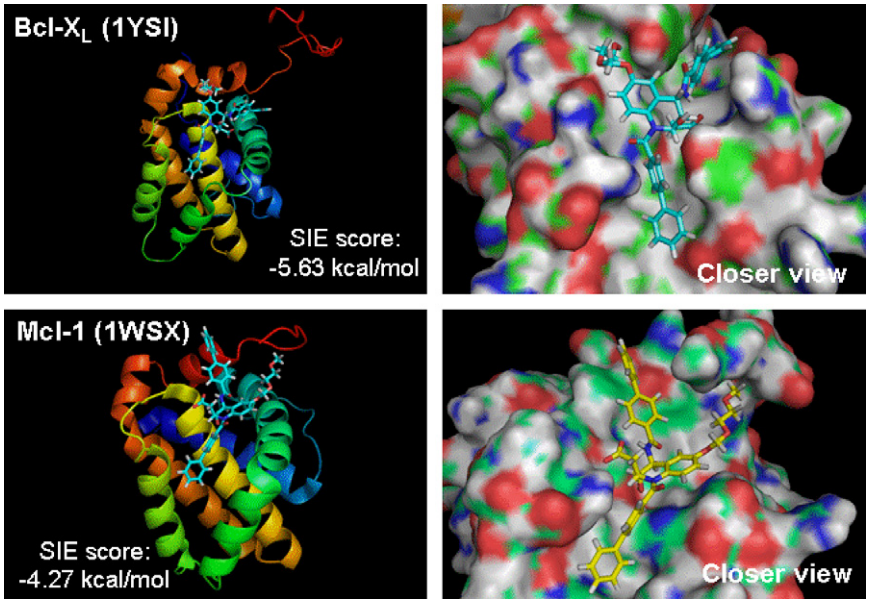


Figure 5. In silico docking of **17** with Bcl-X_L and Mcl-1.

1YSI and 1YSN),[6] (ii) Bcl-2 (pdb entry 1YSW),[6] and (iii) Mcl-1 (pdb entry 1WSX).[27] Blocking groups are added to C- and N-termini ends of both proteins, NH_3^+ and COO^-, respectively. Hydrogen atoms are also added for whole proteins. As binding assay occurs at neutral pH, histidine side-chains are kept neutral. Besides histidine residues, any other ionizable residue side-chains were systematically charged (Lysine, Aspartate and Glutamate). For the Bcl-X_L and Bcl-2 structures, the small molecule inhibitor was removed from the structure to allow docking program to explore the whole protein including the active site. Each protein was care-

fully prepared for force-fields and scoring function compatibility, that is, partial atomic charges, atom-typing, bond-stretching, angle-bending and torsion angles. Only hydrogen atoms are submitted to a short minimization to optimize their orientation and remove VdW clashes.

3.4. Small molecules preparation

The ligands were built in Isis Base and converted from 2D to 3D using concord program within sybyl (Tripos Inc.). The structures were then used to generate GAFF atom types using the antechamber module of AMBER.[28] This facility automatically generates parameters that are compatible with the AMBER force-field (atom types, bond stretch, and angle bend, torsional and improper torsional parameters). The automatically assigned GAFF atom types were then manually checked and corrected as necessary. Partial charges for the ligands were calculated using the AM1-BCC method of assigning partial charges.[29]

3.5. Docking and scoring

All the members of the library were used in a virtual screening on two anti-apoptotic proteins: Bcl-X_L Protein protein (1YSI and 1YSN) and Bcl-2 (1YSW). We were pleased to find several compounds from the library showing good scores. For Bcl-X_L (1YSI), the two compounds **A** and **B** as well as for Bcl-X_L (1YSN), the compound **C** was found as lead compounds (Fig. 1). Concerning the protein Bcl-2 (1YSW) the same compound **C** was found as a lead compound but with a lower-binding affinity (Fig. 2).

3.6. NMR studies: protein sample preparation and NMR spectroscopy

Mouse Bcl-X_L containing deletion in the C-terminus (Δ197–233) and the internal loop (Δ45–84) and mouse Mcl-1 (152–308 amino acids) were prepared as described earlier for Bcl-X_L[30] and Mcl-1.[27] The pET-29b+ plasmid for Bcl-X_L and pGEX-6P-1 for Mcl-1 were used, and proteins were expressed in *Escherichia coli* BL21 cells. For NMR studies, cultures were grown in M9 media supplemented with ^{15}N ammonium chloride to produce uniformly ^{15}N-labeled proteins. Soluble Bcl-X_L protein was purified by Ni^{2+}-affinity chromatography, and GST-Mcl-1 protein was purified by affinity chromatography using glutathione–Sepharose 4B and cleaved with PreScission Protease. NMR samples contained 0.1 mM protein in 80% H_2O/10% DMSO-d_6/10% D_2O, 20 mM sodium phosphate (pH 6.8), 5 mM EDTA, and 3 mM DTT.

NMR spectra were recorded on Bruker DRX 600 MHz spectrometer equipped with triple-resonance cryoprobe. ^{15}N–^1H HSQC spectra were recorded at 1:2, 1:1, 3:1, and 10:1 drug to protein ratios and temperature 35 °C for Bcl-X_L or 25 °C for Mcl-1. Values of the amide chemical shift changes were calculated as $[(\Delta^1H \text{ shift})^2 + (\Delta^{15}N \text{ shift} \times 0.2)^2]^{1/2}$ in ppm. Dissociation constants (K_D) were determined from the changes of chemical shifts versus drug concentration with precision ~50% of K_D value.

Acknowledgments

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Supplementary data

Experimental details and full characterization data for all new compounds are provided. This material is available free of charge. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.06.023.

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