Targeting Bcl-2 and Bcl-X_L With Nonpeptidic Small-Molecule Antagonists

Shaomeng Wang, Dajun Yang, and Marc E. Lippman

Members of the Bcl-2 family of proteins are crucial regulators of programmed cell death or apoptosis. This family of proteins now includes both anti-apoptotic molecules such as Bcl-2 and Bcl-X_L, and pro-apoptotic molecules such as Bax, Bak, Bid, and Bad. The majority of human cancers are found to have overexpression of Bcl-2, Bcl-X_L, or both. Bcl-2 and Bcl-X_L may play a critical role in cancer progression. Cancers with high levels of Bcl-2 or Bcl-X_L or both proteins are resistant to a wide spectrum of chemotherapeutic agents and radiation therapy. Bcl-2 and Bcl-X_L have become attractive targets for designing new anticancer drugs. Small-molecule inhibitors that are capable of inhibiting the activity of Bcl-2 and Bcl-X_L may have great therapeutic potential as an entirely new class of anticancer drugs for treating many forms of cancers in which Bcl-2 and/or Bcl-X_L proteins are overexpressed and for which traditional therapies are ineffective. Design of small-molecule inhibitors of Bcl-2 and Bcl-X_L is a very new and exciting area for current anticancer drug design and development. In this article we will provide a brief review on the strategy and recent progress in designing small-molecule antagonists targeting Bcl-2 and Bcl-X_L.
Semin Oncol 30 (suppl 16):133-142. ***© 2003 Elsevier Inc. All rights reserved.***

Bcl-2 AND Bcl-X_L AS CRUCIAL REGULATORS OF APOPTOSIS

APOPTOSIS, or programmed cell death, is a cell suicide mechanism that enables metazoans to control cell number in tissues and to eliminate unwanted individual cells. In multicellular organisms, individual cells are often eliminated for the common good by apoptosis. Apoptosis is important for normal development, host defense, and suppression of oncogenesis. Apoptosis not only plays an important role in tissue sculpting during development, but is also the primary defense against cells that may pose a threat to the well-being of the whole organism, such as cancer progenitor cells. As a result, cancerous cells must first undergo cellular alteration during tumorigenesis to enable unregulated proliferation without undergoing apoptosis. Evasion of apoptosis is now recognized as one of the major molecular mechanisms for cancer progression and resistance of cancer to chemotherapeutic drugs and radiation therapy.[1-5]

Bcl-2 was originally identified at the chromosomal breakpoint of t(14;18)-bearing B-cell lymphomas.[6,7] Bcl-2 belongs to a growing family of proteins that regulate apoptosis.[1-4] The Bcl-2 family includes both death antagonists such as Bcl-2 and Bcl-X_L, and death agonists such as Bax, Bak, Bid, and Bad.[1-4] The selective and competitive dimerization between pairs of antagonists and agonists determines how a cell will respond to an apoptotic signal.[1-4]

ROLES OF Bcl-2 and Bcl-X_L IN HUMAN CANCERS

The precise roles of Bcl-2 and Bcl-X_L in human cancers remain far from understood. However, several lines of evidence strongly suggest that Bcl-2 and Bcl-X_L not only contribute to cancer progression by preventing normal cell turnover, but also play an important role in the resistance of cancer cells to current cancer treatments.[5] Overexpression of Bcl-2 and/or Bcl-X_L renders cancer cells resistant to a wide spectrum of chemotherapeutic drugs and radiation therapy.[5,6,8,9] Not surprisingly, the majority of human cancers are found to overexpress Bcl-2, Bcl-X_L, or both proteins.

In Table 1, we summarize some representative data on the overexpression status of Bcl-2 and Bcl-X_L proteins in several major human cancers based on immunohistochemical analyses of clinical samples.[10-30] In several types of cancers, both Bcl-2 and Bcl-X_L are found to be overexpressed in a high percentage of tumor samples, such as prostate, breast, and colorectal cancers, and melanoma. Interestingly, in some other types of cancer, only one protein is found to be overexpressed in the majority of patients. For example, in head and neck cancer, Bcl-X_L is found to be overexpressed in 52% to 75% of patients, while Bcl-2 is overexpressed only in approximately 10% to 15% of patients.[27,28]

From the Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI.

Address reprint requests to Marc E. Lippman, MD, Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan Medical School, 3101 Taubman Center, Box 0368, 1500 E Medical Center Dr, Ann Arbor, MI 48109.
© 2003 Elsevier Inc. All rights reserved.
0093-7754/03/3005-1615$30.00/0
doi:10.1053/S0093-7754(03)00448-2

Table 1. Overexpression Status of Bcl-2 and Bcl-X_L in Several Types of Cancer

Cancer Type	Bcl-2 Expression (%)	Bcl-X_L Expression (%)	References
Prostate cancer	20-40 (at diagnosis)	100	10, 11
(hormone-resistant)	80-100		
Breast cancer	60-80	40-60	12-16
(estrogen receptor-positive)	80-90		
Non–small cell lung cancer	20-40		17,18
Small cell lung cancer	60-80		19
Colorectal cancer	50-100	83	20-23
Melanoma	65	90	24,25
Multiple myeloma		29	26
(at relapse)		77	
Head and neck cancer	13	52-75	27,28
Pancreatic cancer	23	90	29
Hepatocellular carcinoma		80	30

Although Bcl-2 and Bcl-X_L are overexpressed in 60% to 80% and 40% to 60% of breast cancer, respectively, there are some differences in their expression patterns in human breast cancer.[12-16] Overexpression of Bcl-X_L protein appears to be associated with higher tumor grade and increased number of positive nodes.[12]In comparison, overexpression of Bcl-2 protein is strongly correlated with estrogen receptor positivity.[12-15] Low expression of Bcl-2 is associated with shorter time to progression and shorter overall survival.[14,15] Bcl-X_L overexpression is correlated with higher tumor grade and an increased number of positive nodes and may be associated with invasive breast cancer.[12] In prostate cancer, Bcl-2 is overexpressed in only 20% to 40% of prostate cancer at diagnosis, but its overexpression increases to 80% to 100% in hormone-refractory disease.[10,11] Bcl-X_L is overexpressed in nearly 100% of hormone-refractory prostate cancer.[10]

Taken together, these observations underscore the need to consider Bcl-2 and Bcl-X_L as two closely related but different molecular targets in the development of new therapeutic agents for treating different forms of cancer.

Bcl-2 AND Bcl-X_L AS ATTRACTIVE MOLECULAR TARGETS FOR ANTICANCER DRUG DESIGN

Bcl-2 and Bcl-X_L have emerged as attractive molecular targets for the design of molecular target-specific new anticancer drugs. Inhibition of the anti-apoptotic function of Bcl-2/Bcl-X_L represents a novel and promising strategy for overcoming the resistance of cancers to chemotherapy or radiation therapy and for developing an entirely new class of anticancer drugs.[5-9]

First, most of the currently available cancer chemotherapeutic agents target cellular DNA integrity or replication, and indirectly trigger apoptosis in tumor cells.[5] Cancers that express high levels of Bcl-2, Bcl-X_L, or both proteins are resistant to chemotherapeutic agents or radiation therapy.[5,6,8] Therefore, a drug that specifically targets the anti-apoptotic function of Bcl-2 and/or Bcl-X_L may restore the sensitivity of cancer cells to chemotherapeutic agents or radiation. Second, Bcl-2 and Bcl-X_L are overexpressed in cancer cells while having very low levels in normal cells and tissues. As a result, cancer cells might develop a strong dependence on the protective effect of Bcl-2 and Bcl-X_L for their survival, while normal cells might not have such dependence. Therefore, a specific inhibitor of Bcl-2 and Bcl-X_L could selectively target cancer cells. Such a specific inhibitor is predicted to have very mild side effects. Third, because Bcl-2 and Bcl-X_L are overexpressed in the majority of human cancers,[10-30] a drug targeting Bcl-2 and Bcl-X_L could be developed for treating many forms of human cancer.

BIOLOGICAL AND PEPTIDE APPROACHES TARGETING Bcl-2 AND Bcl-X_L

Several biological approaches have been sought to target Bcl-2 and Bcl-X_L. One approach is to inhibit their expression levels. This is the basic

idea behind *bcl-2* and *bcl-X_L* antisense therapies. Antisense *bcl-2* oligonucleotides have been shown to induce apoptosis and increase sensitivity of chemotherapeutic drugs in a variety of human cancer cell lines.[31-35] Antisense *bcl-2* has been shown to suppress tumor growth in vivo, either alone or in combination with chemotherapeutic agents in xenograft models of human cancers.[31-35] Currently, bcl-2 antisense (Genasense, or G3139; Genta Pharmaceuticals Inc, Berkeley Heights, NJ) is in several phase II-III clinical trials for the treatment of chronic lymphocytic leukemia, malignant melanoma, multiple myeloma, non–small cell lung cancer, acute myeloid leukemia, mantle cell lymphoma, and prostate cancer in combination with chemotherapeutic drugs. Antisense *bcl-X_L* oligonucleotide has been shown to specifically decrease protein levels of Bcl-X_L in prostate and bladder cancers and sensitize cancer cells to chemotherapy.[36] A bispecific *bcl-2/bcl-X_L* antisense oligonucleotide has also been developed and was shown to inhibit cancer cell growth in human tumor cell lines of diverse histologic origins in vitro.[37] It also statistically significantly inhibited the in vivo growth of breast and colorectal carcinoma xenografts, relative to those treated with a control oligonucleotide.[37] A monospecific *bcl-xL* and a bispecific *bcl-2/bcl-X_L* antisense oligonucleotide were shown to induce apoptosis in primary cell cultures derived from different stages of melanomas and a cell line, and the bispecific *bcl-2/ bcl-X_L* antisense oligonucleotide was found to be superior to the monospecific *bcl-xL* in reducing the cell viability in all melanoma stages.[38] An intracellular anti-Bcl-2 single-chain antibody was shown to increase drug-induced cytotoxicity in the MCF-7 breast cancer cell line.[39] An anti–*bcl-2* ribozyme was developed and has been shown to rapidly degrade *bcl-2* mRNA in vitro.[40] Recently, a synthetic cell-permeable Bad BH3 peptide that binds to Bcl-2 has been shown to induce apoptosis in vitro and have in vivo activity in inhibiting the growth of human myeloid leukemia in severe combined immunodeficient mice.[41]

Taken together, these studies using antisense oligonucleotides, single-chain antibody, ribozyme, and synthetic peptides suggest that Bcl-2 and Bcl-x_L represent promising molecular targets for the design of an entirely new class of anticancer drugs by targeting apoptosis resistance of cancer cells.

STRATEGY IN DESIGNING NONPEPTIDIC, CELL-PERMEABLE SMALL-MOLECULE ANTAGONISTS OF Bcl-2/Bcl-X_L

Nonpeptidic, cell-permeable small-molecule inhibitors of Bcl-2 and Bcl-X_L are valuable tools to study the functions of Bcl-2 and Bcl-X_L. Importantly, potent and cell-permeable small-molecule inhibitors may have great therapeutic potential to be developed as an entirely new class of anticancer drugs. There are potentially several major advantages for small-molecule inhibitors over biological and peptide approaches, including better bioavailability, better stability, low cost, and the ability to penetrate through the blood-brain barrier of the central nervous system.

Several lines of evidence indicate that it is possible to design small-molecule inhibitors targeting Bcl-2 and Bcl-X_L. The anti-apoptotic function of Bcl-2/Bcl-X_L is attributed, at least in part, to their ability to heterodimerize with pro-apoptotic members such as Bid, Bim, Bad, and Bax and antagonize their pro-apoptotic function.[1-6,42-45] It has been shown that only the BH3 domains of these pro-apoptotic members are required for binding to Bcl-2 and Bcl-X_L and for inducing apoptosis.[43] A synthetic Bad BH3 peptide has been shown to induce apoptosis in vitro and have in vivo activity in human myeloid leukemia growth in severe combined immunodeficient mice.[41] The experimental structures of Bcl-2 and Bcl-X_L have provided atomic detailed structural information about the binding of these proteins to BH3 peptides. The experimental structures showed that the BH1, BH2, and BH3 domains of Bcl-2 and Bcl-X_L form a hydrophobic binding pocket (the BH3 binding pocket) into which the Bak or Bad BH3 domain binds (Figs 1 and 2).[46-50] This binding pocket in Bcl-2/Bcl-X_L is essential for its anti-apoptotic function.[42-45] Therefore, it has been hypothesized that nonpeptide small molecules that bind to the BH3 binding pocket in Bcl-2 and Bcl-X_L can block the interaction between Bcl-2/Bcl-X_L and pro-apoptotic members such as Bak, Bax, and Bad simply through direct competition and physical exclusion, thus functioning as antagonists of Bcl-2/Bcl-X_L.

SMALL-MOLECULE ANTAGONISTS TARGETING Bcl-2/Bcl-X_L DISCOVERED BY OTHER INVESTIGATORS

Despite the successful development of several screening assays, nonpeptidic small-molecule

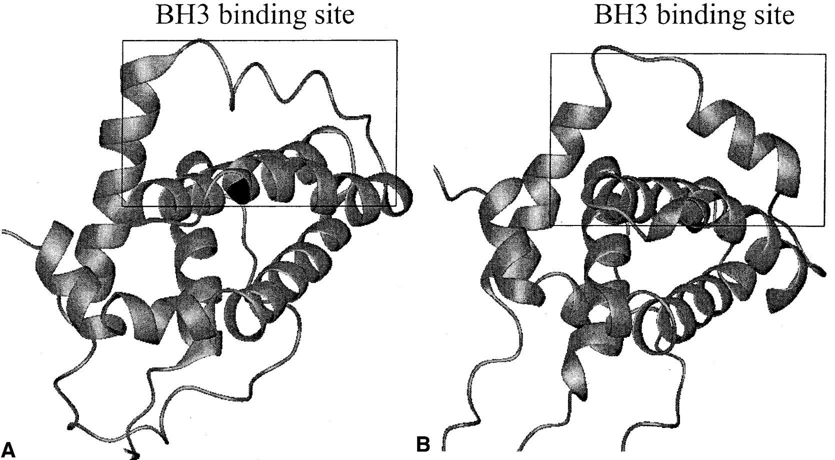

Fig 1. Ribbon representation of the solution structures of (A) Bcl-2 and (B) Bcl-X_L, as determined by Fesik and colleagues.[46,50] Note that Bcl-2 and Bcl-X_L have a well-defined binding site, which mediates the binding of Bcl-2 and Bcl-X_L to the BH3 domain of pro-apoptotic Bak and Bad proteins.

inhibitors of Bcl-2 and Bcl-X_L remained elusive for several years.[51] However, several recent independent reports have shown that it is possible to design nonpeptide small-molecule antagonists that bind to the BH3 binding site of Bcl-2/Bcl-X_L.[52-56]

The first report of a small-molecule inhibitor of Bcl-2 was contributed by Wang et al.[52] Using a computerized structure-based database screening strategy, these investigators screened the Available Chemical Directory of more than 200,000 small organic compounds and reported the discovery of one class of small organic molecule (called HA14-1) that binds to the BH3 binding site in Bcl-2 (no. 1 in Table 2). HA14-1 was shown to bind to the Bcl-2 protein and to effectively inhibit the binding between Bcl-2 and Bak BH3 peptide with a 50% inhibitory concentration (IC_{50}) of 9 μmol/L. HA14-1 also induced apoptosis in human acute myeloid leukemia (HL-60) cells overexpressing Bcl-2 protein, associated with the decrease in mitochondrial membrane potential and activation of caspase-9 followed by caspase-3. The significance of this study was that, for the first time, a nonpeptide small organic compound was discovered that is capable of inhibiting the binding between Bak BH3 peptide and Bcl-2 protein. This small-molecule inhibitor is also cell permeable because it displays a fairly potent (μmol/L) cellular activity in inhibition of cell growth and induction of apoptosis.

Following the discovery by Wang et al in 2000,[52] two independent reports were published in 2001 disclosing the discovery of three new classes of small-molecule inhibitors of Bcl-2 or Bcl-X_L.[53,54] Using a high-throughput screening assay based on fluorescence polarization (FP), Degterev

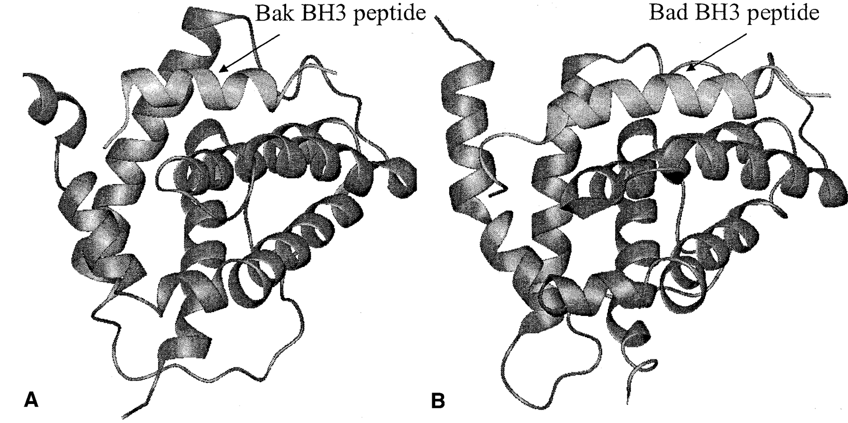

Fig 2. Ribbon representation of the solution structures of Bcl-X_L in complex with the Bak BH3 peptide (A) and in complex with the Bad BH3 peptide (B) as determined by Fesik and colleagues.[48,49] Note that the Bad BH3 peptide has more extensive interactions with Bcl-X_L than the Bak BH3 peptide, which may explain its much higher binding affinity to Bcl-X_L. The binding of the Bak or Bad BH3 peptide also causes significant conformational changes in the binding site of Bcl-X_L as compared with the free form of Bcl-X_L protein.

Table 2. Chemical Structures and Binding Affinities of Small-Molecule Inhibitors of Bcl-2 and/or Bcl-X_L

	Chemical Structure	Binding affinity to Bcl-2 (IC_{50} or K_d, μmol/L)	Binding affinity to Bcl-X_L (IC_{50} or K_d, μmol/L)	Reference
1 (HA14-1)		9	NT	52
2 (BH3I-1)		NT	2.4 ± 0.9	53
3 (BH3I-2)		NT	3.3 ± 0.3	53
4 (Antimycin A3)		2 ± 0.3	NT	54
5		NT	0.114	56
6 (BL-11)		10.4 ± 0.3	7	57
7		10.4 ± 1.2	NT	57
8		1.6 ± 0.1	NT	57
9		5.8 ± 2.2	NT	57
10		7.7 ± 4.5	NT	57

Abbreviation: NT, not tested.

et al screened a library of 16,320 chemicals from ChemBridge Corporation (San Diego, CA). They reported the identification of two classes of small-molecule inhibitors of Bcl-X_L.[53] The most potent representative compounds (nos. 2 and 3 in Table 2) have IC_{50} values of 2.4 and 3.3 μmol/L, respectively. These compounds induce apoptosis and inhibit cell growth. Using nuclear magnetic resonance (NMR) methods, they conclusively showed that these small-molecule inhibitors bind to the BH3 binding site in Bcl-X_L. Further, they convincingly showed that these inhibitors specifically block the BH3 domain-mediated heterodimerization between Bcl-2 family members in cell-free and in cellular systems.

Tzung et al[54] identified antimycin A3 (no. 4 in Table 2), an antibiotic, as a small-molecule inhibitor of Bcl-2/Bcl-X_L using a very different approach.

They hypothesized that agents with mitochondrial effects might directly trigger apoptotic changes in mitochondria, bypassing the protective effects of Bcl-2–related proteins. To test this hypothesis, they screened inhibitors of mitochondrial respiration for the ability to induce apoptosis in isogenic hepatocyte cell lines with graded expression of Bcl-X_L. They found that high levels of Bcl-X_L not only failed to protect against antimycin A, an inhibitor of electron transfer at complex III, but markedly enhanced antimycin A-induced apoptosis. Further investigations into the mechanism of sensitivity to antimycin in Bcl-X_L^{high} cells showed that antimycin A binds to the hydrophobic groove of Bcl-2/Bcl-X_L proteins. It is worth noting that the one significant difference between the results obtained by Yuan et al and those obtained by Tzung et al is the effect of these small-molecule inhibitors on the proposed pore-forming activity of Bcl-X_L. While Yuan et al reported that neither Bak BH3 peptide nor small-molecule inhibitors had any effect,[53] Tzung et al showed that both antimycin A and Bak BH3 peptide were able to block Bcl-X_L–mediated pore formation.[54]

Very recently, a small molecule was designed to mimic the interface functionality of the BH3 Bak in complex with Bcl-X_L using primarily three hydrophobic groups and one carboxylic acid.[56] A potent small-molecule inhibitor was obtained with a K_d value of 114 nmol/L binding to Bcl-X_L (Table 2, compound 5), similar to that potency of the Bak BH3 peptide under the assay conditions (the K_d value for the Bak BH3 peptide is 120 nmol/L). Although it is not known if the compounds are cell permeable, this study for the first time showed that it is possible to design small-molecule inhibitors with a binding affinity to Bcl-x_L as potent as the Bak BH3 peptide.[56]

SMALL-MOLECULE INHIBITORS OF Bcl-2 AND Bcl-X_L DISCOVERED IN OUR LABORATORIES

Our laboratories (University of Michigan Medical School, Ann Arbor, MI) have been pursuing the discovery of small-molecule inhibitors of Bcl-2 and Bcl-X_L using a computational structure-based approach. To date, we have discovered more than 10 chemical classes of small-molecule inhibitors of Bcl-2 and Bcl-X_L. Several of these have recently been reported.[57]

Computerized structure-based 3-dimensional (3-D) database screening, using computational docking aimed at identification of potential small organic molecules from large chemical 3-D databases that can bind to a specific binding site of the target protein, has become a powerful tool for discovery of new lead compounds. Potential inhibitors are subsequently confirmed through biochemical and biological assays. Compared with random screening, structure-based 3-D database searching is effective and has a very low cost. Over the years, our laboratories have extensively and successfully used this approach for the discovery of novel leads. Importantly, the experimental 3-D structures of Bcl-2 and Bcl-X_L alone, or in complex with Bak and Bad peptides, provide a solid basis for structure-based discovery and design of small-molecule inhibitors of Bcl-2 and Bcl-X_L.[46-50]

Our initial efforts have focused on identification of small-molecule inhibitors of Bcl-2. Because the experimental Bcl-2 structure was not available when we performed the computational structure-based screening, we have modeled the structure of Bcl-2 using the experimental 3-D structures of Bcl-X_L as the template protein structures.[46-49] Because of the high degree of homology between Bcl-2 and Bcl-X_L (45% sequence identity, 56% sequence similarity), it was expected that a fairly accurate 3-D structural model of Bcl-2 could be obtained using this homology modeling approach.[58,59] Indeed, when the recently published Bcl-2 NMR structure[50] was compared with our modeled Bcl-2 structure, we found that they were essentially the same with respect to both the overall folding and binding site conformation. The root-mean-square deviation between the NMR structure and our modeled structure is 1.0 Å for all the nonhydrogen atoms for residues that form the BH3 binding site.

Using the modeled 3-D structure of Bcl-2, we have performed structure-based database screening of the National Cancer Institute's 3-D database of 206,000 small molecules and natural products,[60] using the computer program DOCK.[61] The top 500 small molecules with the best-predicted docking scores identified by DOCK were considered as potential small-molecule inhibitors of Bcl-2 for biological testing. Using an established sensitive and quantitative in vitro FP-based method, we initially tested 35 candidate compounds for their

ability to compete with the Bak BH3 peptide binding to Bcl-2. Seven of these compounds were found to have binding affinities (IC_{50} values) ranging from 1.6 to 14.0 μmol/L using the FP-based binding assay.[41]

To verify the chemical structures of these small-molecule inhibitors, we performed mass spectroscopy and ^{1}H NMR analyses on the chemical samples. The mass spectroscopy and ^{1}H NMR data for five small-molecule inhibitors are consistent with their chemical structures recorded in the National Cancer Institute database but are inconsistent for two other active compounds. The chemical structures of these five small-molecule inhibitors with confirmed structures are shown in Table 2 (nos. 6 through 10).

Compound 8 displays the most potent binding affinity among the small-molecule inhibitors identified in our study (IC_{50} = 1.7 μmol/L) but this compound does not have any significant cellular activity in inhibition of cell growth and induction of apoptosis among the cancer cell lines we examined up to 100 μmol/L. Of these inhibitors, 6 (BL-11) and 7 display a fairly potent activity in inhibition of cell viability and cell growth in the HL-60 cell line, which has the highest level of Bcl-2 protein among all the cancer cell lines we have examined. The IC_{50} value (concentration required to inhibit 50% of cancer cell growth versus untreated cells) for compound BL-11 in HL-60 cancer cell is 4 μmol/L using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.

It has been hypothesized 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 BL-11 in inducing apoptosis in cancer cells with high- or low-level Bcl-2 expression. We used the Annexin-V (R & D Systems Inc, Minneapolis, MN) flow cytometry assay to obtain a quantitative assessment on the ability of BL-11 to induce apoptosis in the HL-60 and human breast cancer MDA-231 cell lines. MDA-MB-231 cells treated with 0 (untreated), 5, and 10 μmol/L of BL-11 for 24 hours exhibited 0%, 13%, and 20.0 % apoptotic cells, respectively, while HL-60 cells treated with 0, 5, 10, and 20 μmol/L of BL-11 for 24 hours had 0%, 24%, 31%, and 67% of apoptotic cells, respectively. Therefore, BL-11 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 having low levels of Bcl-2 protein, no significant apoptotic cells were detected at 20 μmol/L of BL-11.

Because Bcl-2 and Bcl-X_L have similar BH3 binding sites, it is expected that small-molecule inhibitors of Bcl-2 could also bind to Bcl-X_L. Indeed, the IC_{50} value of BL-11 to Bcl-X_L as determined by a similar FP-based binding assay is 9 μmol/L, similar to the binding affinity of BL-11 to Bcl-2. Therefore, BL-11 is a dual inhibitor of both Bcl-2 and Bcl-X_L.

Using the FP-based assay, we showed that BL-11 and other inhibitors we have identified bind to Bcl-2. However, the in vitro FP-based method simply shows that addition of a small-molecule inhibitor reduces the intensity of FP. The most straightforward interpretation is that the small-molecule inhibitor binds to Bcl-2 and displaces the binding of the fluorescence-labeled Bak BH3 peptide. However, it is also possible that addition of the small-molecule inhibitor simply causes the unfolding of the protein and thus reduces the binding of the fluorescence-labeled Bak BH3 peptide to the protein. To rule out the latter possibility and to conclusively demonstrate the binding of BL-11 to the BH3 binding site in Bcl-2 and Bcl-X_L, we used NMR methods to further investigate the binding to BL-11.

BL-11 binds to both Bcl-2 and Bcl-X_L with similar affinities, and Bcl-2 and Bcl-X_L have very similar BH3 binding sites. Because Bcl-X_L behaves much better than Bcl-2 in solution, we chose to use Bcl-X_L for our NMR experiments. We measured the hetereo-nuclear single quantum correlation spectrum of ^{15}N-labeled Bcl-X_L without and with BL-11. The hetereo-nuclear single quantum correlation spectrum is also called a fingerprint spectrum because of its sensitivity to structural changes. Our experiment showed that the binding of BL-11 caused the peak shifts of only a few residues in the BH3 binding site of Bcl-X_L, clearly indicating that BL-11 binds to the BH3 binding site in Bcl-$X_L$and does not unfold the protein.[57]

CHALLENGES IN DESIGNING SMALL-MOLECULE INHIBITORS OF Bcl-2 AND Bcl-X_L

The independent discovery of nonpeptide, cell-permeable small-molecule inhibitors of Bcl-2 and Bcl-X_L by several research groups clearly suggests that the approach using small molecules to inhibit the function of Bcl-2 and Bcl-X_L is indeed feasible. It is expected that these cell-permeable, small-molecule inhibitors will provide an invaluable research tool to further elucidate the function of Bcl-2/ Bcl-X_L in vitro and in vivo.

Of note, these small-molecule inhibitors reported to date are not yet useful clinical candidates because of the moderate potency in their binding affinities to Bcl-2 or Bcl-X_L and in inhibition of cell growth. Compound 5 appears to be the most potent small-molecule inhibitor of Bcl-X_L reported with a K_d value of 114 nmol/L, but it is not known if this inhibitor is cell-permeable. Thus, one major challenge scientists are facing in the development of small-molecule inhibitors of Bcl-2 and Bcl-X_L is how to significantly improve the potency of these initial lead compounds. This challenge is not trivial because there are still very few examples that small-molecular-weight ligands with high affinity can be designed to disrupt protein-protein interactions.

Analysis of the 3-D structure of Bcl-X_L in complex with the Bad BH3 peptide may provide some clues (Fig 2B). The Bad BH3 peptide binds to Bcl-X_L with a K_d value of 0.6 nmol/L, which is at least 1,000 times more potent in its binding affinity than those cell-permeable small-molecule inhibitors reported (Table 2). The interactions between Bad and Bcl-X_L are mediated by at least six hydrophobic residues in Bad BH3 peptides, which spans 30 Å in length. All of these reported small-molecule inhibitors are only half the size of the Bad BH3 peptide, suggesting that additional modifications with improved specific interactions with Bcl-X_L and Bcl-2 in these small-molecule inhibitors could significantly improve their binding affinities.

Bcl-2 and Bcl-X_L have similar BH3 binding pockets, but there are differences in the structural topology and electrostatic potential of the binding groove. The selectivity of small-molecule inhibitors of Bcl-2 and Bcl-X_L has not been fully characterized. It will be interesting to develop highly selective small-molecule inhibitors for Bcl-2 and Bcl-X_L because such inhibitors will undoubtedly be useful pharmacologic tools for studying the function of Bcl-2 and Bcl-X_L. On the other hand, because many types of cancers overexpress both Bcl-2 and Bcl-X_L, a small-molecule inhibitor that potently binds to both proteins may have additional therapeutic benefits as compared with an inhibitor of only one of these proteins.

CONCLUSION

The intensive research in the last decade into the molecular mechanism of apoptosis has shown that Bcl-2 and Bcl-X_L play a crucial role in controlling apoptosis in cancer cells and in resistance of cancer to current chemotherapy and radiation therapy. Although still in its very early stages, designing nonpeptidic, cell-permeable, small-molecule antagonists of Bcl-2 and Bcl-X_L holds promise for developing an entirely new class of anticancer drugs by targeting the fundamental molecular mechanism of resistance of cancer cells to apoptosis. We expect that designing small-molecule antagonists of Bcl-2 and Bcl-X_L will continue to be a very exciting area for years to come, and may ultimately lead to the development of a novel class of molecular target-specific new anticancer therapy.

ACKNOWLEDGMENT

The authors thank members of our laboratories (past and present) for their contributions to this project. The financial support from the Susan G. Komen Breast Cancer Foundation (BCTR 0100301), the Cap CURE Foundation, and the Department of Defense Breast Cancer Program (DAMD17-01-1-0256) is greatly appreciated.

REFERENCES

1. Chao DT, Korsmeyer SJ: Bcl-2 family: Regulators of cell death. Annu Rev Immunol 16:395-419, 1998

2. Reed JC: Bcl-2 family proteins. Oncogene 17:3225-3236, 1998

3. Minn AJ, Swain RE, Ma A, et al: Recent progress on the regulation of apoptosis by bcl-2 family members. Adv Immunol 70:245-279, 1998

4. Adams JM, Cory S: The Bcl-2 protein family: Arbiters of cell survival. Science 281:1322-1326, 1998

5. Reed JC: Bcl-2 family proteins: Strategies for overcoming chemoresistance in cancer. Adv Pharmocol 41:501-553, 1997

6. Reed JC, Miyashita T, Takayama S, et al: Bcl-2 family proteins: Regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60:23-32, 1996

7. Tsujimoto Y, Gorham JJ, Cossman E, et al: The t(14;18)

chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229:1390-1393, 1985

8. DiPaola RS, Aisner J: Overcoming bcl-2- and p53-mediated resistance in prostate cancer. Semin Oncol 26:112-116, 1999

9. Strasser A, Huang DCS, Vaux DL: The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy. Biochim Biophys Acta 1333:F151-F178, 1997

10. Krajewska M, Krajewski S, Epstein JI, et al: Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol 148:1567-1576, 1996

11. Furuya Y, Krajewski S, Epstein JI, et al: Expression of bcl-2 and the progression of human and rodent prostatic cancers. Clin Cancer Res 2:389-398, 1996

12. Olopade OI, Adeyanju MO, Safa AR, et al: Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. Cancer J Sci Am 3:230-237, 1997

13. Alsabeh R, Wilson CS, Ahn CW, et al: Expression of bcl-2 by breast cancer: A possible diagnostic application. Mod Pathol 9:439-444, 1996

14. Sjostrom J, Blomqvist C, von Boguslawski K, et al: The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res 8:811-816, 2002

15. Hellemans P, van Dam PA, Weyler J, et al: Prognostic value of bcl-2 expression in invasive breast cancer. Br J Cancer 72:354-360, 1995

16. Hurlimann J, Larrinaga B, Vala DL: bcl-2 protein in invasive ductal breast carcinomas. Virchows Arch 426:163-168, 1995

17. Jiang SX, Kameya T, Sato Y, et al: Bcl-2 protein expression in lung cancer and close correlation with neuroendocrine differentiation. Am J Pathol 148:837-846, 1996

18. Pezzella F, Turley H, Kuzu I, et al: bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 329:690-694, 1993

19. Higashiyama M, Doi O, Kodama K, et al: High prevalence of bcl-2 oncoprotein expression in small cell lung cancer. Anticancer Res 15:503-505, 1995

20. Manne U, Myers RB, Moron C, et al: Prognostic significance of Bcl-2 expression and p53 nuclear accumulation in colorectal adenocarcinoma. Int J Cancer 74:346-358, 1997

21. Schneider HJ, Sampson SA, Cunningham D, et al: Bcl-2 expression and response to chemotherapy in colorectal adenocarcinomas. Br J Cancer 75:427-431, 1997

22. Watson AJ, Merritt AJ, Jones LS, et al: Evidence of reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. Br J Cancer 73:889-895, 1996

23. Maurer CA, Friess H, Buhler SS, et al: Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. Dig Dis Sci 43:2641-2648, 1998

24. Ramsay JA, From L, Kahn HJ: bcl-2 protein expression in melanocytic neoplasms of the skin. Mod Pathol 8:150-154, 1995

25. Leiter U, Schmid RM, Kaskel P, et al: Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. Arch Dermatol Res 292:225-232, 2000

26. Tu Y, Renner S, Xu F, et al: Bcl-X expression in multiple myeloma: Possible indicator of chemoresistance. Cancer Res 1998 58:256-262, 1998

27. Wilson GD, Saunders MI, Dische S, et al: bcl-2 expression in head and neck cancer: An enigmatic prognostic marker. Int J Radiat Oncol Biol Phys 49:435-441, 2001

28. Pena JC, Thompson CB, Recant W, et al: Bcl-xL and Bcl-2 expression in squamous cell carcinoma of the head and neck. Cancer 85:164-170, 1999

29. Miyamoto Y, Hosotani R, Wada M, et al: Immunohistochemical analysis of Bcl-2, Bax, Bcl-X, and Mcl-1 expression in pancreatic cancers. Oncology 56:73-82, 1999

30. Takehara T, Liu X, Fujimoto J, et al: Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 34:55-61, 2001

31. Reed JC, Stein C, Subasinghe C, et al: Antisense-mediated inhibition of Bcl-2 protooncogene expression and leukemic cell growth and survival: Comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. Cancer Res 50: 6565-6570, 1990

32. Cotter FE, Johnson P, Hall P, et al: Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. Oncogene 9:3049-3055, 1994

33. Jansen B, Schlagbauer-Wadl H, Brown BD, et al: bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat Med 4:232-234, 1998

34. Koty PP, Zhang H, Levitt ML: Antisense bcl-2 treatment increases programmed cell death in non-small cell lung cancer cell lines. Lung Cancer 23:115-127, 1999

35. Zangemeister-Wittke U, Schenker T, Luedke GH, et al: Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. Br J Cancer 78:1035-1042, 1998

36. Leech SH, Olie RA, Gautschi O, et al: Induction of apoptosis in lung-cancer cells following bcl-xL anti-sense treatment. Int J Cancer 86:570-576, 2000

37. Gautschi O, Tschopp S, Olie RA, et al: Activity of a novel bcl-2/bcl-xL-bispecific antisense oligonucleotide against tumors of diverse histologic origins. J Natl Cancer Inst 93:463-471, 2001

38. Olie RA, Hafner C, Kuttel R, et al: Bcl-2 and bcl-xL antisense oligonucleotides induce apoptosis in melanoma cells of different clinical stages. J Invest Dermatol 118:505-512, 2002

39. Piche A, Grim J, Rancourt C, et al: Modulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity in the breast cancer cell line MCF-7. Cancer Res 58:2134-2140, 1998

40. Gibson SA, Pellenz C, Hutchison RE, et al: Induction of apoptosis in oral cancer cells by an anti-bcl-2 ribozyme delivered by an adenovirus vector. Clin Cancer Res 6:213-222, 2000

41. Wang JL, Zhang ZJ, Choksi S, et al: Cell permeable Bcl-2 binding peptides: A chemical approach to apoptosis induction in tumor cells. Cancer Res 60:1498-1502, 2000

42. Yin X-M, Oltval ZN, Korsmeyer SJ: BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369:321-323, 1994

43. Cosulich SC, Worrall V, Hedge PJ, et al: Regulation of apoptosis by BH3 domains in a cell-free system. Curr Biol 7:913-920, 1997

44. Letai A, Bassik M, Walensky L, et al: Distinct BH3 domains either sensitize or activate mitochondrial apoptosis,

serving as prototype cancer therapeutics. Cancer Cell 2:183-192, 2002

45. Shangary S, Johnson DE: Peptides derived from BH3 domains of Bcl-2 family members: A comparative analysis of inhibition of Bcl-2, Bcl-xL and Bax oligomerization, induction of cytochrome c release, and activation of cell death. Biochemistry 41:9485-9495, 2002

46. Muchmore SW, Sattler M, Liang H, et al: X-ray and NMR structure of human Bcl-X_L, an inhibitor of programmed cell death. Nature 381:335-341, 1996

47. Aritomi M, Kunishima N, Inohara N, et al: Crystal structure of rat Bcl-X_L Implications for the function of the Bcl-2 protein family. J Biol Chem 272:27886-27892, 1997

48. Michael S, Heng L, David N, et al: Structure of Bcl-X_L-Bak peptide complex: Recognition between regulators of apoptosis. Science 275:983-986, 1997

49. Petros AM, Nettesheim DG, Wang Y, et al: Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. Protein Sci 9:2528-2534, 2000

50. Petros AM, Medek A, Nettesheim DG, et al: Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A 98:3012-3017, 2001

51. Oltersdorf T, Fritz LC: The Bcl-2 family: Targets for regulation of apoptosis. Annu Reports Med Chem 33:253-262, 1998

52. Wang J-L, Dongxiang L, Zhang Z-J, et al: Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci U S A 97:7124-7129, 2000

53. Degterev A, Lugovskoy A, Cardone M, et al: Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-x_L. Nat Cell Biol 3:173-182, 2001

54. Tzung S-P, Kim KM, Basanez G, et al: Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. Nat Cell Biol 3:183-191, 2001

55. Zheng TS: Death by design: The big debut of small molecules. Nat Cell Biol 3:E1-E3, 2001

56. Kutzki O, Park HS, Ernst JT, et al: Development of a potent Bcl-x_L antagonist based on α-helix mimicry. J Am Chem Soc 124:11838-11839, 2002

57. Enyedy IJ, Ling Y, Nacro K, et al: Discovery of small molecule inhibitors of Bcl-2 through structure-based computer screening. J Med Chem 44:4313-4324, 2001

58. Sali A, Potterton L, Yuan F, et al: Evaluation of comparative protein modeling by MODELLER. Proteins 23:318-326, 1995

59. Sali A: Modeling mutations and homologous proteins. Curr Opin Biotech 6:437-451, 1995

60. Milne GWA, Nicklaus MC, Driscoll JS, et al: The NCI Drug Information System 3D Database. J Chem Inf Comput Sci 34:1219-1224, 1994

61. Makino S, Kuntz ID: Automated flexible ligand docking method and its application for database search. J Comput Chem 18:1812-1825, 1997