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# Structure-based discovery of a new class of Bcl-$x_L$ antagonists

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**Abstract**

Apoptosis, or programmed cell death, plays a key role in normal tissue homeostasis ensuring a proper balance between cell production and cell loss. Anti-apoptotic Bcl-2-family proteins are central regulators of the apoptotic pathway and due to their ability to confer tumor resistance to chemotherapy or radiation, have been recently validated as targets for cancer drug discovery. Since the crucial interaction between pro- and anti-apoptotic members occurs via a conserved region located on the surface of the protein, a viable way to inhibit the anti-death activity of Bcl-2 proteins is to design small molecule inhibitors that occupy this cavity. Here, we describe a structure-based approach that led to the identification of four small molecule inhibitors directed at the hydrophobic groove on the surface of the Bcl-2 family protein Bcl-$x_L$. The compounds were characterized in a number of assays including in vitro binding using $^{15}$N-labeled protein, a displacement DELFIA assay, and a cell-based viability assay with human cancer cells.


*Keywords:* Apoptosis; Bcl-2; Bcl-$x_L$; Structure-based; NMR; DELFIA

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## 1. Introduction

Apoptosis represents an efficient mechanism adopted by multicellular organisms in order to eliminate superfluous or damaged cells [1]. Disruption of this mechanism is implicated in several human malignancies [2,3]. Central regulators of the apoptotic pathway are proteins belonging to Bcl-2 (B-cell lymphocyte/leukemia-2) family. Anti-apoptotic Bcl-2 proteins, such as Bcl-$x_L$, are over-expressed in most human cancer types and therefore are very attractive targets for the development of anticancer agents [4–13]. While Bcl-$x_L$ and its closest relatives such as Bcl-2, Bfl-1, Mcl-1, Bcl-W and Bcl-B, promote cell survival, the structurally similar pro-apoptotic members such as Bak, Bax, Bad, Bim or Bid, promote cell death. The critical mechanism by which pro- and anti-apoptotic members interact, modulating the apoptotic machinery, involves the hydrophobic groove on the surface of the anti-apoptotic members and the BH3 dimerization domain of the pro-apoptotic counterparts [3]. Currently the most followed approach aimed at designing inhibitors of anti-apoptotic Bcl-2 family proteins is the discovery of small molecules BH3 mimics. We have recently investigated and reported the ability of several natural and semisynthetic compounds [12–14] to antagonize the binding of BH3 peptides to Bcl-2 and Bcl-$x_L$. Here, we report the design and the exploration of the binding mode of new synthetic compounds targeting Bcl-$x_L$.

## 2. Materials and methods

### 2.1. Virtual docking

Docking studies were performed using the crystal structure of Bcl-$x_L$ in complex with BAK-derived peptide [17], Protein Data Bank code 1BXL. Docking geometries were obtained by using FlexX (five solutions per molecule) implemented on a $32 \times 3.2$-GHz CPUs Linux cluster. Sixteen thousand compounds were subsequently docked and ranked according to the software FlexX using a modified scoring function as we have recently reported [15]. Top 500 ranking compounds were further investigated to check for H-bonding with Bcl-$x_L$. Subsequent visual inspection reduced potential candidates with improbable binding geometries from 500 to 320 compounds. The selected 320 compounds (Maybridge) were experimentally tested by NMR. Hit compounds were further tested in additional NMR binding, in vitro displacement and cell-based assays. Comparison between the docked geometries of our hit compounds, Compound **31** (PDB ID 1YSI) and ABT-737 were obtained with SYBYL (TRYPOS, St. Louis). Molecular surfaces were computed with MOLCAD.

### 2.2. DELFIA (dissociation enhanced lanthanide fluoro-immuno assay)

To each well of 96-wells streptavidin coated plates (Perkin-Elmer), 100 μL of a 10 ng/mL, 4 nM, concentration of biotin-labeled BH3 peptide (Biotin-lc-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R; where lc indicates a hydrocarbon chain of 6 –CH2– groups) is added. After incubation for 1 h, unbound Biotin-BH3 peptide is eliminated with three washing steps. Subsequently, to each well are added 80 μL solution of anti-His Eu-antibody conjugate (100 ng/well; 7.2 μM), 10 μL solution containing a test compound, and

10 μL solution containing His6-Bcl-$x_L$ at concentrations of 150 nM. After 1 h of incubation, each well is washed five times to eliminate unbound protein (and so the Eu-antibody if displaced by the test compound).

The assay buffer from Perkin-Elmer was used in each step. Subsequently, to each well, 200 μL of enhancement solution (Perkin-Elmer) is added, and fluorescence is measured after 30 min incubation (excitation wavelength, 340 nm; emission wavelength, 615 nm). Note that measurements are made in time-resolved mode given the relaxation properties of Eu. Controls include unlabeled peptide, Gossypol and blanks receiving no compounds [16].

### 2.3. NMR experiments

NMR-based screening has been conducted by acquiring $^{13}$C-filtered $^{1}$H, 1D experiments with 500 μL solution of uniformly $^{13}$C-labeled Bcl-$x_L$ at 50 μM concentration, in absence and presence of added compounds, each at 250 μM concentration. The binding mode has been characterized by recording [$^{15}$N, $^{1}$H]-HSQC experiments with 500 μL solution of uniformly $^{15}$N-labeled Bcl-$x_L$ (250 μM concentration) in absence and presence of added compounds, each at 1000 μM concentration. $^{15}$N, $^{13}$C-labeled and unlabeled Bcl-$x_L$ samples were prepared and purified as described previously [17]. Briefly, *Escherichia coli* strain BL21 was transformed with the pET-21b plasmid (Novagen) carrying the gene coding for Bcl-$x_L$ ΔTM (Bcl-$x_L$ deletion mutant lacking the transmembrane domain). To obtain $^{15}$N and $^{15}$N/$^{13}$C labeled protein, bacteria were grown on M9 minimal media supported with 2 g/L of $^{13}$C-Glucose and/or 0.5 g/L of $^{15}NH_4Cl$. Induction of protein expression was carried out at $OD_{600} = 0.6$ with 1 mM IPTG for 4 h at 37 °C. Following cell harvest and lysis by sonication, the protein was purified using a Ni-affinity column (Amersham). The eluate was extensively dialyzed against 40 mM phosphate buffer (pH = 7.5) and 150 mM NaCl. Dose–response curve for compound BI-21C5 has been obtained by monitoring the chemical shift of several protein (50 μM) resonance lines upon titration with the compound. The dissociation constant was obtained by nonlinear fit of the data with PRISM according to the equation $p = \{([To] + [Lo] + K_d) - \sqrt{(([To] + [Lo] + K_d)^2 - 4[Lo][To])}\}/2[To]$, where the parameter $p$ represents the fractional population of bound versus free species at equilibrium, which for fast exchanging ligands is measured as $p = (\delta_{obs} - \delta_{free})/(\delta_{sat} - \delta_{free})$, and $[To]$ and $[Lo]$ represent the concentration of target and ligand, respectively [18].

All experiments were performed with a 600 MHz spectrometer Bruker Avance 600 equipped with four rf channels and $z$-axis pulse-field gradients.

### 2.4. Cell viability assay

For this assay we used the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). ZR-75-1 cells were grown in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and humidified 5% $CO_2$. Cells were seeded at $5 \times 10^3$/well in a 96-well plate in triplicates. On day 2, compounds were added. On day 4, 20 μL of MTS reagent was added to 100 μL of medium. Optical density was then read directly at 490 nM using the automated Victor plate reader. Assays were performed in triplicate.

## 3. Results and discussion

Starting from the 3D structure of Bcl-$x_L$, by using a molecular docking approach, we have selected a small subset of 320 compounds out of a large library of compounds, for their predicted ability to bind to the BH3 binding pocket of Bcl-$x_L$. In order to test the ability of these compounds to bind to Bcl-$x_L$, a $^{13}$C-labeled sample of the protein was prepared and 1D-$^{13}$C-filtered $^{1}$H NMR spectra were collected in absence and presence of test compounds. By observing the aliphatic region of the spectra, binding could be readily detected in these simple experiments due to chemical shift changes in active site methyl groups of Ile, Leu, Thr, Val or Ala (region between −0.8 and 0.3 ppm Fig. 1A). While the $^{13}$C-filter eliminates resonances from solvent and test compounds, the latter rarely display NMR signals in the upfield region of the spectrum. Therefore, a similar screening strategy could be obtained simply by working with unlabeled protein. The advantage of these simple 1D NMR screenings versus more traditional 2D [$^{15}$N–$^{1}$H] or even [$^{13}$C–$^{1}$H]-HSQC experiments is that a relatively low protein concentration (5–50 μM) could be used to obtain spectra in a reasonably short measurements times (10–40 min). Using $^{13}$C-filtered $^{1}$H, 1D NMR experiments and $^{13}$C-labeled Bcl-$x_L$, we have screened the above subset of selected molecules and we found four small molecules (Table 1) capable to significantly modify signals coming from amino acids in the active site of our protein. The first quantitative binding data has been achieved for compound BI-21C5, by executing an NMR titration using $^{13}$C-filtered $^{1}$H, 1D NMR experiments (Fig. 1B). In order to asses whether these compounds were capable of displacing the interactions between Bcl-$x_L$ and its pro-apoptotic counterparts, we have tested them in a heterogeneous lanthanide-based assay that we have recently developed [16] based on the DELFIA (dissociation enhanced lanthanide fluoro-immuno assay) technology (Perkin-Elmer). In our format, a biotin-labeled BH3 peptide (Biotin-lc-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R) from the protein Bak is bound to streptavidin-coated plates. Then His6-Bcl-$x_L$ and a solution containing Eu-labeled anti-His6 antibody (Perkin-Elmer) are added together with a test inhibitor and then incubated to promote binding. After several washing steps to eliminate unbound Bcl-$x_L$, an enhancing solution is added to deliver the lanthanide from the antibody to the solution. Residual Eu fluorescence is then detected with an excitation at 340 nm and emission at 615 nm. If the test compound is capable of dissociating Bcl-$x_L$ from the biotinylated-Biotin-BH3 peptide, the antibody carrying the label (Eu) will be washed out and reduced fluorescence will be detected. A typical binding curve of Biotin-BH3 as a function of the concentration of Bcl-$x_L$, that gives the dissociation constant for the complex, is reported in Fig. 1C. The $k_d$ value for this peptide in such assay is 11.8 nM as we previously reported [16]. At this point, by using a Bcl-$x_L$ concentration corresponding to the flex point in the latter curve, hit compounds have been tested. A typical displacement curve is reported in Fig. 1D. $IC_{50}$ values obtained with this method compare very well with displacement values obtained by Fluorescence Polarization Assay [12,19] (Fig. 1E and F). All compounds displaced the BH3 peptide with $IC_{50}$ values in the low micromolar range, thus comparably well to most of the currently available Bcl-$x_L$ inhibitors [4–14, 20–23]. Once we have established the BH3-displacement activity of our hits, in order to study the binding mode of these compounds to Bcl-$x_L$ we have used [$^{15}$N,$^{1}$H]-HSQC NMR experiments in the presence and absence of added compounds (Fig. 2A). Based on the available resonance assignments we have also mapped chemical shift changes induced by the compounds into the 3D structure of Bcl-$x_L$ in

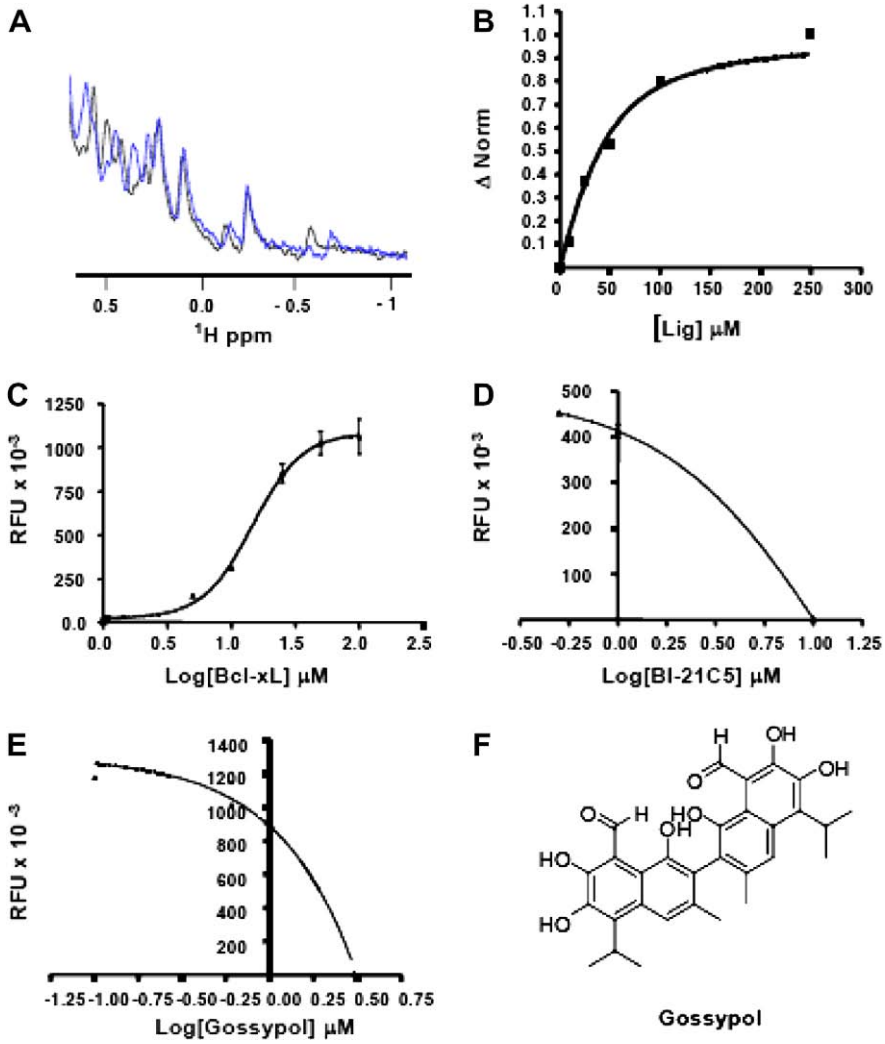
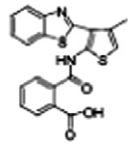
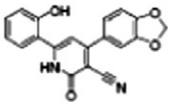
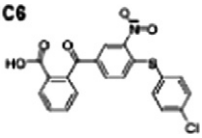
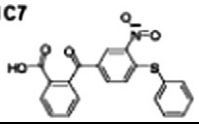



Fig. 1. *In vitro* characterization of Bcl-$x_L$ inhibitors. (A) Aliphatic region of the $^{13}C$-filtered 1D $^{1}H$ NMR spectrum of Bcl-$x_L$ reported in absence (black) and presence (blue) of a test compound; (B) binding curve for compound BI-21B5 obtained by following the chemical shift changes in the $^{13}C$-filtered 1D $^{1}H$ NMR spectrum of Bcl-$x_L$ upon titration; (C) dose–response curve of the binding of Bcl-$x_L$ to a Bak BH3 peptide in the TR-FRET assay; (D) displacement curve for compound BI-21B5 in the TR-FRET assay; (E) displacement curve and chemical structure (F) of Gossypol. The $IC_{50}$ value obtained with the DELFIA is 1.1 μM, which compares well with the value reported by Fluorescence Polarization Assay [12,19]. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

complex with BAK peptide [17]. This data demonstrates that most of the resonances that are significantly affected by the compound are indeed located in the BH3 binding pocket of Bcl-$x_L$ (Fig. 2B).

When compared with the predicted docked geometries of the compounds, the mapping data also reveal a relatively good agreement (Fig. 3). Interestingly, in agreement with the

Table 1
Chemical structures, inhibition (DELFIA) and binding constants (NMR) relative to the discovered Bcl-$x_L$ antagonists

| CMPD ID | $IC_{50}$ μm |
|---|---|
| **BI-21C4**  | 3.3 |
| **BI-21C5**  | 5.1 $K_d$19.8 μM |
| **BI-21C6**  | 0.5 |
| **BI-21C7**  | 5.7 |

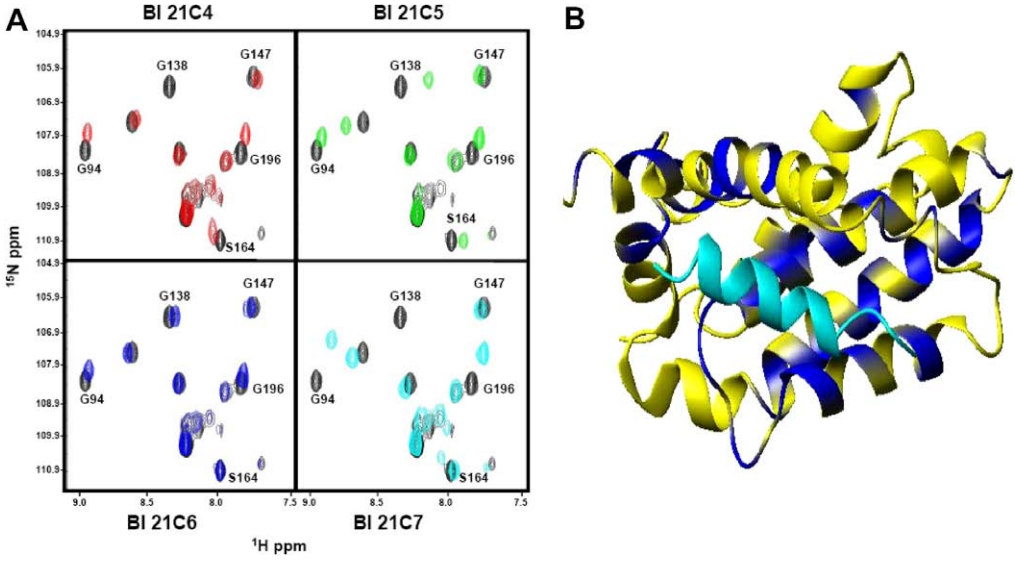



Fig. 2. Chemical shift mapping studies. (A) Portions of the [$^{15}$N, $^{1}$H]-HSQC spectra of Bcl-$x_L$ recorded in absence (black) and in presence of each of the four molecules (in colors). Resonance assignments for amino acid residues that exhibit large shifts are reported; (B) structure of Bcl-$x_L$ in complex with the BH3 peptide from Bak [17] (PDB code 1BXL) showing the chemical shift changes in Bcl-$x_L$ upon ligand binding (blue, large shifts; yellow, no shifts; the Bak peptide is reported in cyan). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

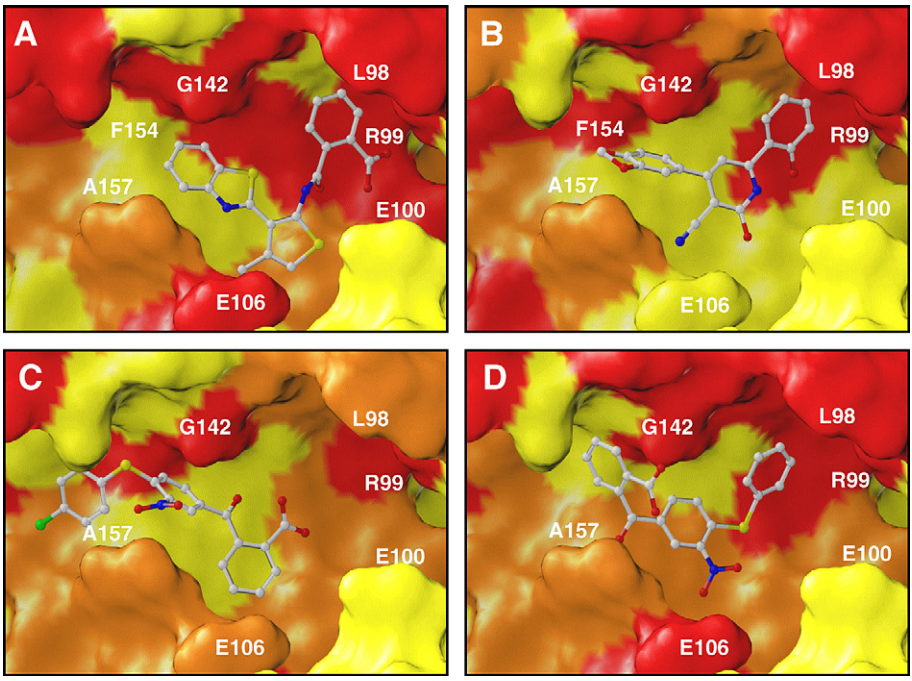



Fig. 3. Comparisons between the docked geometries of the compounds and their induced chemical shift perturbations. The docked structure of each compound is reported and the surface of Bcl-$x_L$ (PDB code 1YSI) is colored according to the magnitude of the chemical shift changes in the amide hydrogen and nitrogen nuclei upon ligand binding (red, large shifts; orange, intermediate shifts; yellow, no shifts). Some of the amino acids located in the BH3 binding pocket of Bcl-$x_L$ are also reported. Mapping studies are reported for compounds BI-21C4, (A); BI-21C5, (B); BI-21C6, (C); BI-21C7, (D). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

predicted geometries, the presence of a p-chlorine group in compound BI-21C6 results in rather different chemical shift perturbation map (Fig. 3C) compared to the parent compound, BI-21C7 (Fig. 3D). These data suggest that there is very good correspondence between the predicted docked geometries of the compounds and the experimental NMR data (Fig. 3).

Recently a potent Bcl-$x_L$ antagonist was derived by using a NMR guided fragment-based approach. Compound **31** [9] and it is optimized version, ABT-737 [10] (Fig. 4A and B), bind on the surface of Bcl-$x_L$ occupying two deep and adjacent pockets, as demonstrated by the NMR structure of the complex [9,10] (Fig. 4A). Compared to this bi-dentate compound, our molecules are predicted to occupy only partially either one or the other subpocket (Fig. 4C–F). For example, compounds BI-21C4, BI-21C5, BI-21C7 all appear to nicely fit into site 2 of the surface of Bcl-$x_L$. These compounds occupy the space covered by the 3-nitro-4-(2-(phenylthio)ethylamino)benzene-sulfonamide moieties present in Compound **31** [9] and ABT-737 [10] (Fig. 4). Therefore, we could envision the design of novel bi-dentate compounds derivatizing our molecules with bi-aryl fragments that occupy site 1. Conversely compound BI-21C6 appears to bind in site 1 in agreement with chemical shift mapping data despite it differs from compound BI-21C7 by the presence of a chlorine

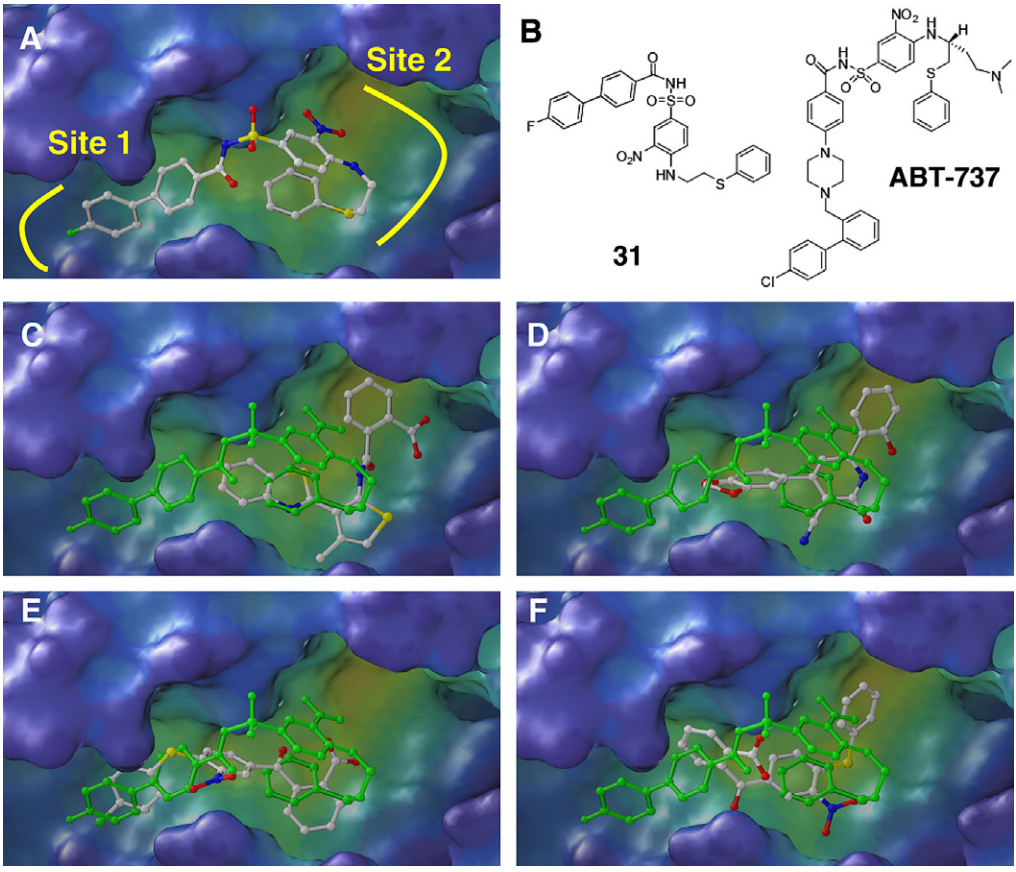



Fig. 4. Docked and chemical structures of Compound **31** and ABT-737 (A and B) and comparison of the docked structures of BI-21B4 (C), BI-21B5 (D), BI-21B6 (E) and BI21-B7 (F) with Compound **31** (PDB ID 1YSI). The figures showing the surface of Bcl-$x_L$ were generated by MOLCAD. The color code is according to cavity depth: blue, shallow; yellow, deep. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

substituent (Table 1 and Fig. 4). It is not surprising that a small modification of chemical structure of a compound results in a very different binding mode [24]. The proposed binding mode for BI-21C6 places the chloro-phenyl group in a position that is very similar to what observed in a similar substructure present in Compound **31** [9] and ABT-737 [10]. Therefore, the docked geometries of our compounds, supported by experimental NMR data, and the comparison with ABT-737, provide a framework that may guide the design of compounds with improved affinities.

Finally, to evaluate also the cytotoxic activity of our compounds on human tumors cells, we tested their biological activities using the ZR-75-1 breast cancer cell line, using as positive controls our previously identified Bcl-$x_L$ inhibitors, gossypol [12] and apogossypol [13] (Fig. 5). Interestingly compound BI-21C5 and BI-21C7 exhibit a cell-based activity as single agents that is approaching that of apogossypol, suggesting that these molecules represent viable leads for the development of more effective agents.

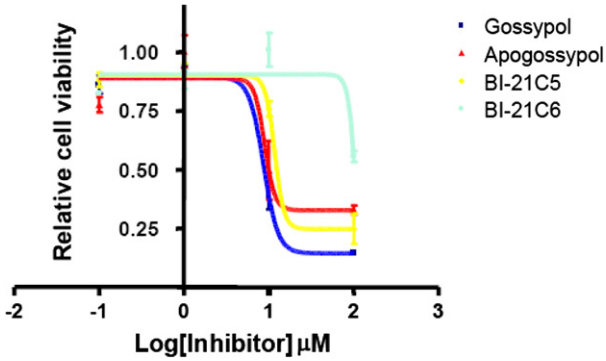



Fig. 5. Cell viability assays. The effect of two of the newly discovered compounds (BI-21B5 $IC_{50}$ 11.7 μM, BI-21B6 117.3 μM) on the viability of the breast cancer ZR-75-1 cell line was assessed by using the MTS assay. The data relative to the known Bcl-$x_L$ antagonists, Gossypol $IC_{50}$ 8.7 μM and Apogossypol $IC_{50}$ 8.9 μM, are also reported.

## 4. Conclusions

In conclusion, we have discovered a new class of compounds that bind to Bcl-$x_L$ in the BH3 binding pocket, that are able to antagonize the binding of BH3 peptides, and that present cellular activity. As such, we believe that the scaffolds validated here, together with structural information on their site of binding and binding mode, may represent valuable starting points for further iterative optimizations aimed at the development of novel apoptosis-based therapies.

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