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Letters

Structure-Based Design of Potent Small-Molecule Inhibitors of Anti-Apoptotic Bcl-2 Proteins

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Abstract: A structure-based approach was employed to design a new class of small-molecule inhibitors of Bcl-2. The most potent compound **5** (TW-37) binds to Bcl-2 with a K_i value of 290 nM and also to Bcl-xL and Mcl-1 with high affinities. Compound **5** potently inhibits cell growth in PC-3 prostate cancer cells with an IC_{50} value of 200 nM and effectively induces apoptosis in a dose-dependent manner.

Bcl-2 is a critical arbiter of apoptosis and functions as a potent anti-death molecule.[1] Bcl-2 is overexpressed in many types of human cancer,[2−4] and such overexpression protects cancer cells from a variety of apoptotic stimuli, including those associated with cancer chemotherapeutic agents, and confers on cancer cells resistance to current therapeutic agents.[2−4] Bcl-2 is a promising molecular target for the design of an entirely new class of anticancer drugs aimed at overcoming resistance of cancer cells to apoptosis.[2−4]

The anti-apoptotic function of Bcl-2 is attributed, at least in part, to its ability to heterodimerize with pro-apoptotic Bcl-2 proteins such as Bad, Bid, and Bim.[1−4] The experimental structures of Bcl-2 and its closely related homologous protein Bcl-xL show that the BH1[a] (Bcl-2 homology domain 1), BH2, and BH3 domains in Bcl-2 and Bcl-xL form a well-defined hydrophobic surface binding groove, known as the BH3 binding groove, into which Bad, Bid, and Bim bind.[5−7] This binding groove in Bcl-2 and Bcl-xL proteins is essential for their anti-apoptotic function.[5−7] We and others have hypothesized that small molecules binding in this BH3 binding groove in Bcl-2/Bcl-xL may be capable of blocking the heterodimerization of Bcl-2 and Bcl-xL with pro-apoptotic members in the Bcl-2 protein family. This in turn may inhibit the anti-apoptotic function of Bcl-2 and Bcl-xL and induce apoptosis in cancer cells in which Bcl-2/Bcl-xL is overexpressed. Design of non-peptide small molecule inhibitors of Bcl-2 and Bcl-xL is currently an exciting research area for the development of new cancer agents.[8−14]

Using structure-based database screening, we discovered that (−)-gossypol[15] (**1**), a natural product isolated from cotton seeds and roots, binds to Bcl-2 and Bcl-xL with quite high affinities. (−)-Gossypol has K_i values of 320 and 480 nM for Bcl-2 and Bcl-xL proteins, respectively, in our competitive FP-based binding assays (Table 1 and Supporting Information). The binding of racemic gossypol to Bcl-xL has been confirmed independently.[12] Interestingly, our competitive binding data show that (−)-gossypol also binds to Mcl-1 protein, another anti-apoptotic Bcl-2 member, with a K_i value of 180 nM (Table 1). (−)-Gossypol is currently being clinically evaluated as an anticancer drug and demonstrates acceptable and manageable toxicity with evidence of single-agent antitumor activity in patients with advanced malignancies.[16] To date, (−)-gossypol is the only orally available small-molecule inhibitor of Bcl-2, Bcl-xL, and Mcl-1 that has advanced into clinical trials. Hence it represents a promising lead compound for the development of non-peptidic small-molecule inhibitors of anti-apoptotic Bcl-2 proteins as a new class of anticancer drugs. Herein, we wish to report our structure-based design of a new class of small-

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[a] Abbreviations: BH1−3, Bcl-2 homology domain 1−3; FP, fluorescence polarization; ELISA, enzyme-linked immunosorbent assay; HSQC, heteronuclear single quantum coherence; TUNEL, terminal dUTP nick-end labeling; WST, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt.

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Table 1. Binding Affinities of Small-Molecule Inhibitors to Bcl-2, Bcl-xL, and Mcl-l as Determined Using a Fluorescence-Polarization-Based Binding Assay (FP) and Enzyme-Linked Immunosorbent Assay (ELISA)[a]

	Bcl-2		Bcl-xL	Mcl-l
compound	FP $K_i \pm$ SD (μM)	ELISA $IC_{50} \pm$ SD (μM)	FP $K_i \pm$ SD (μM)	FP $K_i \pm$ SD (μM)
(−)-gossypol	0.32 ± 0.02	0.5 ± 0.1	0.48 ± 0.04	0.18 ± 0.01
2	24.1 ± 2.1	53.1 ± 0.1		
3	8.3 ± 1.1	7.2 ± 1.6		
4	0.93 ± 0.11	1.6 ± 0.5		
5	0.29 ± 0.06	0.7 ± 0.3	1.11 ± 0.4	0.26 ± 0.01
6	25[b]			
Bid BH3 peptide[c]	0.011 ± 0.001		0.007 ± 0.001	0.012 ± 0.001
Bim BH3 peptide[d]		0.011 ± 0.003		

[a] Three to seven independent experiments were performed for each compound. [b] Partial binding was observed at 100 μM, and the K_i value was an estimate. [c] Residues 79−99 (also see Supporting Information). [d] Residues 81−106 (also see Supporting Information).

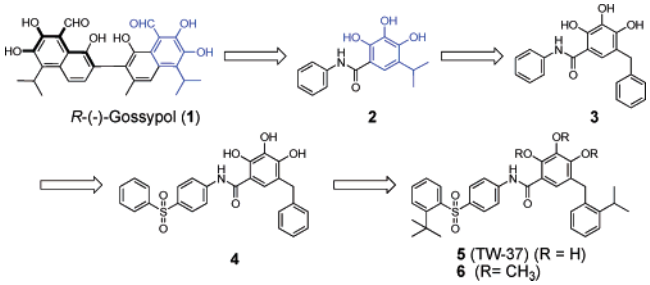

Figure 1. Structure-based design of a new class of Bcl-2 inhibitors.

molecule inhibitors of Bcl-2, using (−)-gossypol as the initial lead compound.

To understand the structural basis of (−)-gossypol binding to Bcl-2, we have performed computational docking of (−)-gossypol into the BH3 binding groove in Bcl-2 (Supporting Information). We have also modeled the binding of a Bim BH3 peptide in a complex with Bcl-2 since this Bim BH3 peptide binds to Bcl-2 with a high affinity (Table 1). Based upon the predicted binding model (Figure 2), (−)-gossypol forms a hydrogen bonding network with residues Arg146 and Asn143 in Bcl-2 through the aldehyde group and the adjacent hydroxyl group on the right naphthalene ring. This mimics the hydrogen bonding network formed by Asp99 and Asn102 in Bim and Arg146 and Asn143 in Bcl-2 (Figure 2). The isopropyl group on the same naphthalene ring inserts into a hydrophobic pocket in Bcl-2, in part mimicking the Phe101 in the Bim peptide. The left half of the (−)-gossypol molecule interacts primarily with Bcl-2 through hydrophobic contacts, mimicking Ile97 in the Bim peptide (Figure 2). This binding model suggests that the two halves of (−)-gossypol interact differently with Bcl-2 and provides a structural basis for the design of novel small-molecule inhibitors of Bcl-2.

Based upon our predicted binding model for (−)-gossypol (Figure 2), we have sought to design completely new structural classes of compounds that mimic the interaction between (−)-gossypol and Bcl-2. The binding model calls for one-half of the (−)-gossypol molecule to form an extensive hydrogen bonding network with Bcl-2. Modeling shows that a polyphenol ring containing three hydroxyl groups can mimic the hydrogen bonding network between (−)-gossypol and Bcl-2, and this led to the design of our initial template compound **2** (Figure 1). In **2**, an isopropyl group was installed on the polyphenol ring to mimic the hydrophobic interaction formed between the isopropyl group on the right naphthalene ring of (−)-gossypol and Bcl-2.

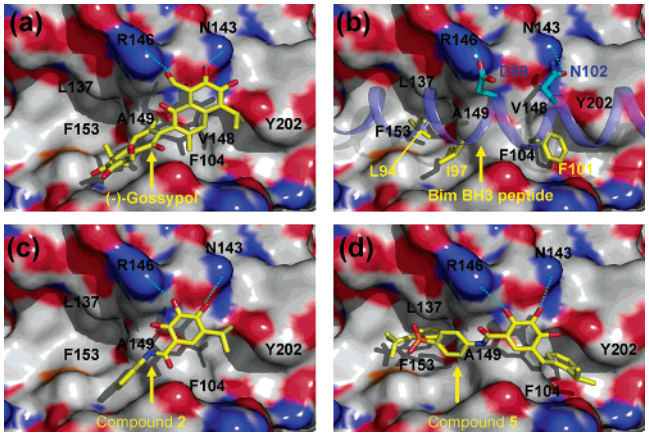

Figure 2. Predicted binding models of Bcl-2 in complex with (a) (−)-gossypol, (b) Bim BH3 peptide, and designed compounds (c) **2** and (d) **5**. Bcl-2 is shown in surface representation where carbon, oxygen, nitrogen, and sulfur atoms are colored in gray, red, blue, and orange, respectively. The carbon and oxygen atoms in gossypol, **2**, and **5** are shown in yellow and red, respectively. The Bim BH3 peptide is shown as a light-blue helix. Hydrogen bonds are depicted as dotted lines in cyan.

We used a simple phenyl ring tethered to the polyphenol ring via an amide linker to mimic the hydrophobic interaction between this part of (−)-gossypol and Bcl-2. Our predicted binding model (Figure 2) shows that **2** indeed mimics most, but not all, of the crucial interactions observed between (−)-gossypol and Bcl-2.

Compound **2** was synthesized (Supporting Information) and determined to bind to Bcl-2 with a K_i value of 24.1 μM in our FP-based binding assay (Table 1). Consistent with our modeling prediction (Figure 2), **2** has a significant binding affinity to Bcl-2 but is 80-times less potent than (−)-gossypol.

Analysis of the binding models for (−)-gossypol and **2** suggests that **2** can be further optimized to enhance its interaction with Bcl-2. For example, the hydrophobic pocket occupied by the isopropyl group in **2** and in (−)-gossypol can accommodate a larger hydrophobic group. Modeling suggests that a benzyl group may be used to replace the isopropyl group to optimize the interaction at this site (Supporting Information), and this led to the design of **3** (Figure 1 and Supporting Information). Compound **3** was synthesized (Supporting Information) and was determined to bind to Bcl-2 with a K_i value of 8.3 μM (Table 1). Hence, **3** is 5-times more potent than **2**, supporting our modeling prediction.

The binding model of the Bim BH3 peptide in the complex with Bcl-2 suggested that the hydrophobic pocket occupied by Leu94 in Bim is not utilized by **3** (Figure 2 and Supporting Information), and it was predicted that capture of this hydrophobic interaction should yield new inhibitors with higher affinities. This led to the design of **4** (Figure 1), which contains a phenyl ring tethered through a sulfone linker to the *para*-position of the phenyl ring in **3**. Modeling studies showed that this additional phenyl group in **4** inserts into the hydrophobic pocket occupied by the Leu94 in the Bim BH3 peptide (Supporting Information) and predicted that **4** should have a higher binding affinity to Bcl-2 than **3**. Compound **4** was synthesized (Supporting Information) and found to bind to Bcl-2 with a K_i value of 0.93 μM (Table 1), an affinity 8-times higher than that of **3**.

Analysis of the binding model of **4** to Bcl-2 (Supporting Information) showed that the hydrophobic interaction between **4** and Bcl-2 as mediated by the two terminal phenyl groups

can be further optimized at these two sites. Guided by molecular modeling, compound **5** was designed, in which an isopropyl and a *tert*-butyl were installed on the *ortho*-positions of these two terminal phenyl rings. Modeling predicted that **5** would bind more potently than **4** (Figure 2 and Supporting Information). Compound **5** was synthesized in eight steps (Supporting Information) and determined to bind to Bcl-2 with a K_i value of 290 nM. Therefore, **5** is 3-times more potent than **4** and 80-times more potent than the initial compound **2**.

Our modeling results suggest that the hydroxyl groups in **5** play an important role in the binding to Bcl-2 through the formation of two hydrogen bonds (Figure 2). To investigate the importance of these hydroxyl groups, we designed and synthesized **6** (Figure 1 and Supporting Information), in which all three hydroxyl groups in **5** are replaced by methoxyls. Our FP-based binding assay determined that **6** has an estimated K_i value of 25 μM and is thus nearly 100-times less potent than **5**, confirming the importance of these hydroxyl groups for the binding of **5** to Bcl-2.

Since (−)-gossypol binds to Bcl-xL and Mcl-1 proteins, we have further evaluated **5** for its binding affinities to these two Bcl-2 members using FP-based assays developed in our laboratory for these two proteins (Supporting Information). It was found that **5** binds to Bcl-xL and Mcl-1 with K_i values of 1100 nM and 260 nM, respectively (Table 1).

FP-based assays can be influenced by the autofluorescence of the tested inhibitors. We have developed an enzyme-linked immunosorbent assay (ELISA) for Bcl-2 (Supporting Information) and determined the binding of our designed inhibitors to Bcl-2. As can be seen from Table 1, the relative binding affinities for these inhibitors in our ELISA assay are highly consistent with those obtained from the FP-based assay, and compound **5** binds to Bcl-2 with an IC_{50} value of 0.7 μM in our ELISA assay.

Compound **5** was designed to bind to the BH3 binding groove in Bcl-2 protein, competing with BH3 peptides derived from Bid, Bim, and Bad proteins. To further probe the mode of action of **5**, we have performed heteronuclear single quantum coherence (HSQC) NMR spectroscopy using uniformly ^{15}N-labeled Bcl-2 protein. ^{15}N HSQC spectra are very sensitive to structural changes in the protein, and subtle changes such as an interaction with a small molecule inhibitor will induce shifts in peak positions in the spectra. Hence, the HSQC NMR spectroscopy provides a convenient way to conclusively confirm the binding of a small molecule inhibitor to Bcl-2 and to identify where the inhibitor binds on the protein.

Using ^{15}N labeled Bcl-2 (isoform 2),[5] ^{15}N HSQC NMR spectra of Bcl-2 were recorded with or without compound **5**. The NMR spectra of Bcl-2 alone showed well-dispersed peaks, indicative of a folded and stable protein. The comparison of the two HSQC spectra showed induced shifts in several peak positions, indicating that compound **5** interacts with several residues on Bcl-2 (Figure 3). Since the backbone chemical shift assignments of Bcl-2 are yet to be completed, we compared the induced shift patterns on Bcl-2 for compound **5** and a Bid BH3 peptide. This Bid BH3 peptide (Table 1) is known to bind to the BH3 binding groove on Bcl-2 and has a K_i value of 11 nM to Bcl-2 in our FP-based competitive binding assay (Table 1). The residues in Bcl-2 affected by **5** overlap nicely with those by the Bid BH3 peptide (Figure 3), strongly suggesting that **5** and the Bid BH3 peptide bind to the same site on Bcl-2. The number of residues on Bcl-2 affected by the Bid BH3 peptide was greater than the number affected by compound **5**, due presumably to the fact that the Bid peptide covers a larger

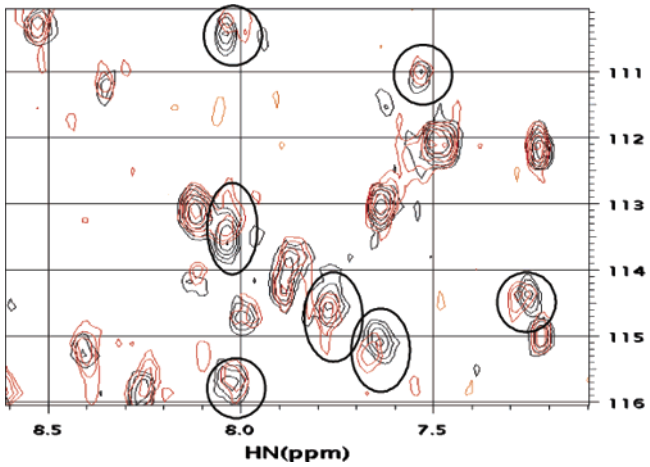

Figure 3. Superposition of two ^{15}N-HSQC spectra of Bcl-2 in free form (black) and with compound **5** (red). Peaks circled in black showed induced shifts when the Bid BH3 peptide was added to BCl-2.

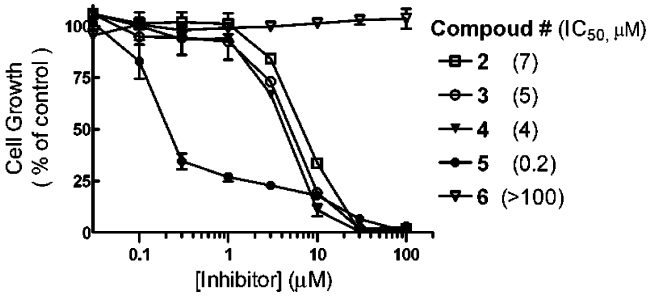

Figure 4. Inhibition of cell growth in human prostate cancer PC-3 cells as determined using a WST-based assay.

binding area than that covered by compound **5** in the BH3 binding groove on Bcl-2.

A major advantage for non-peptide small-molecule inhibitors of Bcl-2 over BH3 peptides is that they may have much superior cell permeability and thus much better cellular activity. We have evaluated our designed small-molecule inhibitors for their ability to inhibit cell growth in PC-3 human prostate cancer cells with a high level of Bcl-2 (Figure 4). Compound **5** inhibits cell growth in PC-3 cancer cells with an IC_{50} value of 200 nM. Significantly, the IC_{50} values of compounds **2**−**6** in inhibition of cell growth in PC-3 cells correlate reasonably well with their binding affinities to Bcl-2.

Since Bcl-2, Bcl-xL and Mcl-1 proteins function as critical apoptosis regulators and potent anti-apoptotic molecules,[1−4] it is predicted that concurrent inhibition of these Bcl-2 members by **5** may effectively induce apoptosis in cancer cells. To test this prediction, we have evaluated **5** for its ability to induce apoptosis in PC-3 cells using the TUNEL assay (Figure 5). Compound **5** effectively and dose-dependently induces apoptosis in PC-3 cells. At 1.25 and 5 μM, 11.2% and 89.5% of cells underwent apoptosis when cells were treated for 4 days, while 2.6% of cells underwent apoptosis in an untreated control.

In summary, our structure-based design has led to the discovery of a potent small-molecule inhibitor of compound **5**. Although (−)-gossypol was used as the starting point for our design, compound **5** belongs to a totally new chemical class different from that of (−)-gossypol. Compound **5** binds to Bcl-2 and Bcl-xL with K_i values of 290 and 1110 nM, respectively. Interestingly, unlike *N*-{4-[4-(4′-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl}-4-((*R*)-3-(dimethylamino)-1-phenylsulfanylmethyl-propylamino)-3-nitro-benzenesulfonamide (ABT-737),[13] a previously reported potent small-molecule inhibitor

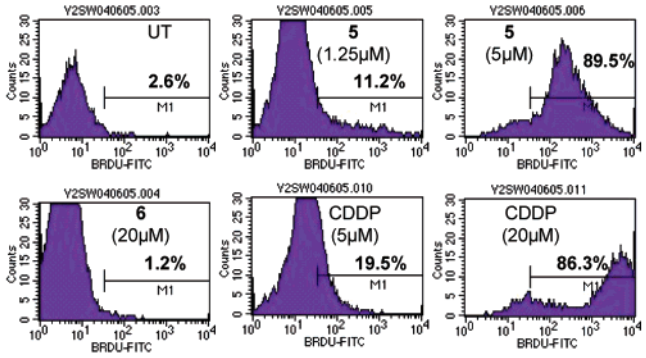

Figure 5. Apoptosis induction by compound 5 and its inactive analogue **6** in human prostate cancer PC-3 cells, in comparison to cisplatin (CDDP). PC-3 cells were treated with **5**, **6**, or CDDP for 4 days. Apoptosis was measured with flow cytometry using a TUNEL assay. A histogram of BrdU-FITC-positive cells (*x*-axis) versus counts (*y*-axis) has been displayed. Cells undergoing apoptosis are represented as percentages (M1 marker).

of Bcl-2 and Bcl-xL, **5** also binds potently to Mcl-1, a Bcl-2 homologous protein, with a K_i value of 260 nM. Thus, **5** and (−)-gossypol represent a new type of small-molecule inhibitors that concurrently target Bcl-2, Bcl-xL, and Mcl-1 proteins. Such inhibitors may be more effective inducers of apoptosis in cancer cells, especially those with high levels of Mcl-1, than compounds that target only Bcl-2 and Bcl-xL. Taken together, our data indicate that **5** is a potent, cell-permeable small-molecule inhibitor that targets multiple anti-apoptotic Bcl-2 members and is therefore a promising lead compound for further optimization and development as a novel therapy for the treatment of human cancer.

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Supporting Information Available: An experimental section including information on the synthesis and chemical data for compounds **2**−**6**, molecular modeling methods and results for **2**−**6**, the experimental procedures for the fluorescence polarization-based binding assays for Bcl-2, Bcl-xL, and Mcl-1 and the enzyme-linked immunosorbent assay for Bcl-2, and details on the cellular growth inhibition and apopposis assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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