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Review Article

Small-molecule inhibitors of Bcl-2 protein

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CONTENTS

Abstract

Approaches to drug discovery are varied and range from high-resolution NMR solution structure of targeted molecules to rational design. This review is focused on the use of small-molecule inhibitors of Bcl-2 as therapeutic agents. Members of the Bcl-2 family of proteins are crucial regulators of apoptotic cell death. Human cancers have been found to overexpress Bcl-2 and Bcl-XL. Cells with high levels of these antiapoptotic molecules are usually resistant to a wide spectrum of chemotherapeutic drugs. Targeting the Bcl-2 family of proteins with small-molecule inhibitors has therefore become an attractive potential therapy for a variety of cancers. The role of Bcl-2 in sabotaging the success of cytotoxic agents suggests that novel treatments should be devised to target Bcl-2-overexpressing tumor cells and induce apoptosis directly. In this article, we will provide a review of potential small-molecule inhibitors as anticancer agents. The deregulated overexpression of Bcl-2 and Bcl-XL is directly related to cancer cell survival and resistance to chemotherapeutic drugs, making antagonists or inhibitors of these proteins very promising candidates for use in cancer therapy

Introduction

Apoptosis is an evolutionarily conserved process of programmed cell death that plays an essential role in organism development and tissue homeostasis. Damaged, unnecessary or aged cells are carefully removed via apoptosis to ensure the overall health of the organism. Apoptotic resistance contributes to the development of several diseases, including cancer, because cellular damage, genomic instability, cytotoxic stress or radiation is allowed to propagate as the affected cells reproduce (1, 2). Several morphological changes are characteristic of apoptotic cells –plasma membrane blebbing, chromatin condensation and DNA fragmentation– and distinguish necrotic cell death from apoptosis.

Several mechanisms exist to allow cells to escape programmed cell death. Especially relevant to tumorigenesis is the overexpression of the antiapoptotic protein Bcl-2. Many groups have been working to develop cancer treatments that block the activity of Bcl-2, thus allowing apoptosis to carry on as programmed by the cell. Methods include the downregulation of Bcl-2 expression or the use of peptides or small organic molecules targeting the Bcl-2 binding pocket, preventing its heterodimerization with and sequestration of proapoptotic proteins such as Bax, Bak, etc. (3). This paper reviews several examples of Bcl-2 inhibitors and their application to the treatment of human cancers.

Mechanisms of apoptosis

Apoptosis is very strictly regulated by competitive dimerization between proapoptotic and antiapoptotic members of the Bcl-2 family (named for B-cell lymphocyte/leukemia 2). Complexation is dependent upon hydrophobic interactions between conserved BH3 domains within each individual protein. The Bcl-2 family is composed of antiapoptotic and proapoptotic agents, all of which share Bcl-2 homology domains (BH1, 2, 3 or 4). Different Bcl-2 proteins are expressed and regulated differently in various tissues, suggesting that there is a level of specificity of function which is not yet clearly understood (4).

The antiapoptotic proteins are Bcl-2 and Bcl-XL, which are both composed of two central hydrophobic α-helices surrounded by five amphipathic helices (5). These proteins act to delay or prevent apoptosis and promote cell survival by blocking the release of cytochrome *c* from the mitochondria and subsequent caspase activation. The BH4 domain is conserved only between Bcl-2 and Bcl-XL and is responsible for their inhibition of the voltage-dependent anion channel (VDAC), which regulates the release of cytochrome *c* from the mitochondrial intermembrane space (6). The BH4 domain is required for the antiapoptotic function of Bcl-2, and a peptide constructed of amino acids 4-23 has been shown to inhibit VDAC opening in HeLa cells (7).

The proapoptotic members include Bak, Bik, Bid, Bax and the BH3-only proteins. These family members function to bind Bcl-2 or Bcl-XL, allowing the induction of apoptosis via the release of cytochrome *c*. Some specificity of heterodimerization is suggested by the fact that Bak has been shown to favor binding Bcl-XL over Bcl-2, with higher affinity than Bax or Bik.

BH3-only proteins, such as Bim, Bmf, Noxa or Puma, are released in damaged cells to neutralize the antiapoptotic proteins (8). p53, which probably functions to sense cell damage, activates the transcription of Noxa and Puma. Bim is sequestered to the microtubules and Bmf to actin, and they are released when cytoskeletal damage is sensed. BH1 and BH2 domains participate in family member dimerization, but only the BH3 domain is absolutely required (9, 10). Even subtle mutations in the BH3 domain result in a significant reduction in binding affinity. It has been discovered that the BH3 domain itself is responsible for inhibiting antiapoptotic proteins, and an excess of BH3 domain peptides is able to induce apoptosis by releasing Bax or Bak from sequestration by Bcl-2. It has also been suggested that BH3 peptides may bind and activate proapoptotic proteins directly (1).

Sattler *et al.* (5) showed that a region of Bcl-XL from amino acids 72 to 87 was responsible for Bak recognition and binding to Bcl-XL. It remains to be proven which amino acid residues are involved in the heterodimerization of every proapoptotic/antiapoptotic protein combination, which would certainly be useful information for the design of drugs targeted to the Bcl-2 family at large.

Apoptosis can be carried out via two distinct pathways. The intrinsic, mitochondria-mediated pathway is activated in response to stresses such as activation of oncoproteins, DNA damage, hypoxia or growth factor deprivation. Stress-sensing proteins (ATM, Chk2, etc.) signal p53 to activate the transcription of proapoptotic proteins, deactivate the transcription of antiapoptotic proteins and inhibit survival pathways (6). Bcl-2 is inhibited and releases Bax, Bak or other apoptotic effectors, leading to the release of cytochrome *c* from the mitochondria, activation of Apaf-1 and subsequent activation of the initiator caspase 9 and the effector caspase 3 (11). Effector caspases are responsible for inducing the morphological changes that are associated with apoptotic cells. It is this mode of apoptosis that is most often disabled in tumorigenesis, thus allowing tumor cells to thrive. The main mechanism of avoiding mitochondria-mediated apoptosis is probably overexpression of Bcl-2 or Bcl-XL, but many cancers also overexpress members of the IAP (inhibitor of apoptosis protein) family, which function to prevent the activation of caspases (12).

The death receptor pathway is activated by the binding of an extracellular ligand to a death receptor, such as TRAIL (Apo-2L) or CD95 (13, 14). Receptor activation leads to activation of caspase 8 and then caspase 3, which proceeds to disassemble the cell. Tumors deactivate this pathway less often than the intrinsic pathway, but deactivation has been shown to occur in certain forms of cancer (6). Although most cytotoxic drugs induce mitochondrial apoptosis, some do activate death receptors, especially the tumor necrosis factor receptor (TNFR) family (14).

Apoptotic resistance – tumorigenesis and chemoresistance

Inhibition of apoptosis contributes to tumorigenesis by preventing normal cell turnover, which is imperative to the elimination of damaged or unneeded cells from the body (15). While cells utilize many different mechanisms to avoid apoptotic death, the overexpression of antiapoptotic proteins is probably the most significant contributor to tumorigenesis. Overexpression of Bcl-2 has been observed in 80% of B-cell lymphomas, 30-60% of prostate cancers, 90% of colorectal adenocarcinomas, and several others (2). Bcl-XL is overexpressed in many breast and lung cancers.

When such mechanisms are employed to prevent successful apoptotic removal of cancer cells, the sort of mutations that allow the development of increasingly invasive disease are allowed to accumulate, promoting hormone or growth factor independence, anchorage independence, nutrient independence, cell cycle checkpoint independence, resistance to immune attack and angiogenesis (6, 16). Cells that have progressed to this level of tumorigenicity are very likely to have simultaneously developed resistance to drug-induced apoptosis.

The dilemma of chemoresistance involves the permissive mutations accumulating in the cancer cells and the mechanism of death induced by a given drug. Because tumor cells are known to reproduce faster than normal cells, many antitumor drugs aim to interfere with DNA replication or cellular metabolism. The lack of tumor cell specificity leads to damage of healthy tissues that are meant to proliferate rapidly, such as bone marrow and gut, resulting in dose-limiting side effects (6). The effectiveness of chemotherapy is also limited by rapid drug metabolism and intrinsic or acquired drug resistance. Because cells interpret most drug-induced stresses the same as intrinsic stresses, alterations in the intrinsic apoptotic pathway have been linked to multidrug resistance (16, 17).

Considering the fact that cytotoxic drugs allow accumulation of DNA damage in cells that are likely to be

resistant to apoptosis, one may suggest that tumor cells are growing more malignant and less chemosensitive, thus indirectly propagating the disease. Additionally, many patients have experienced treatment-related leukemia, with new primary tumors arising after treatment of the original cancer with alkylating agents or topoisomerase inhibitors (6). It is generally accepted that tumor cell death is more favorable than cytostasis, because dead tumor cells are no longer able to contribute to further disease progression. Apoptotic death is preferable to death by necrosis, because there is no necrotic inflammatory reaction that can damage surrounding healthy tissues, and treatment that directly induces apoptosis should be less mutagenic. Therefore, successful cancer therapy should consider both the tumor genotype and the mechanism of drug action, and should attempt to induce apoptosis in tumor cells while avoiding normal cells.

The effectiveness of chemotherapy-induced apoptosis, whether direct or indirect, is dependent upon the balance of pro- and antiapoptotic proteins in a cell, and the abundance of Bcl-2 or Bcl-XL is associated with resistance to such treatments (2, 18). Amundson *et al*. (19) tested a panel of 122 chemotherapeutic agents for their ability to induce apoptosis in a library of 60 various cancer cell lines obtained from the National Cancer Institute (NCI). It was discovered that cells with elevated Bcl-XL levels were resistant to every class of compound tested. Many other studies have demonstrated that overexpression of Bcl-2 and Bcl-XL in a wide range of cancer types –breast, pancreas, leukemia, colorectal, lung, renal, thyroid, etc.– is linked to their resistance to chemotherapy-induced apoptosis (15, 16, 20, 21). The role of Bcl-2 in sabotaging the success of cytotoxic chemotherapies suggests that novel treatments should be devised to target Bcl-2-overexpressing tumor cells and induce apoptosis directly (2, 12).

Small-molecule inhibitors of Bcl-2 protein

The deregulated overexpression of Bcl-2 and Bcl-XL is directly related to cancer cell survival and resistance to cytotoxic chemotherapies, making these proteins very promising candidates for suppression and/or inhibition.

Due to the fact that the BH3 domain is responsible for Bcl-2 sequestration of Bax, Bak and others, it has become a goal to fine-tune drug action even further by inhibiting the BH3 binding domain itself. Because intact proapoptotic proteins are able to form mitochondrial membrane pores, the introduction of exogenous protein or transcriptional upregulation could result in nonspecific toxicity to all cells (1). The BH3 domain can be mimicked by small molecules that are easier to introduce into cells than whole proteins and are expected to selectively kill only cells with Bcl-2 overexpression. A feature of Bcl-2 that makes it an even more promising target for inhibition is the hydrophobic binding pocket into which Bax, Bak and others fit very snugly. While the majority of protein-protein interactions occur at relatively flat surfaces, Bcl-2's deep binding groove should make the process of identifying potential inhibitors much easier (2). Already, BH3-domain peptides and small organic mimetics of the BH3 domain have been used to treat various cancers with some success.

Bcl-2 and Bcl-XL differ by only three amino acids, but the result is a significantly different electrostatic potential at one end of the BH3 binding groove of one protein compared to the other (2). This fact could be utilized to identify inhibitors that bind both Bcl-2 and Bcl-XL similarly, which would be effective against cancers overexpressing both, or inhibitors that prefer one over the other, which would provide additional binding specificity in the treatment of cancers that overexpress only one or the other.

Bak and Bax are believed to bind Phe61, Leu94, Gly102, Ala106 and Tyr159 of Bcl-2 in a manner that stabilizes its "open cleft" conformation (15). NMR images suggest that the BH3 domain of the ligand actually targets and interacts with the top portion of the hydrophobic binding groove, without contacting the area of variable electrostatic potential (2). Also, it appears as though the BH3 domain of Bak and Bax is hidden inside the proteins during their resting state and probably requires an undefined conformational change to make it available for substrate binding (13). These mechanistic details must be better understood to successfully disrupt BH3-mediated protein-protein interactions in an attempt to attenuate apoptotic resistance in different types of cancers.

Many methods of drug discovery and design have been employed to identify specific inhibitors of Bcl-2 and Bcl-XL, and the substantial diversity of chemical libraries available for screening has been a good source for novel drug leads. Libraries of natural products and their derivatives have revealed the most promising leads, compounds derived by nature to interact with specific protein targets (22). The enhancement of ligand-protein interactions requires a greater understanding of the binding mechanism on a molecular level, such that the drug compound can recognize the specific protein surface that activates a biological response and bind the surface so that the association with its natural ligand is sterically blocked. In seeking compounds that can bind strongly to a given protein target with high specificity, there are several factors that must be analyzed, such as the steric complementarity of the shapes of the interaction sites, electrostatic interactions, hydrogen bonding and the role of solvent kinetics in the stabilization of interactions (23). Once promising compounds have been selected for further study, the ability of the ligand to identify and fit into the substrate's binding pocket must be deciphered via protein docking assays, now often conducted rapidly using computer software. Although new programs are currently being developed, there is no one program capable of simultaneously estimating the binding energy of various docked states, enumerating the possible docking orientations at high resolution, and considering the mobility of the docking surfaces and conformational changes that may occur during interaction. Thus, entirely

computer-based analysis of efficient Bcl-2 inhibitors has been a time-consuming and imprecise undertaking.

Antisense oligonucleotide inhibitors of Bcl-2 translation

Antisense oligonucleotides are chemically synthesized and purified single-strand DNA sequences, 15-25 bases long, which are complementary to specific target regions of mRNA (24). When administered to a cell, the goal is hybridization of the oligonucleotide to the target sequence, which triggers degradation of cellular mRNA and a subsequent decrease in target protein translation. The first study of Bcl-2 antisense therapy in human beings was conducted in 1997, with an 18-base, fully phosphorylated oligonucleotide administered subcutaneously to 9 patients with Bcl-2-overexpressing lymphomas (25). Four patients exhibited a partial response, suggesting that antisense treatment may be better used to sensitize chemoresistant tumors than as a primary chemotherapeutic agent. Side effects included anemia, thrombocytopenia, hyperglycemia and local skin infection, but the treatment did not appear to affect normal memory B-cells, T-cells or neuronal tissue.

An antisense oligonucleotide for Bcl-XL mRNA was transfected into PANC-1, PancTU1, and COLO 357 pancreatic cancer cell lines, and was shown to decrease Bcl-XL expression and overall cell survival following treatment with death receptor ligands, TRAIL and CD95 antibodies (14). Another group synthesized an 18-base phosphorothioate oligonucleotide compound (augmerosen) to bind the first 6 codons of Bcl-2 mRNA, resulting in decreased expression of Bcl-2 and increased apoptosis in *Xenopus* oocytes. Augmerosen treatment exhibited evident antitumor activity in 6 of 14 patients with metastatic melanoma, and none had major hematological side effects. No patient had toxicity suggestive of antisense-related Bcl-2 alterations in normal tissues or immune stimulation.

Oblimerosen sodium (G-3139) is another antisense oligonucleotide targeting Bcl-2 and has been shown to enhance the efficacy of cytotoxic chemotherapy in patients with chronic lymphocytic leukemia, multiple myeloma, malignant melanoma and non-small cell lung cancer (26).

An antisense inhibitor of Bid was bound to a 2*′-O-*(2-methoxy)ethyl group to facilitate its entrance into tumor cells (4). Administration to BALB/c mice resulted in an 80% decrease in the expression of Bid without affecting the levels of Bad or Bax. The group combined this inhibitor with an antisense oligonucleotide against Bcl-XL, resulting in the first simultaneous inhibition of two specific proteins via antisense therapy.

While antisense oligonucleotides have had partial success in reversing chemoresistance in Bcl-2-overexpressing cells, the method has considerable shortcomings. Favorable results from *in vitro* experiments are often not repeatable *in vivo* or in a human patient. Additionally, there is no way to ensure oligonucleotide delivery to the entire population of tumor cells *in vivo* (6), and there is no efficient method of confirming whether any apparent antitumor effect is due to the presence of the antisense oligonucleotide in the target cell population.

Small peptide inhibitors of Bcl-2 protein

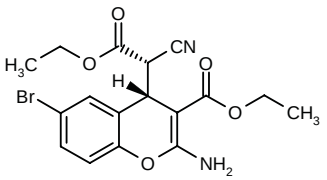
Small peptides have been designed to bind and inhibit specific protein-protein interactions, including Bcl-2 heterodimerization with proapoptotic proteins. While using small peptides composed only of the significant binding domains of a certain protein is preferred over the entire intact protein, some problems remain regarding the delivery of small peptides to their target cells. The biggest problems with using small peptide inhibitors *in vivo* include directing them to tumor cells, getting them to cross specific cell membranes and finally locating and inhibiting intracellular targets (13). A decanoic fatty acid bound to a synthetic peptide representing the Bad BH3 domain, termed CPM-1285, resulted in strong nanomolar-range binding to Bcl-2 in human myeloid leukemia HL-60 cells (27). Fluorescence polarization (FP) assays showed that over 90% of the cells had significant CPM-1285 uptake, followed by chromatin condensation, DNA ladder formation and other indicators of apoptosis. *In vivo* experiments suggested that CPM-1285 was able to slow tumor growth without damaging normal peripheral blood cells.

Another group used the internalization domain of the Antennapedia (Ant) protein to assist the entrance of small peptides into tumor cells (12). The Ant domain was linked to the Bak BH3 domain (Ant-Bak-BH3) and was able to induce apoptosis in HeLa cervical carcinoma cells. Ant-Bak-BH3 was also shown to inhibit Bcl-XL selectively relative to Bcl-2.

The problem with current methods employed to design synthetic peptides is the difficulty of simulating the *in vivo* conditions to which the naturally occurring proteins are exposed, such as the effects of solvation and conformational changes upon activation. Under normal circumstances *in vivo*, folded proteins recognize their binding partners by signal-induced formation of extended surfaces of complementary shape and electrostatic potential, usually undergoing some conformational change during the process (2). Due to the largely unstructured nature of BH3-domain peptides, the energy associated with the folding transition during binding Bcl-2 limits their affinity and effectiveness. The ideal synthetic ligand would be designed as a preorganized functional epitope, allowing increased affinity and specificity. Other difficulties pertaining to the use of small peptides to treat cancer include their poor oral availability, poor stability and/or high cost (28).

Organic small-molecule inhibitors of Bcl-2 protein

Organic molecules and their derivatives may prove to be a promising resource for the targeted inhibition of



HA-14-1

specific cellular proteins such as Bcl-2 and Bcl-XL. These low-molecular-weight compounds, less than 750 daltons, usually have high membrane permeability, making their use *in vivo* more feasible, and possibly more cost-efficient, than oligonucleotides or small peptides.

HA-14-1

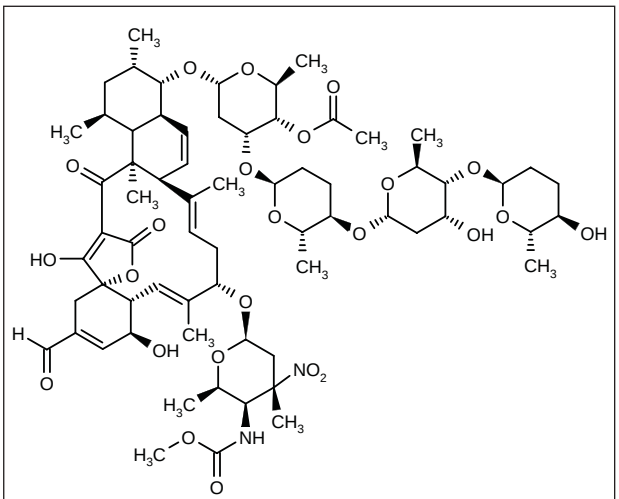
A model structure for Bcl-2 was derived from the DISCOVER program and used in a computational screen of over 190,000 compounds from the Molecular Design Limited three-dimensional database (San Leandro, CA). The search resulted in the discovery of HA-14-1 as a molecule with shape complementarity, high virtual affinity and potential for hydrogen bonding with Bcl-2 (29). The discovery was confirmed with an *in vitro* competitive FP assay in which the amino terminus of the Bak BH3 peptide was labeled with 5-carboxyfluorescein, allowing any displacement of ligand from Bcl-2 to be monitored. The results demonstrated that HA-14-1 was binding directly to Bcl-2 to inhibit the interaction with the labeled Bak peptide. At 50 μM, HA-14-1 was able to induce apoptosis in 90% of HL-60 cells, visualized by the common hallmarks of DNA ladder formation, cleavage of caspase 9 and 3, and activation of PARP (poly[ADP-ribose] polymerase, NAD+ ADP-ribosyltransferase). These effects were almost completely blocked by addition of the caspase inhibitor zVAD-fmk, which suggests that HA-14-1 may be a poor treatment choice for tumors overexpressing members of the IAP family of caspase inhibitors. HA-14-1-induced apoptosis was also shown to require expression of Apaf-1, further illustrating the mitochondrial signaling pathway utilized by the compound. This study was one of the first to portray organic small-molecule inhibitors as a promising, cell-permeable, nonimmunogenic cancer therapy. HA-14-1 has since been shown to induce apoptosis in MDA-MB-468 breast cancer, PCB and LNCaP prostate cancer, and primary acute myelogenous leukemia cells (12).

The mechanism of HA-14-1-induced apoptosis suggests potential for its use in combination therapy. Synergistic induction of apoptosis occurred when HA-14-1 was combined with the MEK inhibitors PD-184352 or BMS-247550 (a cytotoxic analogue of epothilone B). BMS-247550 triggers apoptosis in human breast cancer cells by inducing a conformational change in Bax and subsequent translocation to the mitochondria, and cell death was significantly amplified after pretreatment with HA-14-1 to inhibit Bcl-2 (30). Treatment of MM.1S and U266 human multiple myeloma cells with the proteasome inhibitor bortezomib followed by treatment with HA-14-1 resulted in a synergistic induction of apoptosis that was significantly greater than after treatment with either agent alone (31). Interestingly, synergistic apoptosis was not achieved by treating cells with both agents simultaneously, suggesting that the use of HA-14-1 in combination with other chemotherapeutic drugs should be timed to best utilize the mechanisms of action of the various drugs.

A study conducted on several malignant hematopoietic cell lines found that HA-14-1 was able to effectively induce apoptosis at concentrations of 5-12.5 μM (32). However, at higher concentrations, HA-14-1 treatment resulted in necrotic death of malignant cells and suppression of normal hematopoietic colony formation. This contradicts data from a previous study in which HL-60 cells were treated with 50 μM of HA-14-1 without necrotic death (29). It is possible that different concentrations of HA-14-1 are required for effective apoptotic induction for different cell types or for different levels of Bcl-2 expression. Therefore, therapeutic doses of HA-14-1 administered to patients should be carefully calculated to ensure that safe concentrations are not exceeded.

Tetrocarcin A

Tetrocarcin A (TC-A) is produced by actinomycetes to protect them from Gram-positive bacterial infections. A screen of natural products able to inhibit Bcl-2 function identified TC-A as a promising drug (33). A concentration of 2.2 μM was able to decrease the viability of HeLa cells transfected with Bcl-2, causing release of cytochrome *c* and a rapid decrease in mitochondrial transmembrane potential, without altering protein expression or subcellular localization. The same results were noted for experiments with Bcl-XL-overexpressing HeLa cells.



Tetrocarcin A

Tetrocarcin A was shown to sensitize the cells to various types of death stimuli, including Fas ligand, tumor necrosis factor-α (TNF-α) and Bax activation. It has been proposed that TC-A may in fact induce apoptotic events by binding and activating proapoptotic proteins, and not by inactivating Bcl-2 (13). Although these results gave promising insight into the use of TC-A in cancer therapy, it may prove to be more effective in synergy with common cytotoxic drugs than given on its own.

To increase the efficiency of TC-A as an anticancer agent against Bcl-2-overexpressing cells, derivatives were sought that may exhibit greater specificity against certain antiapoptotic proteins. It was found that a group of 21-acetoxy-9-glycoslyoxy derivatives of TC-A potently inhibited Bcl-2 function in a cell-free assay (34). However, it remains to be seen how these derivatives would behave *in vitro,* or whether they do in fact specifically target cells with overexpression of Bcl-2 more that those overexpressing Bcl-XL.

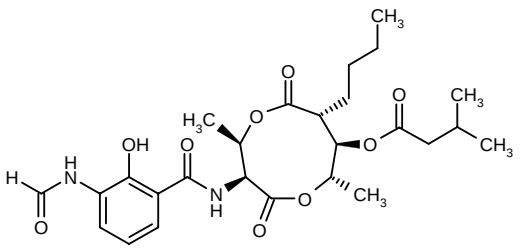
More recently, it was found that TC-A probably acts directly on the mitochondria to reduce transmembrane potential, independent of Bcl-2 overexpression (35). T-cell acute lymphocytic leukemia (T-ALL) cells were transfected with either a vector control or a Bcl-2 plasmid, with both cell types experiencing the same extent of apoptosis following TC-A exposure. Bcl-2-overexpressing cells took slightly longer to present morphological changes associated with apoptotic death, presumably due to the higher amount of Bcl-2. Jurkat cells treated with TC-A experienced upregulation of four transcription factors and six members of the heat shock protein (HSP) family, followed by activation of the endoplasmic reticulum (ER) stress-induced apoptotic pathway. These findings reveal the possibility that TC-A-induced mitochondrial changes may be secondary to its interference with ER activity.

B-cell chronic lymphocytic leukemia (B-CLL) cells are defective in cell death signaling mechanisms, and were therefore chosen to further observe the effects of TC-A on the ER. To understand the role played by Bcl-2 in this reaction, B-CLL cells were compared to normal B-cells and showed no significant differences in apoptotic characteristics induced by TC-A (36). Tetrocarcin A caused upregulation of HSP70, indicative of ER disruption, which was positively correlated with the number of apoptotic cells in the population. One drawback of using a drug that triggers the ER stress-induced cell death pathway is the simultaneous activation of immune responses, and the release of interleukins and TNF-α, which can damage neighboring healthy cells.

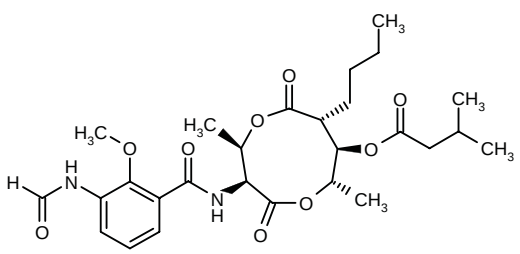
Antimycin A

Antimycin A is an antibiotic derived from *Streptomyces* and is known to bind cytochrome *b* to inhibit electron transport at complex III. It was recently found to be capable of mimicking various proapoptotic BH3 peptides during a screen of known inhibitors of mitochondrial respiratory complexes I, II and III and ATP synthase (13), and has been shown to compete with Bak BH3 for binding to the hydrophobic surface pocket of Bcl-2 and Bcl-XL. Specific induction of apoptosis in Bcl-XL-overexpressing TAMH murine hepatocytes was observed via nuclear chromatin condensation and swollen mitochondria, which was not blocked following treatment with the broad-spectrum caspase inhibitor zVAD-FMK (17). This suggests that antimycin A may be especially useful for the treatment of tumors overexpressing the IAP family of caspase inhibitors. To confirm that the induction of apoptosis was due to direct binding of antimycin A to Bcl-XL, DOCK was used to identify the ligand binding site as overlapping the hydrophobic pocket on Bcl-XL to which BH3 domains naturally bind. Additionally, a synthetic phenacylether derivative used during repeat assays was unable to induce cytotoxicity or to displace antimycin A from Bcl-XL. A study conducted on the effects of Bcl-XL mutations found that antimycin A favors binding to the wild-type protein, with low affinity for mutated Bcl-XL (37). While the tertiary structure remained intact, three site-directed mutations in the hydrophobic binding groove decreased antimycin binding affinity 8-20-fold, suggesting that antimycin A treatment may not be beneficial in tumors expressing mutated Bcl-XL. No experiments were conducted on mutated forms of Bcl-2, but the conserved nature of the binding grooves of the two proteins leads to the conclusion that antimycin would also favor the wild-type form of Bcl-2 over certain mutated forms.

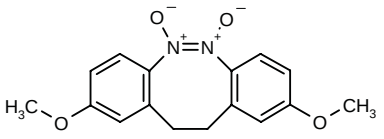
Another group demonstrated the specificity of antimycin A and several of its analogues for Bcl-2 over Bcl-XL, although the use of different cell lines may have contributed to the discrepancy (38). One analogue of interest, 2-methoxyantimycin A3, exhibited significant



Antimycin A



2-Methoxyantimycin A3



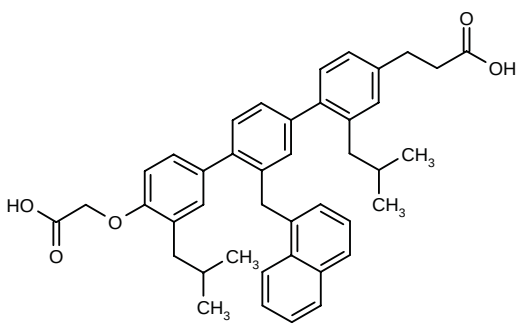
Compound **6**

specificity towards Bcl-2, suggesting the use of antimycin A and its derivatives as templates for the design of highly specific inhibitors of Bcl-2 family members without systemic mitochondrial toxicity, such as interference in oxidative phosphorylation (12, 17).

A study in A-172 human glioblastoma cells used antimycin A to relate drug-induced generation of reactive oxygen species and ATP depletion to the simultaneous induction of cell death (39). While cell death corresponded with the appearance of common characteristics of chemical hypoxia, the experiments suggested that death was more dependent upon antimycin-induced increases in mitochondrial permeability, probably due to its inhibition of Bcl-2. This further substantiates the design of antimycin A-derived compounds that retain the Bcl-2-inhibitory function and lack systemic mitochondrial toxicity.

*Compound **6***

DOCK was used to search 200,000 nonpeptide small molecules from the NCI's three-dimensional database for compounds with the ability to target and inactivate Bcl-2, resulting in the identification of 35 potential inhibitors (28). *In vitro* FP assays isolated 7 candidates showing active inhibition of Bak binding to Bcl-2, followed by trypan blue exclusion and MTT assays to determine the effects of the compounds on apoptotic resistance. Human myeloid leukemia HL-60 cells were chosen because of their high Bcl-2 expression, and compound **6** was found to be the most potent inhibitor of cell viability, with apoptosis induction correlating to the amount of Bcl-2 expressed. A lack of significant activity in MDA-MB-453 and T-47D breast cancer cells, which express very low levels of Bcl-2, illustrates the specificity of compound **6** for Bcl-2 and implies therapeutic potential for the inhibitor alone or in combination with cytotoxic drugs or radiation.



Compound **4**

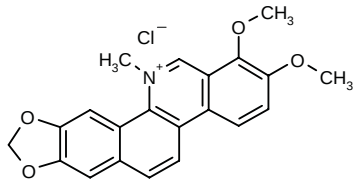
*Compound **4***

To design a small organic mimic of the BH3 domain, the crystal and solution structures of the Bak/Bcl-XL complex were analyzed, revealing four hydrophobic residues of Bak responsible for the binding of its helical edge to Bcl-XL: Val74, Leu78, Ile81 and Ile85 (40). This information was used to design a terphenyl scaffold with substituents that mimicked key hydrophobic characteristics of the helical exterior of Bak, referred to as compound **4**. This proteomimetic was able to competitively displace a 16-mer Bak peptide from the hydrophobic ligand binding cleft of Bcl-XL, with a binding affinity 1,000-fold stronger than small-molecule inhibitors previously discovered by library screening. A similar study was conducted on a different type of organic scaffolding molecule, the trispyridylamide scaffold (41). It was hoped that this scaffold design would provide a more efficient side-chain functionality with more accurate distance and angular constraints to mimic the natural Bcl-XL ligands. The α-helical secondary structure of Bak was considered to create a synthetic compound with a highly preorganized conformation accomplished through a stabilizing bifurcated hydrogen-binding network. This compound was shown to bind the same hydrophobic cavity as Bak and to inhibit Bak BH3/Bcl-XL complexation at a lower concentration than compound **4**.

Further experiments investigating the effectiveness of these compounds in the inhibition of apoptotic resistance mechanisms should include *in vitro* assays to measure the characteristics of apoptosis after addition of the compound(s) to tumor cell cultures. Additionally, it would be beneficial to examine any specificity of the compound(s) for either Bcl-2 or Bcl-XL.

Chelerythrine chloride

Chelerythrine chloride is a natural benzophenanthridine alkaloid extracted from the stems of *Bocconia vulcanica.* The compound was discovered to indirectly induce apoptosis in SQ-20B cells by inhibiting protein kinase C, thus inducing the ceramide production pathway



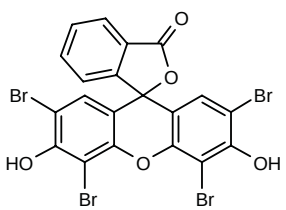
Chelerythrine chloride

of cell death (42). Chelerythrine was also able to delay tumor growth in SCID mouse xenograft models compared to an untreated control group.

More recently, in a high-throughput screening of 100,000 compounds extracted from plants, fungi and microbes, chelerythrine chloride was identified as an inhibitor of the interaction between the Bak BH3 domain and Bcl-XL (43). In an FP assay, chelerythrine was found to displace Bak from both Bcl-XL and Bcl-2 and to induce apoptosis in human neuroblastoma SH-SY5Y, colon carcinoma HCT 116 and breast adenocarcinoma MCF7 cells. SH-SY5Y cells overexpressing Bcl-XL were susceptible to chelerythrine-induced apoptosis but were resistant to the effects of etoposide and staurosporine. It would be interesting to study the effects of chelerythrine chloride on cells known to be resistant to common chemotherapeutic drugs and whether chelerythrine has the ability to induce tumor growth delay *in vivo.*

*Compound **A5***

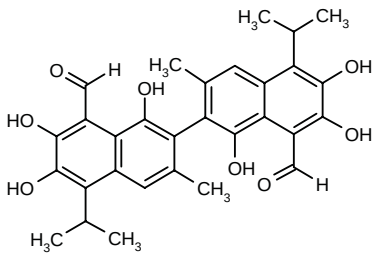
The BIAcore assay was used to screen 10,000 compounds for the ability to inhibit the interaction between Bax and Bcl-XL, revealing only 1 compound able to induce more than 50% inhibition (44). In Bcl-XL-transfected MCF7 cells compound **A5**, 2',4',5',7'-tetrabromofluorescein, was found to have binding affinities in the nanomolar range, while HA-14-1 had affinities in the micromolar range. However, treatment with **A5** also inhibited the homodimerization of Bax, which is an important step in the apoptotic cascade. Analogues of **A5** should be tested for their ability to interrupt Bax-Bcl-XL binding without affecting the homodimerization of Bax, which may prove to be a useful antitumor strategy for cells overexpressing Bcl-XL.



Compound **A5**

Preclinical studies of BL-193 and TW-37

The incidence of non-Hodgkin's lymphoma (NHL) has been increasing over the past three decades and is now the leading cause of death in males aged 15-54. Diffuse large cell lymphoma (DLCL) is the most common subtype of NHL. The CHOP regimen (a combination of cyclophosphamide, doxorubicin, vincristine and prednisone) remains the standard therapy despite the fact that only 30-40% of DLCL cases are cured using this regimen. The design of nonpeptide, drug-like, cell-permeable, potent small-molecule inhibitors that bind to the BH3 binding site in Bcl-2/Bcl-XL is a new and exciting area of research for the therapy for lymphomas, > 80% of which overexpress Bcl-2/Bcl-XL. Results using FP-based methods indicate that BL-193 (Gossypol) and TW-37 are very potent nonpeptide, small-molecule inhibitors with K_i values of 464 and 158 nM, respectively, for Bcl-2, and respective values of 570 and 684 nM for Bcl-XL. Both BL-193 and TW-37 demonstrated antitumor activity in our lymphoma model similar to clinically employed chemotherapeutic agents such as vincristine and cyclophosphamide. We hypothesized that these compounds have therapeutic potential and are capable of sensitizing human lymphoma cells to CHOP therapy through inhibition of the antiapoptotic function of Bcl-2/Bcl-XL proteins.



BL-193

The antitumor activity of BL-193 and TW-37 was evaluated *in vivo* and *in vitro* in the WSU-$DLCL_2$ lymphoma model. *In vitro*, cells were treated with 0-4.0 μg/ml over 4 days and viability and apoptosis were determined. BL-193 and TW-37 resulted in a concentration- and time-dependent inhibition of cell proliferation. The IC_{50} values for BL-193 and TW-37 were 2 μM and 300 nM, respectively. BL-193 and TW-37 induced apoptosis in this cell line (40-52%) and cells from lymphoma patients (n=3; 30-55% apoptosis), with no toxicity against normal peripheral blood lymphocytes. *In vivo*, WSU-$DLCL_2$-bearing SCID mice were treated with BL-193, TW-37 or their combination with CHOP. Tumor growth inhibition for BL-193 + CHOP and TW-37 + CHOP was statistically significantly greater than with BL-193, TW-37 or CHOP alone ($p < 0.5$). TW-37, both as a single agent and in combination with CHOP, appears to be less toxic than BL-193. The use of this inhibitor alone or in combination with active cytotoxic agents should be further explored clinically for the treatment of lymphoma.

Potential of small-molecule Bcl-2 inhibitors as anticancer agents

One of the most promising aspects of small-molecule inhibitors in treating cancer is that their targets and mechanisms of action are different from those of cytotoxic

drugs and radiation. This makes it feasible to combine them with other treatments, creating a synergistic therapy unlikely to be associated with the development of cross-resistance or increased toxicity (45). For example, a recent study conducted on OCI acute myelogenous leukemia (OCI-AML) cells with constitutive MAPK (mitogen-activated protein kinase) activation showed successful synergy between PD-184352, an MAPK inhibitor, and HA-14-1 (46). The cells are normally resistant to PD-184352, but when combined with the small-molecule Bcl-2 inhibitor HA-14-1, significant apoptosis was measured 24-48 h following treatment. This is especially interesting because 74% of AML patients have constitutively activated MAPK, which promotes cell growth and survival, and inhibition of MAPK is usually cytostatic in cancers, preventing cell proliferation without inducing apoptosis (47). Simultaneous inhibition of survival mechanisms and antiapoptotic mechanisms may be a sound strategy for the treatment of a variety of cancers exhibiting resistance to cytotoxic drugs or to apoptosis indirectly triggered by cytostatic drugs.

The main hurdles in the development of small-molecule inhibitors of Bcl-2 as a common component of cancer therapy regimens are the need to discover highly specific inhibitors that can be targeted to tumor cells and the low binding affinity often seen in solution and *in vivo* (2, 8, 48). Common molecular modeling approaches such as DOCK and GRID use stochastic applications that: 1) do not account for all possible relative configurations and/or conformational changes of the molecules involved; and 2) do not consider the possibility of ligand dimerization before or during binding to the substrate (15). Furthermore, the solvation effects on the target protein or possible ligands are not taken into consideration by any of the currently popular screening methods (49). Several virtual screening techniques require solubility in aqueous solution, and thus the lack of aqueous solubility of certain compounds is another stumbling block that needs to be addressed in order to incorporate as many compounds in the screening process as possible. A better geometric understanding of protein-ligand complexes is imperative to subsequent chemical optimization techniques, such as increasing interaction affinity and eliminating drug molecule aggregation or nonspecific binding, to fine-tune drug action against the target (22). New strategies are currently being explored to make drug discovery and/or design more efficient and accurate.

Techniques for the discovery and design of small-molecule Bcl-2 inhibitors

The most popular high-throughput screening method for monitoring the effects of a library of molecules on a given substrate (*i.e.*, inhibition of Bcl-2 heterodimerization) is probably the FP assay, which was incorporated into the study of small peptides, HA-14-1, compound **6** and chelerythrine as Bcl-2 inhibitors (27-29, 43). The assay is informative but time-consuming and has often produced too many false-positives to be an efficient method of screening libraries of 100,000 compounds or more (12, 50). More recently, FP assays have been combined with virtual computer-based screening methods to compensate for the shortcomings of each separate method. For example, one group used direct NMR to map the interaction surfaces of Bcl-2 and a library of possible inhibitors, then TreeDock software to analyze each relative orientation of the ligand and substrate, and finally validated *in vitro* binding affinity of the lead compounds with FP and NMR titration (15). TreeDock searches for clash-free docking orientations of a multidimensional representation of a substrate and a compound library, a significant improvement over its predecessor, DOCK, which searched databases of ligands against negative two-dimensional images of the substrate (23). This combination proved effective in quickly eliminating the most improbable compounds while isolating a small group of the most interesting leads for specific study.

An additional technique recently employed to complement high-throughput screening is SAR, or structure-activity relationships, in which NMR data are used to correlate the structural elements of small-molecule inhibitor leads with their inhibition potentials and to define the topology of the substrate's binding pocket (51). SAR (sometimes referred to as SAR by NMR) gives high-sensitivity results for weak binding interactions and few false-positives, although large amounts of protein are required and must be soluble at micromolar concentrations in aqueous solution (52, 53). No articles using SAR by NMR to identify small-molecule inhibitors of Bcl-2 are currently available, but this does seem a promising technique to add to the repertoire of discovery assays.

Protein targets other than Bcl-2

While Bcl-2 plays a very important role in the regulation of apoptosis, the inhibition of proteins other than Bcl-2 may be a more effective treatment for certain types of cancers. Several proteins function to induce expression of Bcl-2 and other survival proteins during cell stress. These proteins may be inhibited to reduce the amount of Bcl-2 being produced, making its inhibition a more feasible task. Cancer cells expressing mutated versions of Bcl-2 or Bcl-XL may not be susceptible to small-molecule inhibitors that are effective against the wild-type proteins, as demonstrated by the lack of apoptotic events following antimycin A exposure to cells expressing mutated Bcl-2 (37). It should also be considered that certain cell types may be more reliant on the activity of proapoptotic proteins, like Bax, than the inhibition of Bcl-2 to respond to chemotherapy-induced apoptosis (18, 54).

Aloe-emodin is an antitumor compound derived from the root of *Rheum palmatum,* and has been reported to induce apoptosis in lung carcinoma NCI-H460 cells via transcriptional reduction of protein kinase C, caspase 3 and Bcl-2 (55). Inhibition of Bcl-2 transcription was also seen with flavopiridol-induced inhibition of cyclin-dependent kinases in head and neck tumor HN30 cells (56, 57). However, flavopiridol recipients showed symptoms of

dose-limiting toxicity, including diarrhea, fever and local tumor pain (58). Both aloe-emodin and flavopiridol may be useful additions to a therapeutic regimen aimed at inhibiting Bcl-2 activity. A third possible target for inhibition of Bcl-2 transcription could be NF-κB, which increases the expression of Bcl-2 upon the receipt of various survival signals (14).

Because caspases are necessary for the apoptotic disassembly of cellular structures into phagocytic bodies, inhibitors of caspase activation such as Smac or the IAP family can rescue several types of cancer cells from apoptosis, whether Bcl-2 is active or under chemical inhibition. A study on IAP expression in several human prostate cancers found significant overexpression of cellular IAP 1 and 2, X chromosome-linked IAP and survivin in all the tumor lines compared to normal prostatic epithelium (59). Simultaneous inhibition of Bcl-2 and caspase may be required to allow induction of apoptotic death in cells exhibiting this type of survival mechanism (60). However, the only reported inhibitors of Smac and IAP are oligonucleotides and small peptides, respectively (12).

Bcl-2 and Bcl-XL are capable of rescuing many cell types from apoptotic cascades that require mitochondrial amplification of death signals. However, some cell lines are more highly influenced by mitochondria-independent apoptotic pathways (14), and may not undergo apoptosis following inhibition of Bcl-2. For example, multiple myeloma and lymphoid cell lines are influenced by TRAIL-induced apoptotic signaling, and are not affected by either overexpression or inhibition of Bcl-2 (61). Tumor cell lines such as these would benefit from the discovery of small molecules that activate proteins functioning at mitochondria-independent points in the apoptotic cascade, or inhibit any proteins functioning to impede cell death, such as the IAP caspase inhibitor family.

Bortezomib, a novel proteasome inhibitor designed by Millennium, was shown to effectively induce apoptosis in NCI-H460 non-small cell lung carcinoma cells by triggering mitochondrial production of reactive oxygen species, which induce cytochrome *c* release (62). This process occurs independently of Bcl-2, making it useful against cancers overexpressing antiapoptotic proteins, but it also triggers a local inflammatory response that can damage healthy tissues near the tumor cells. A recent study with various multiple myeloma cells utilized a sequential treatment consisting of bortezomib followed by HA-14-1, which resulted in synergistic induction of apoptotic events (31).

Possibly even more significant than mitochondria-independent apoptotic mechanisms are the cell survival pathways. Even if apoptosis is chemically activated, a cell with constitutively activated survival pathways could still be able to avoid cell death. Overexpression of growth factors or constitutive activation of their receptors contributes to cell proliferation, inhibition of apoptosis, resistance to hormonal or cytotoxic therapies, angiogenesis and metastasis in many tumor types, especially epithelial cells (45). Iressa™ (gefitinib, ZD-1839) is a quinazoline derivative used to inhibit ligand activation and tyrosine kinase activity of epidermal growth factor receptors (EFGRs) in a variety of cancers, such as colon, ovarian, breast and non-small cell lung carcinomas. ZD-1839 has been shown to exhibit antitumor activity both by suppressing EGFR-mediated survival pathways and activation of Akt, and by inhibiting the expression of Bcl-2 and Bcl-XL. ZD-1839 was recently found to increase the expression of the proapoptotic protein Bad, which may be its major mechanism for triggering apoptotic death (63). Both ZD-1839 and OSI-774, another small-molecule EGFR inhibitor currently under investigation, have shown activity against non-small cell lung cancer (64-66). In addition to EGFR, other growth factor receptors should be considered as drug targets, especially the vascular endothelial growth factor (VEGF) receptor, which is required for tumor angiogenesis (67).

The survival pathway mediated by phosphatidylinositol 3-kinase (PI3-K) and its effector Akt has been under investigation as a target for sensitizing cells to chemotherapy-induced apoptosis. The nonspecific tyrosine kinase inhibitor staurosporine and its 7-hydroxy analogue UCN-01 have antiproliferative activity against several tumor lines, probably via their inhibition of PI3-K. However, troublesome side effects associated with these agents include vomiting and hyperglycemia with insulin resistance, and the resultant cell death exhibited characteristics of necrosis rather than apoptosis (58, 68). While a promising target for cancer therapies, these PI3-K inhibitors require modifications to reduce toxicity to the patient and may be more useful in combination with non-cytotoxic triggers of apoptosis.

The Ras proteins are transducers of proliferative and survival signals to downstream effectors such as PI3-K and Bad and may represent another promising target for inhibition by small molecules (69). Oncogenic mutation of Ras has been shown to simultaneously activate cell growth and suppress apoptotic signaling. A synthetic *trans*-farnesylthiosalicylic acid molecule was used to displace Ras from its membrane binding site in 607B human melanoma cells (70). This resulted in accelerated Ras degradation and 80% inhibition of melanoma xenograft growth in SCID mice, with no adverse effects observed. Considering the prevalence of Ras mutations in pancreatic (90%), lung (30%) and colorectal (50%) carcinomas (71), inhibition of Ras function with resultant apoptosis will be a welcome addition to the therapeutic regimens for several types of cancers.

Conclusions

A 2003 study conducted by the NCI on data obtained from the National Center for Health Statistics estimated that over 1 million new cases of cancer will be diagnosed this year (72). The probability of developing cancer in one's lifetime is 43.5% for men and 38.5% for women. These facts only begin to illustrate the urgent need for the discovery of more effective cancer therapies.

The inhibition of Bcl-2 as a means of overcoming apoptotic resistance in tumor cells has advantages over commonly used cytotoxic drugs. Many chemotherapeutic drugs act on cellular proteins that are required for the function of healthy, noncancerous cells, thus causing systemic toxicity in the patient (58, 73). Because the function of Bcl-2 is not absolutely necessary for normal cellular activities, it is predicted that its inhibition should not adversely affect normal cells (12). DNA methylation of antiapoptotic genes has been attempted to alleviate drug resistance, but the histone deacetylases used in the process also alter expression of genes required for healthy functioning and produce systemic toxicity (6). Small peptides and antisense oligonucleotides have been designed to inhibit Bcl-2 function with variable success, but the treatments are difficult to administer efficiently and produce problematic side effects, mostly due to a lack of target cell specificity (44, 74). Much work has been done to design small organic Bcl-2 inhibitors that will bind and inhibit only Bcl-2, which will assist in the targeting of the small-molecule inhibitors to tumor cells and further reduce systemic toxicity.

While much has been learned about the inhibition of Bcl-2 and Bcl-XL by small organic molecules, there are still questions to be answered before these new cancer treatments can be utilized to their full potential. For instance, the heterodimerization of Bcl-2 with its binding partners is still somewhat unclear (2). All currently known organic small-molecule inhibitors of Bcl-2 also bind Bcl-XL and do so with similar affinity, presumably due to the highly conserved BH3 domains shared by the two proteins (12). Additionally, the proapoptotic proteins Bax and Bid appear to undergo conformational changes to expose their BH3 domains for heterodimerization, and a clearer understanding of the process may shed light on the requirements for strong binding of a small-molecule inhibitor to Bcl-2 (13). Learning more about the mechanistic details of these heterodimerization reactions and the factors governing binding specificity may make it possible to design small-molecule inhibitors of Bcl-2 with higher binding affinities at lower drug concentrations. Another feat will be the design of a more efficient protocol for screening libraries of potential Bcl-2 inhibitors, possibly involving multiple methods combined in a way that compensates for the shortcomings of each separate method. The design of virtual screening software that allows for protein flexibility and conformational changes during the screening process will alleviate some of the inaccuracy currently hindering the progress of drug discovery.

It has been suggested that Bcl-2 ligands with extremely high affinity could be synthesized by linking two molecules that bind different domains of the target protein with moderate affinity (75). Presumably, the binding affinities of the components of the synthetic ligand would combine synergistically to create an inhibitor with a more potent effect than either of the molecules alone.

Finally, the design of more informative *in vitro* and *in vivo* assays to better explore the function of small-molecule inhibitors of Bcl-2 would be very beneficial (8). Important information regarding small-molecule inhibitor specificity could be gathered from a comparison of their effects on a cell line expressing basal levels of Bcl-2 *versus* the same line in which the Bcl-2 gene is knocked out, silenced or overexpressed. Several small-molecule Bcl-2 inhibitors have been tested for antitumor function *in vitro,* which is an important step in the drug development process.

In conclusion, the inhibition of apoptosis contributes significantly to both tumorigenesis and chemotherapeutic resistance. The development of drugs able to induce apoptosis in tumor cells without damaging healthy cells in the vicinity of the tumor or anywhere else in the body is an important endeavor in the fight against cancer. There is substantial evidence to support the use of Bcl-2 inhibitors, especially small organic inhibitors, to overcome apoptotic resistance in a variety of human cancers. Much remains to be deciphered regarding the mechanism of inhibition of Bcl-2 and Bcl-XL, but there is potential to use this information for the design of more potent and specific small-molecule inhibitors of Bcl-2 to treat stubborn cancers on their own or in combination with other cytotoxic drugs.

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