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Task 1. Molecular modeling, structure-based database searching, and computational docking (**1-30 months**).

During the first 12 months of this project, we have largely accomplished the tasks outlined in Task 1 of the original proposal.

Task 1.1. Extensive molecular dynamics simulation of Bcl-2 through molecular dynamics simulations.

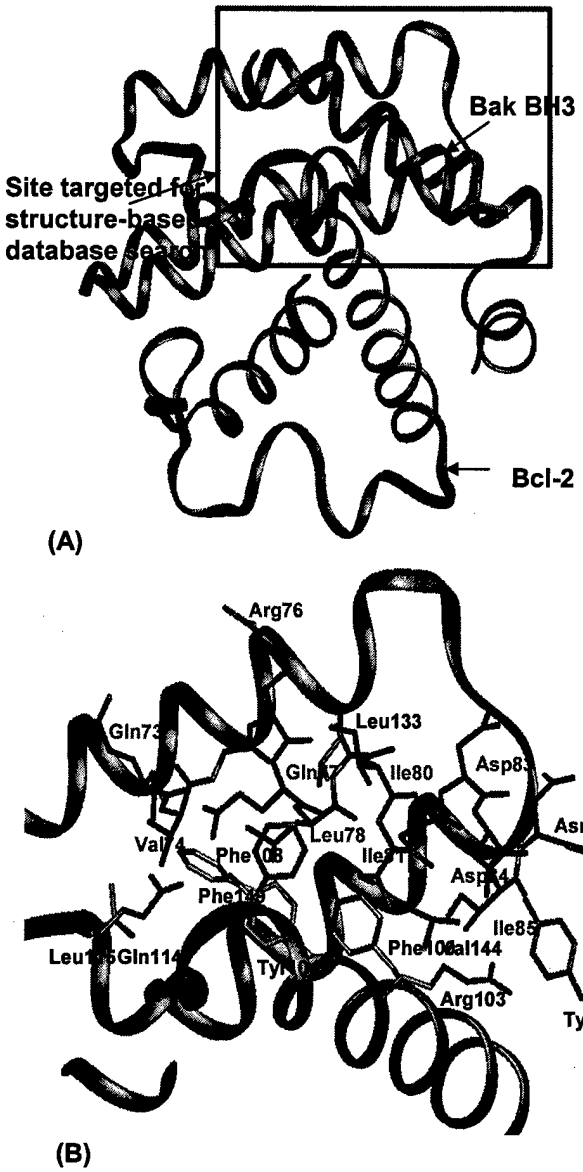
This task is essentially completed.

Structure-based database searching requires an accurate three-dimensional (3D) structure of Bcl-2. The experimental 3D structures of Bcl-2 have been recently determined. However, when we started this project, the experimental Bcl-2 structure was not determined. Fortunately, high resolution experimental 3D structures of Bcl-X_L alone and in complex with a Bak BH3 (Bcl-2 homology domain 3) peptide have been determined. Bcl-2 and Bcl-X_L share a high degree of homology in their amino acid sequences (45% of identity and 56% of similarity). It has been demonstrated that when there is a sequence identity of more than 30% between the target protein (Bcl-2) and the template protein (Bcl-X_L), current computational homology modeling methods, such as MODELLER, can provide an accurate 3D structure for the target protein. Therefore, computational homology modeling was employed to model the 3D structure of Bcl-2 (the target protein) based upon the experimental 3D structural coordinates of Bcl- X_L (the template protein) in this study.

The sequence alignment between Bcl-2 and Bcl-X_L was obtained using the BLAST program, which was used in our homology modeling. Since the Bak BH3 peptide binds to both Bcl-2 and Bcl-X_L with good affinities, the 3D structure of Bcl-2 in complex with the Bak BH3 peptide was modeled based upon the experimental NMR structure of Bcl-X_L in complex with the Bak BH3 peptide. Using the MODELLER program, 10 different models were generated. It was found that these 10 models were very similar, with a root-mean-square deviation (RMSD) within 1 Å for all the non-hydrogen atoms of residues that form the BH3 binding site. To further refine the side chain conformations, the modeled 3D complex structure was extensively simulated through molecular dynamics (MD) simulation in explicit water for 3 ns. Comparison of our modeled Bcl-2 structure with the recently published experimental high-resolution Bcl-2 NMR structure showed that they are essentially the same with respect to both the overall fold and binding site conformation. The root-mean-square deviation (RMSD) is 1.0 Å for all the non-

hydrogen atoms of residues that form the BH3 binding site between the NMR structure and our modeled structure. Thus, computational homology modeling provided us with an accurate 3D structure of Bcl-2 for our structure-based 3D-database searching. The refined structure of Bcl-2 in complex with the Bak BH3 peptide is depicted in **Figure 1**.

Figure 1. Modeled 3D structure of Bcl-2 in complex with Bak BH3 domain based upon the experimental structure of Bcl-XL in complex with Bak-BH3 peptide complex (pdb code: 1BXL). (A) Ribbon representation of the overall Bcl-2 structure in complex with the Bak BH3 peptide. (B). Detailed representation of the binding site. The carbon atoms in the Bak BH3 peptide are in magenta, while the carbon atoms in the Bcl-2 protein are in green, the oxygen atoms are in red and the nitrogen atoms are in blue.



Task 1.2. Structure-based 3D-database searching on four 3D-databases containing more than 650,000 small organic compounds and natural products to identify most promising small molecule inhibitors that effectively interact with the Bcl-2 surface-binding pocket. **(1-30 months)**.

During the first 12 months, we have completed the 3D-database searching of the National Cancer Institute's 3D-database of more than 250,000 synthetic organic compounds and natural products.

Since 1955, the National Cancer Institute (NCI) at the National Institutes of Health, USA has conducted extensive testing of materials for possible activity against different forms of cancer. Most of the substances tested have been pure organic compounds. The program examined an extraordinarily eclectic assembly of organic structures. Currently, more than 500,000 compounds have been tested. Of these compounds, about half (250,000 compounds) are classified as "open" compounds, whose structures and biological data can be accessed by the public. Because samples of compounds were needed for testing, it was necessary to develop a large acquisition effort and a repository, both of which are still functioning today. Continual scanning of the chemistry literature allows identification of compounds that are novel and of interest to the program, and the authors are approached for a sample, typically under 100 mg. Currently, the NCI repository has physical samples of about 60% of the registered compounds. Recently, analysis of several large chemical databases showed that the NCI database has by far the highest number of compounds that are unique to it. Approximately 200,000 of the NCI structures are not found in any of the other six analyzed databases. Therefore, the NCI "open" database provides a large number of unique synthetic compounds and natural products and is an excellent resource for drug lead discovery.

Using the modeled 3D structure of Bcl-2, we searched the NCI 3D-database of 250,000 small molecules using the program DOCK. In the database search, conformational flexibility of the small molecules was taken into account. The small molecules were ranked according to their scores as calculated using the energy scoring function in the DOCK program. The top 500 candidate small molecules with the best scores were considered as potential Bcl-2 inhibitors. Only non-peptide molecules were selected for testing. We have obtained samples of more than

150 compounds from the National Cancer Institute and tested their binding affinity to Bcl-2 using a sensitive and quantitative *in vitro* fluorescence polarization (FP) based binding assay.

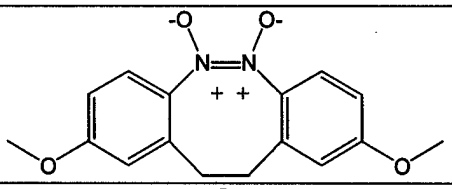
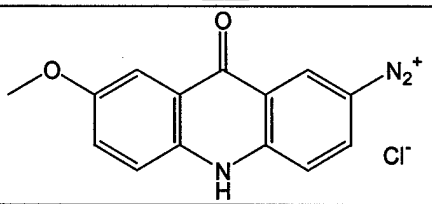
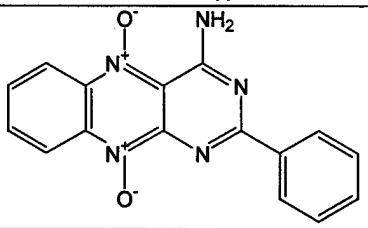
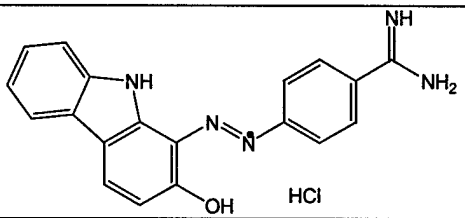
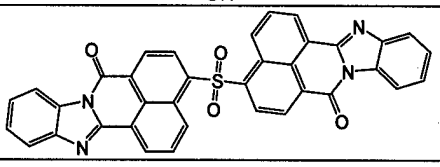
Task 2. *In vitro* biological confirmation of potential Bcl-2 inhibitors and mechanism investigations (**3-30 months**).

Task 2.1. Testing of potential small molecule inhibitors of Bcl-2 using an *in vitro* fluorescence polarization (FP) based binding assay.

We have used a sensitive and quantitative *in vitro* fluorescence polarization (FP) based binding assay to test the binding of these potential small molecule inhibitors to Bcl-2. The basic principle behind this assay is competition: a fluorescent peptide tracer (Flu-Bak-BH3) and a non-fluorescent small molecule inhibitor compete for binding to the target protein (Bcl-2). In a reaction mixture containing no small molecule inhibitor, the fluorescent tracer (Flu-Bak-BH3) is bound to the target protein (Bcl-2) and the emission signal is polarized; however, in a reaction mixture containing a small molecule inhibitor, the tracer is displaced by the small molecule inhibitor from the target protein and the emission signal becomes depolarized. The resulting change in the FP signal is directly related to the inhibitory activity of the small molecule inhibitor. Using this method, a binding affinity of 0.3 μM (IC_{50}) was obtained for the Bak-BH3 peptide to the Bcl-2 , which is consistent with the value reported in literature.

The binding affinity of these 150 candidate small molecules was tested initially at a dose of 100 μM in this binding assay. Of which, 30 compounds showed inhibitory activity more than 50% at the initial 100 μM dose level and were classified as active. Further dose dependent binding experiments showed that most of these 30 compounds displayed a dose dependent inhibition of the Bak peptide binding to Bcl-2. The chemical structures and IC_{50} values of 5 representative small molecule inhibitors are shown in Table 1.

Table 1. Chemical structures and binding affinities of representative small molecule inhibitors of Bcl-2 discovered in this project.

	Chemical structure	Binding affinity to Bcl-2 (IC_{50} μM)
1		10.4 ± 0.3
2		10.4 ± 1.2
3		1.6 ± 0.1
4		5.8 ± 2.2
5		7.7 ± 4.5

As can be seen from Table 1, these active compounds have an IC_{50} value better than 20 μM. Compound **3** is the most potent compound in the binding assay, with an IC_{50} value of 1.6 μM. The other active compounds have an IC_{50} value from 5.8 to 10.4 μM. It is of note that these active compounds belong to different chemical classes and their structures are also different from other reported Bcl-2 inhibitors.

Our results thus suggest that computational structure-based 3D-database screening is quite effective for the discovery of small molecule inhibitors of Bcl-2.

Task 2.2. Testing the activity of small molecule inhibitors of Bcl-2 in human breast cancer cells.

Binding experiments showed that these active compounds are capable of competing with the Bak BH3 peptide binding to Bcl-2 *in vitro*. Since we are interested in identifying cell permeable Bcl-2 small molecule inhibitors, we have tested the inhibitory activity of these active compounds on cell viability and proliferation using two different assays. First, the trypan blue exclusion method was used to determine the effect of an inhibitor on cell viability in which cells were treated with the inhibitor for 24 hours. Second, the MTT assay was used to determine the

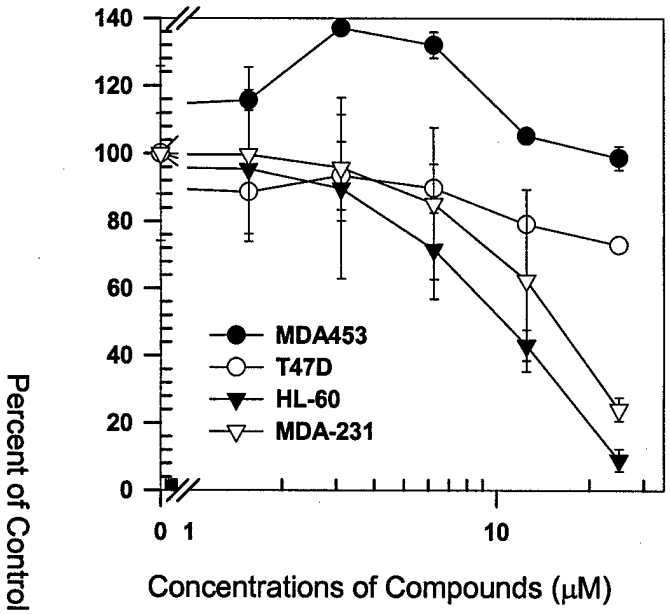


Figure 2. Inhibition of cell growth of compound **1** in human breast cancer and other cancer cell lines.

activity of an inhibitor on cell proliferation where cells were treated for four days. It is important to keep in mind that an active compound in the binding assay could fail to show any cellular activity simply because of its poor cellular permeability. We first tested these active compounds using the HL-60 cell line. HL-60 is a human myeloid leukemia cell line and expresses the

highest level of Bcl-2 protein among all the cancer cell lines examined in our laboratories. We then tested these active compounds using human breast cancer cell lines with different levels of Bcl-2 protein expression.

Using the trypan blue exclusion assay, these compounds were screened for their activity in inhibition of cell viability. More than half of the active compounds in the binding assay also have quite potent activity in cancer cells. For example, compound **1** is the most potent compound in the cellular assay, with an IC_{50} value of 10 μM. We have further tested compound **1** for its ability to inhibit cell growth. In the MTT assay where cells were treated for 4 days, **1** showed a potent inhibition in cell growth with an IC_{50} value of 4 μM. It was found that compound **1** potently inhibits cell growth in human breast cancer cell lines MDA-MB-231 with a high level of Bcl-2 (**Figure 2**). Compound **1** only has a weak activity in human breast canner cell lines MDA-MB-453 and T-47 with low levels of Bcl-2 protein. Therefore, compound **1** has selectivity between cancer cell lines with high level of Bcl-2 and low levels of Bcl-2

It is predicted that a potent small molecule that binds to the BH3 binding site of Bcl-2 will block the anti-apoptotic function of Bcl-2, which in turn would induce apoptosis in cancer cells with Bcl-2 protein overexpression. To test this hypothesis, we evaluated the ability and importantly the specificity of **1** in inducing apoptosis in cancer cells with high or low level of Bcl-2 expression. We used the Annexin-V flow cytometry assay to obtain a quantitative assessment on the ability of **1** in induction of apoptosis in HL-60 and human breast cancer MDA-231 cell line. MDA-MB-231 cells treated with 0 (untreated), 5 and 10 μM of compound **1** for 24 hours exhibited 0, 13% and 20.0 % apoptotic cells, respectively, while HL-60 cells treated with 0, 5, 10 and 20 μM of **1** for 24 hours had 0, 24%, 31% and 67% of apoptotic cells, respectively. Therefore, compound **1** induced apoptosis in a dose-dependent manner in MDA-MB-231 and HL-60 cell lines with Bcl-2 protein overexpression. In the human breast cancer cell line MDA-MB-453 and the normal fibroblast cell line WI-38 with low levels of Bcl-2 protein, no significant apoptotic cells were detected at 20 μM of compounds **1**.

Task 3. *In vivo* testing of 2-3 most promising lead compounds (**24-36 months**).

We have synthesized gram quantity of two our most potent small molecule inhibitors we have discovered from our structure-based database searching and we are poised to perform ant-tumor activity of these most potent small molecule inhibitors during the second year of this

project.

Task 4. Preparing scientific publications **(6-36 months)**.

We have completed a manuscript, disclosing the discovery of structurally diverse and potent small molecule inhibitors of Bcl-2 and are preparing our second manuscript. These manuscripts will be submitted for publication within the next 6-12 months.