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Letters

Pyrogallol-Based Molecules as Potent Inhibitors of the Antiapoptotic Bcl-2 Proteins

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Abstract: We report herein a new class of small-molecule inhibitors of antiapoptotic Bcl-2 proteins. The most potent compound, **7**, binds to Bcl-2, Bcl-xL, and Mcl-1 proteins with K_i of 110, 638, and 150 nM, respectively. Compound **7** is highly effective in induction of cell death in breast cancer cells with high levels of Bcl-2, Bcl-xL, and Mcl-1 proteins and represents a promising lead compound for the design of new anticancer drugs.

Apoptosis, or programmed cell death, is a critical cell process in normal development and homeostasis of multicellular organisms. Inappropriate regulation of apoptosis has been implicated in many human diseases, including cancer.[1−3] Targeting critical apoptosis regulators is an attractive therapeutic approach for the development of new classes of therapies for the treatment of cancer and other human diseases.[1]

The Bcl-2[a] family proteins are a class of central arbiters of apoptosis and consist of antiapoptotic members such as Bcl-2, Bcl-xL, and Mcl-1 and proapoptotic members such as Bim, Bid, Bak, and Bax.[4−7] The antiapoptotic proteins in the Bcl-2 family are overexpressed in many cancer cell lines and human cancer tissues. This overexpression protects cancer cells from the induction of apoptosis by current anticancer therapies and plays a role in the failure of conventional anticancer drugs.[4−7] Consequently, these antideath Bcl-2 proteins are considered to be promising molecular targets for the design of novel anticancer drugs.

Although the precise mechanism by which Bcl-2 proteins regulate apoptosis in cells is still under intense investigation,[8] it is very clear that these antiapoptotic Bcl-2 proteins effectively inhibit apoptosis, at least in part, by directly binding to proapoptotic Bcl-2 proteins such as Bim, Bid, Bak, and Bax and blocking their proapoptotic activity. Experimentally determined three-dimensional structures of Bcl-2, Bcl-xL, and Mcl-1 by NMR or X-ray crystallography showed that the BH1 (Bcl-2 homology domain 1), BH2, and BH3 domains in these proteins form a well-defined, hydrophobic surface binding groove, known as the BH3 binding groove, into which Bad, Bid, and Bim bind.[9−12] Hence, small molecules that are designed to target the BH3 binding groove in these antiapoptotic Bcl-2 proteins are predicted to promote apoptosis in cancer cells by antagonizing their antiapoptotic function. Design of nonpeptidic, cell-permeable, small-molecule inhibitors that bind to the BH3 binding groove in these antideath Bcl-2 proteins is being intensely pursued as a new anticancer therapeutic strategy.[13−22]

Design of nonpeptidic, small-molecule inhibitors to target protein−protein interactions (PPIs) is considered one of the most challenge tasks in modern drug discovery and medicinal chemistry. Nevertheless, significant progress has been made in the past few years in the design of small-molecule inhibitors to target the Bcl-2 PPIs,[13−22] and a number of classes of potent small-molecule inhibitors, shown in Figure 1, have been reported. Among them, **1** binds to Bcl-2, Bcl-xL, and Bcl-w proteins with a very high affinity but does not bind to Mcl-1.[18] Compound **2**, a natural product isolated from cotton seeds, concurrently targets Bcl-2, Bcl-xL, and Mcl-1 proteins with similar affinities[21] and is currently in clinical trials as an orally administered agent for the treatment of multiple forms of human cancer.[22] Using a structure-based strategy, we have recently reported the design of **3** as a new class of potent, cell-permeable small-molecule inhibitor of Bcl-2, Bcl-xL, and Mcl-1 proteins.[21]

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[a] Abbreviations: Bcl-2, B-cell leukemia/lymphoma 2; Mcl-1, (myeloid cell leukemia sequence 1; Bid, BH3 interacting domain death agonist; Bim, Bcl-2 interacting mediator of cell death; Bax, Bcl-2-associated X protein; Bak, Bcl-2 homologous antagonist/killer.

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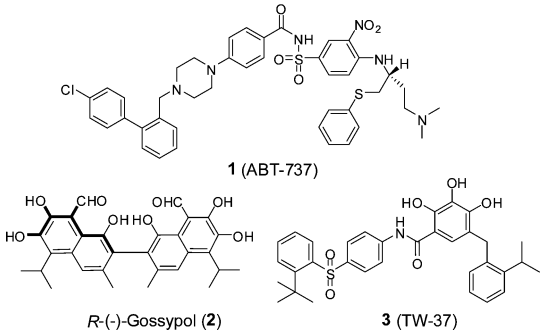


Figure 1. Representative small-molecule inhibitors of Bcl-2 proteins.

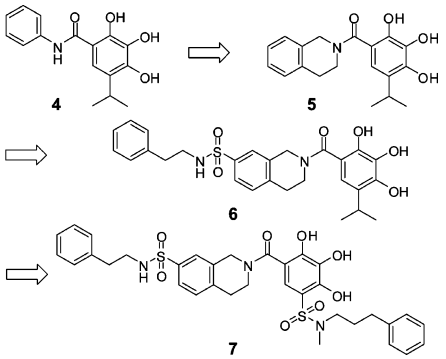


Figure 2. Structure-based design of novel small-molecule inhibitors of Bcl-2 proteins.

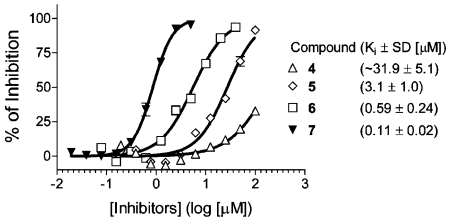


Figure 3. Competitive binding curves of designed small-molecule inhibitors to Bcl-2 as determined using a fluorescence polarization based binding assay. Recombinant human Bcl-2 protein and fluorescently tagged Bid BH3 peptide were employed in this competitive binding assay.

Although Bcl-2 and Bcl-xL proteins have been the primary focus for the design of small-molecule inhibitors to target these proteins,[13−22] recent studies have demonstrated that the Mcl-1 protein plays a crucial role in protecting cancer cells from induction of apoptosis by a variety of anticancer agents. Compound **1** had potent activity only in cancer cells with low levels of Mcl-1 protein but a much weaker activity in cancer cells with high levels of Mcl-1.[23] Knocking down Mcl-1 using siRNA in cancer cells greatly sensitizes the activity of **1**. Hence, small-molecule inhibitors that target not only Bcl-2 and Bcl-xL but also Mcl-1 could be highly effective in induction of cell death in cancer cells with high levels of these proteins. Herein, we report the structure-based design, synthesis, and initial evaluations of pyrogallol-based compounds as novel small-molecule inhibitors of Bcl-2, Bcl-xL, and Mcl-1.

In our previous study,[21] **4** was designed as an initial lead starting from **2** (Figure 2). In our fluorescence polarization (FP) based binding assay, **4** binds to Bcl-2 with a K_i of 31.9 μM (Figure 3). Analysis of its binding model to Bcl-2 (Figure 4 and Supporting Information) showed that two of the hydroxyl groups in its phenyl ring form hydrogen bonds with R146 and N143 in Bcl-2, mimicking the key residue D99 in the Bim BH3 peptide. Its phenyl ring mimics I97, and its isopropyl group partially fills the hydrophobic pocket occupied by F101 in the

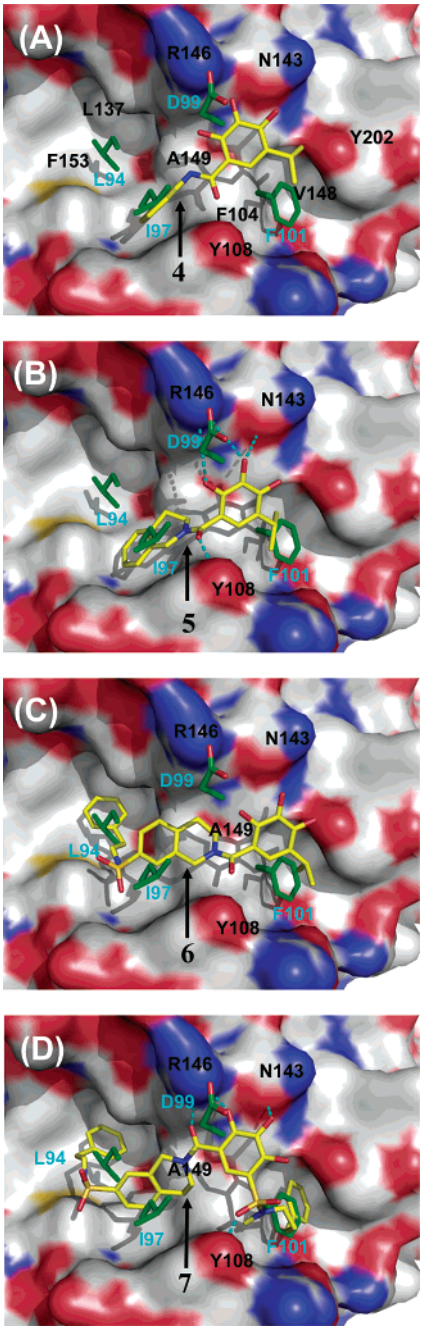
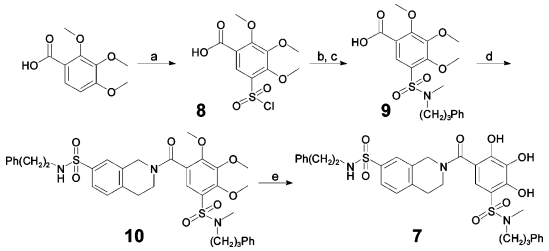


Figure 4. Predicted binding models of designed small-molecule inhibitors **4**−**7** to Bcl-2. For inhibitors, carbon atoms are shown in yellow, oxygen atoms in red, nitrogen atoms in blue, and sulfur atoms in yellow. For protein, carbon atoms are shown in yellow, oxygen atoms in red, nitrogen atoms in blue. Side chains of crucial residues L94, I97, D99, and F101 in the Bim BH3 peptide are displayed with carbon atoms shown in green and oxygen atoms in red.

Bim peptide. The predicted binding model for **4** in complex with Bcl-2 forms the structural basis for our current design and optimization.

On the basis of the binding model for **4**, we designed **5** (Figure 2), in which a fused, conformationally constrained bicyclic system was used to replace the phenyl ring in **4**. Our modeling showed that **5** maintains all the hydrogen bonding observed for **4** binding to Bcl-2 but has enhanced hydrophobic contacts mediated by the tetrahydroisoquinoline ring (Figure 4). Modeling predicted that **5** may bind to Bcl-2 with a higher affinity than **4** (Supporting Information). Compound **5** was synthesized (Supporting Information) and was determined to bind to Bcl-2

Scheme 1. Synthesis of Designed Inhibitor **7**[a]



[a] Reagents and conditions: (a) $ClSO_3H$, room temp, 91%; (b) 3-phenylpropylamine, NEt_3, CH_2Cl_2, 97%; (c) (i) MeI, K_2CO_3, acetone, reflux, overnight; (ii) KOH, MeOH, 60 °C, 3 h, 92%; (d) substituted tetrahydroisoquinoline, EDAC, HOBt, DiPEA, CH_2Cl_2, 85%; (e) BBr_3 (15 equiv), CH_2Cl_2, −78 to −20 °C, 70%.

with a K_i of 3.1 μM (Figure 3). Compound **5** is thus 10 times more potent than **4**, supporting our modeling prediction.

Comparison of the predicted binding models for **5** and the Bim BH3 peptide suggested that the hydrophobic pocket occupied by I94 in the Bim peptide is not utilized by **5** (Figure 4). Taking advantage of this key hydrophobic interaction should further improve the binding affinity. We have thus designed **6** (Figure 2), in which a phenethyl group is attached to the 7-position of the fused bicyclic system through a sulfonamide linker. This facilitates the synthesis and enhances the solubility of the resulting compound. Modeling predicted that the phenethyl group in **6** is inserted into the hydrophobic pocket in Bcl-2 occupied by the I94 residue in the Bim peptide (Figure 4). Compound **6** was synthesized (Supporting Information) and determined to bind to Bcl-2 with a K_i of 590 nM (Figure 3). It is thus 5 times more potent than **5**, providing further support for our design strategy.

Analysis of the predicted binding model for **6** in complex with Bcl-2 showed that its isopropyl group inserts into the hydrophobic pocket occupied by F101 (Figure 4). It is clear, however, that this hydrophobic pocket can accommodate a larger hydrophobic group. Although one could simply replace the isopropyl group with a larger hydrophobic group, the resulting compounds would become too hydrophobic and insoluble. To address this problem, we replaced the isopropyl group with *N*-methyl-*N*-(3-phenylpropyl)sulfamoyl, as in **7**. Modeling predicted that the phenylpropyl group in **7** should significantly improve the hydrophobic interactions with Bcl-2 compared to the isopropyl group in **6**. Importantly, the use of a sulfonamide as the linker leads to a compound with a much improved solubility. Interestingly, our modeling also suggested that the sulfonyl group in **7** forms a hydrogen bond with Y108 (Figure 4).

Compound **7** was synthesized (Scheme 1) and was found to bind to Bcl-2 with a K_i of 110 nM (Figure 3). It is therefore 5 times more potent than **6** and 290 times more potent than the initial lead compound **4**.

Since FP-based assays can be influenced by autofluorescence of the tested inhibitors, we have developed and validated an enzyme-linked immunosorbent assay (ELISA) for Bcl-2 (Supporting Information). Using this ELISA assay, we have determined the binding of our designed inhibitors to Bcl-2. The IC_{50} values for **5**, **6**, and **7** are 38.2 ± 6.2, 6.6 ± 2.7, and 0.33 ± 0.05 μM, respectively, obtained from three independent experiments. Hence, the relative binding affinities for these inhibitors, obtained from the ELISA assay, are consistent with those obtained from the FP-based assay.

We next evaluated **7** for its binding affinities to Bcl-xL and Mcl-1 proteins using our established FP-based binding assays (Supporting Information). Compound **7** has K_i of 638 and 150 nM to Bcl-xL and Mcl-1 proteins, respectively.

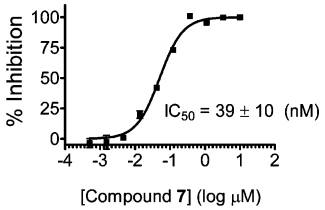


Figure 5. Binding curve of **7** to Mcl-1 protein in competition with a biotinylated Bim BH3 peptide as determined using an ELISA assay.

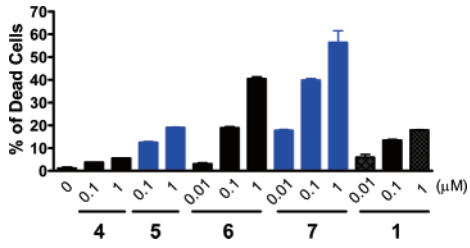


Figure 6. Induction of cell death in the MDA-MB-231 (2LMP) human breast cancer cell line by designed small-molecule inhibitors. Cells were treated with each inhibitor for 96 h, and cell viability was determined using Trypan blue exclusion assay.

To further confirm the binding affinity of **7** to Mcl-1, we have developed and validated an ELISA assay using recombinant Mcl-1 protein and a biotinylated Bim peptide (Supporting Information). In this assay, **7** has an IC_{50} of 39 ± 10 nM to Mcl-1 from three independent experiments (Figure 5). Hence, the FP and the ELISA-based assay showed that **7** binds to Mcl-1 protein with high affinity.

Potent, cell-permeable, small-molecule inhibitors that concurrently target Bcl-2, Bcl-xL, and Mcl-1 proteins are predicted to be highly effective in inducing cell death in cancer cells.[23] We evaluated these compounds for their ability to induce cell death in the MDA-MB-231 (2LMP) human breast cancer cell line, which has high levels of Bcl-2, Bcl-xL, and Mcl-1 proteins (Supporting Information). Each inhibitor induces cancer cells to undergo cell death in a dose-dependent manner (Figure 6). Compound **7** effectively induces cell death at concentrations as low as 10 nM and is much more potent than other compounds. For example, **7** at 100 nM is as effective as **6** at 1000 nM and is much more effective than **4** and **5** at 1000 nM. In direct comparison, **1**, which does not bind to Mcl-1,[18] is 100 times less effective than **7** in cell death induction in the MDA-MB-231 cell line (Figure 6).

In summary, using a structure-based design strategy, we have successfully designed a new class of potent small-molecule inhibitors targeting Bcl-2, Bcl-xL, and Mcl-1 proteins. Our most potent inhibitor, **7**, which was named TM-1206, binds to Bcl-2, Bcl-xL, and Mcl-1 proteins with K_i of 110, 639, and 150 nM, respectively. The binding of **7** to Bcl-2 and Mcl-1 proteins has further been confirmed using ELISA-based assays. Compound **7** is effective in induction of cell death in MDA-MB-231 cancer cells with high levels of Bcl-2, Bcl-xL, and Mcl-1 proteins in a dose-dependent manner. Our present study indicates that concurrently targeting Bcl-2, Bcl-xL, and Mcl-1 proteins using a small-molecule inhibitor is potentially a promising therapeutic strategy for the design of novel anticancer drugs.

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G. Komen Foundation, Ascenta Therapeutics, Inc., and the Intramural Research Program of the National Cancer Institute, Center for Cancer Research.

Supporting Information Available: Experimental details including chemical data for compounds **5**−**7** and details of the fluorescence polarization-based and ELISA binding assays, molecular modeling, and the cell viability assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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