



Synthetic peptides and non-peptidic molecules as probes of structure and function of Bcl-2 family proteins and modulators of apoptosis

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The Bcl-2 family includes a growing number of proteins that play an essential role in regulating apoptosis or programmed cell death. Members of this family display diverse biological functions and can either inhibit or promote cell death signals. Abnormal gene expression of some Bcl-2 family members such as Bcl-2 that inhibits apoptosis is found in a wide variety of human cancers and contributes to the resistance of tumor cells to conventional therapies through interfering with the cell death signals triggered by chemotherapeutic agents. As such, elucidating the structure-function and mechanism of the Bcl-2 family is important for understanding some of the fundamental principles underlying the death and survival of cells and of practical value for developing potential therapeutics to control apoptosis in pathological processes. Synthetic peptides derived from homologous or heterogeneous domains in Bcl-2 family proteins that might mediate different biological activities provide simplified and experimentally more tractable models as compared to their full-length counterparts to dissect and analyze the complex functional roles of these proteins. Non-peptidic molecules identified from random screening of natural products or designed by rational structure-based techniques can mimic the effect of synthetic peptides by targeting similar active sites on a Bcl-2 family member protein. In this article, we review recent progress in using these synthetic peptides and non-peptidic mimic molecules to obtain information about the structure and function of Bcl-2 family proteins and discuss their application in modulating and studying intracellular apoptotic signaling.

Keywords: apoptosis; Bcl-2 family; cancer; caspases; drug design; mitochondria; peptides; structure and function.

Introduction

Apoptosis or programmed cell death is the prevalent mechanism complementary to proliferation that is critical

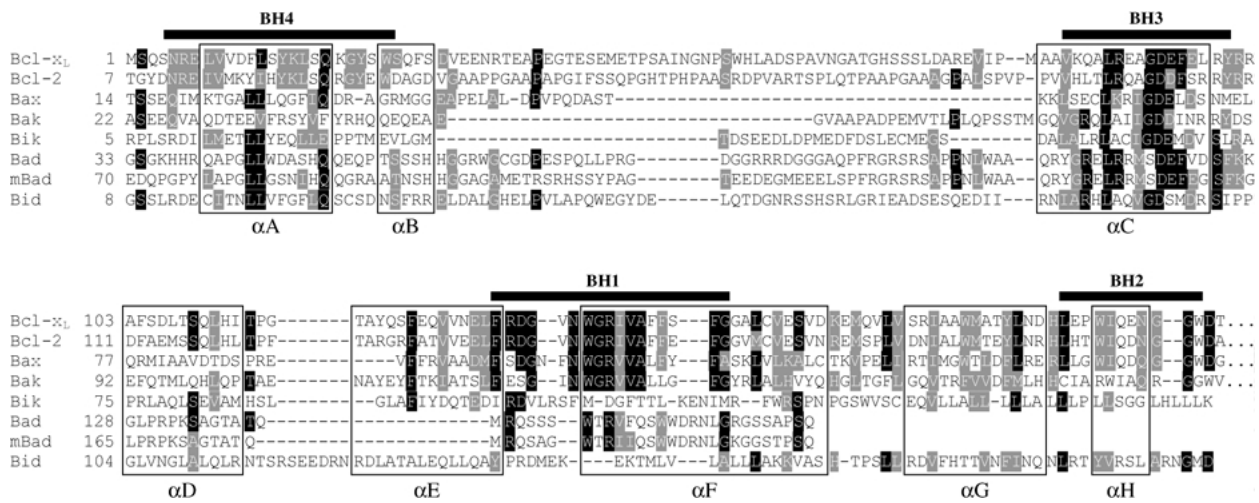
for the normal development and function of multicellular organisms. The dysregulation of apoptosis is implicated in a number of human diseases, including cancer, autoimmune diseases, viral infections, neurodegenerative diseases, AIDS and cardiovascular diseases.¹ There are two major pathways for apoptosis that have been elucidated so far, both of which involve a family of cysteine proteases with aspartate specificity called caspases as the executioners of apoptosis.² The mitochondria provide one of the apoptotic pathways, from which cytochrome *c* is released upon the stimulation by a variety of cell death triggers.³ This leads to activation of Apaf-1, caspase-9 and caspase-3. Another apoptotic pathway involves the ligation of death receptors such as Fas and activation of caspase-8 and subsequently caspase-3.⁴

The Bcl-2 family proteins are key regulators of the apoptotic pathway involving the mitochondria and cytochrome *c*.^{5,6} By controlling the release of apoptogenic factors such as cytochrome *c* from the mitochondria, members of the Bcl-2 family can either suppress or promote the cell death signal. These proteins share at least one of four homologous regions termed Bcl homology (BH) domains (BH1 to BH4) (Figure 1). Based on their biological function and sequence homology, Bcl-2 family members can be classified into three main categories: anti-apoptotic proteins such as Bcl-2 and Bcl-x_L that inhibit cell death, pro-apoptotic proteins such as Bax and Bak that promote cell death, and "BH3-only" proteins such as Bad and Bid that are pro-apoptotic and share sequence homology only in the BH3 domain. Despite the opposing biological functions and wide differences in amino acid sequences, experimentally determined structures of Bcl-2,⁷ Bcl-x_L,^{8,9} Bax¹⁰ and Bid^{11,12} are surprisingly similar (Figure 2). This structural similarity observed so far for these four proteins representing all three Bcl-2 subfamilies is in sharp contrast to the functional and sequence diversity that is used to classify them into different categories.

The mechanism by which Bcl-2 family proteins regulate apoptosis has been a subject of intensive research. Currently it remains controversial and several models have

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Figure 1. Aligned amino acid sequences of representative members of the Bcl-2 family. The conserved BH1-4 domains are indicated for the anti-apoptotic Bcl-2 and Bcl-x_L whereas pro-apoptotic Bax and Bak and members of the BH3-only subfamily Bik, Bad, mBad and Bid show homology in one or more of these regions. The boxed regions represent the helical segments observed in the structures of Bcl-x_L,^{8,9} Bcl-2,⁷ Bax¹⁰ and Bid.^{11,12}



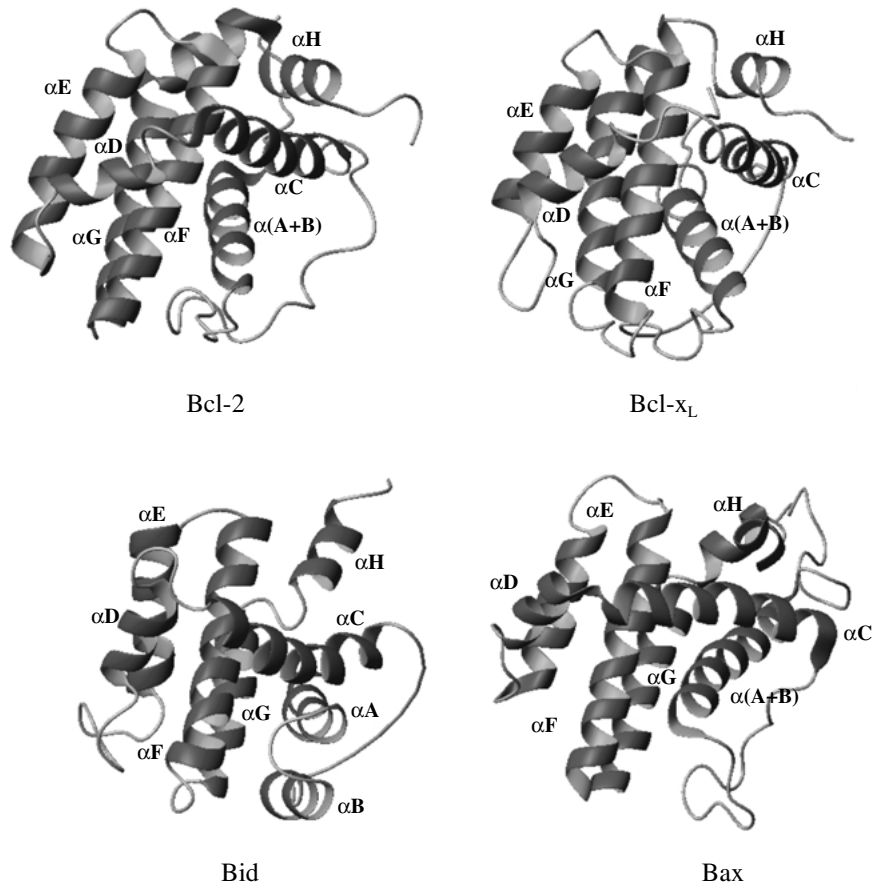
been proposed. Homo- and heterotypic dimmers are observed among members of the Bcl-2 family. Through heterodimerization anti-apoptotic and pro-apoptotic proteins neutralize the biological activity of opposing partners and thus the fate of a cell is determined by the ratio of these proteins and the different combinations of their complexes.¹³⁻¹⁵ Some information about the interactions between anti-apoptotic and pro-apoptotic Bcl-2 family members is provided by the three-dimensional structure of Bcl-x_L in complex with a peptide derived from the BH3 domain of Bak.¹⁶ The structure reveals a hydrophobic surface pocket on Bcl-x_L formed by the BH1-3 domains bound by the Bak BH3 domain peptide in helical conformation. Since the BH3 domain is buried in the structures of pro-apoptotic proteins Bid^{11,12} and Bax,¹⁰ this raises the speculation that conformational changes are necessary for the exposure of the BH3 domain of a pro-apoptotic protein and its inhibition of the functional pocket on anti-apoptotic partners. In the cell environment, pro-apoptotic Bcl-2 family members are suggested to undergo such conformational changes¹⁷ triggered by dephosphorylation¹⁸ or proteolytic cleavage by caspases.¹⁹⁻²¹

In addition to the regulation of each other's activity through heterodimerization, Bcl-2 family proteins can function independently as observed in transgenic and knockout mice experiments.²² Some family members such as Bcl-2, Bcl-x_L, Bax, and Bid can form ion channels in lipid bilayers *in vitro*²³⁻²⁷ although the existence of these channels *in vivo* and their functional relevance to apoptosis remain to be established. Another mechanism by which Bcl-2 family proteins regulate apoptosis independently is suggested to be involved with their direct interactions with the voltage-dependent anion channel (VDAC).²⁸ It is suggested that cytochrome c is released from the mito-

chondria through VDAC channel and that anti-apoptotic and pro-apoptotic Bcl-2 family members exert different effects of closing and opening the channel, respectively.²⁹

As described above, Bcl-2 family proteins display multiple functions and interactions among themselves and with other proteins. Presumably these activities are mediated by different regions of Bcl-2 family proteins including the highly conserved BH1-BH4 domains as well as those of a high degree of heterogeneity. Many studies have been reported using synthetic peptides derived from specific functional domains of pro-apoptotic and anti-apoptotic Bcl-2 members to dissect and characterize the role of these regions in regulating apoptosis. As compared with full-length proteins, these peptides devoid of other domains provide simplified and more tractable models for structural and functional studies *in vitro* or in cell free systems. When properly modified to incorporate cell permeable moieties, these peptides can also be used to study mechanisms of Bcl-2 function in living cells. Another strategy for studying and controlling the function of Bcl-2 family proteins *in vitro* and *in vivo* is to use small non-peptidic organic molecules either discovered by random screening procedures or designed by structure-based rational techniques that target certain functional sites of the Bcl-2 family such as the surface pocket described above. Cell permeable synthetic peptides and non-peptidic molecules targeted to a specific Bcl-2 family member can be used to control and modulate the function of that particular protein instantaneously. This allows the dissection and determination of the kinetic time-course, not possible with genetic techniques, of complex molecular interactions and signaling events involving multiple Bcl-2 family members and other proteins during apoptotic processes.

Figure 2. The structures of Bcl-2,⁷ Bcl-x_L,⁸ Bax¹⁰ and Bid^{11,12} displaying the similarity in their overall structure and folding pattern despite their opposing biological functions and wide differences in amino acid sequences (see Figure 1). Note that the C-terminal helix of Bax which is a putative transmembrane domain and packed against the surface pocket on Bax is not shown in the figure. Also note that Bid possesses two helical segments (α A and α B, see Figure 1) in BH4 region whereas Bcl-2, Bcl-x_L and Bax display a continuous helix combining these two segments as denoted by α (A + B).



Synthetic peptides and non-peptidic molecules targeted to the Bcl-2 family also provide the leads for development of potential anticancer therapeutics. Abnormal expression of Bcl-2 gene is commonly found in a wide variety of human cancers and contributes to the resistance of tumor cells to conventional therapies by interfering with the cell death signals triggered by chemotherapeutic agents.^{15,30} The functional blockade of Bcl-2 or Bcl-x_L should either restore the apoptotic process in tumor cells or sensitize tumors for chemo- and radiotherapies. Consistent with this notion, antisense oligonucleotides targeting Bcl-2 gene have been found to inhibit non-Hodgkin's lymphoma in human.³¹ Oligonucleotides³² and single-chain antibodies³³ against Bcl-2 have also been shown to improve the chemosensitivity of tumor cells. In this article, we review recent studies and progress in using synthetic peptides and non-peptidic organic molecules to study the structure and function of Bcl-2 family proteins. The application of these agents for modulating apoptosis in tumor

cells and the implication for anticancer drug development are also discussed.

Structure and function of peptides derived from the Bcl-2 family

The BH3 domains of pro-apoptotic Bcl-2 family members (Figure 3) are suggested to interact with the surface pocket of Bcl-2 or Bcl-x_L and neutralize their anti-apoptotic activity.³⁴ How these BH3 domains recognize their anti-apoptotic partners and the mechanism by which they trigger apoptosis have been the subject of much investigation. Sattler *et al.* synthesized peptides containing the BH3 domain of several Bcl-2 family proteins including Bcl-2, Bcl-x_L, Bax, Bik, and Bak.¹⁶ These synthetic BH3 peptides displayed widely different binding affinities to Bcl-x_L with the peptide derived from Bak BH3 domain (residues 72–87, see Figure 3) showed relatively high binding affinity ($K_D = 0.34 \mu\text{M}$). The difference in

Figure 3. The aligned amino acid sequences of the BH3 domain in representative members of the pro-apoptotic and BH3-only subfamilies. The amino acid sequence of the BH3 domain of mouse Bad (mBad) is shown because it has been used for peptide design. All other BH3 sequences are from human proteins. The numbering of residues corresponds to that in the native protein (see Figure 1).

	16-amino acid core of BH3
Bax (52-76)	Q D A S T K K L S E C L K R I G D E L D S N M E L
Bak (67-91)	P S S T M G Q V G R Q L A I I G D D I N R R Y D S
Bik (50-74)	C M E G S D A L A L R L A C I G D E M D V S L R A
Bad (103-127)	N L W A A Q R Y G R E L R R M S D E F V D S F K K
mBad (140-164)	N L W A A Q R Y G R E L R R M S D E F E G S F K G
Bid (79-103)	Q E D I I R N I A R H L A Q V G D S M D R S I P P

binding affinity of these BH3 peptides derived from different Bcl-2 family members and Ala-substituted analogs of Bak BH3 peptide was explained by the NMR structure of Bcl-x_L bound by Bak BH3 peptide.¹⁶

Compared with other BH3 peptides described above, the BH3 peptide derived from mouse Bad protein (residues 140–165 of mBad, Figure 3) showed higher affinity for Bcl-x_L with the K_D value of 6 nM.³⁵ The structure of Bcl-x_L protein complexed with a 25-residue peptide derived from human Bad BH3 domain (NLWAAQRYGRELRRMSDEFVDSFKK) was determined by using NMR spectroscopy.³⁶ The overall structure is similar to the complex of Bcl-x_L bound by a 16-residue Bak BH3 peptide. The N- and C-termini of Bad BH3 peptide form additional interactions with Bcl-x_L protein. However, such contacts were suggested to play no major role in the increase of receptor binding based on results from mutant peptides containing Ala-substitutions at N- and C-termini. Rather, it was argued that the high Bcl-x_L binding affinity was contributed by the enhanced helical stability of the longer Bad BH3 peptide sequences as compared with other shorter 16-amino acid BH3 peptides.

Using an *in vitro* protein-protein binding assay, Otilie, Diaz and coworkers evaluated the effects of peptides (either 16 amino acids or longer) derived from the BH3 domains of Bak, Bax and Bad in blocking the heterodimerization of Bcl-x_L with death agonists.^{37,38} These BH3 peptides were shown to inhibit Bcl-x_L-Bax and Bcl-x_L-Bad interactions in a dose-dependent manner. Consistent with its high binding affinity to Bcl-x_L,³⁵ peptides derived from Bad BH3 (26 amino acids) were more potent than other BH3-derived peptides in blocking protein-protein interactions involving Bcl-x_L.

Cosulich *et al.* studied the biological activity of synthetic BH3 peptides in triggering apoptosis in a cell-free system based on extracts of *Xenopus* eggs.³⁹ Peptides of 16 amino acids derived from the BH3 domains of Bak, Bax or Bid were found to induce apoptosis by causing rapid activation of caspases, whereas a Bak BH3 mutant peptide containing Ala substitution at Leu-78, which is critical for Bcl-x_L binding¹⁶ did not show any effect. Since

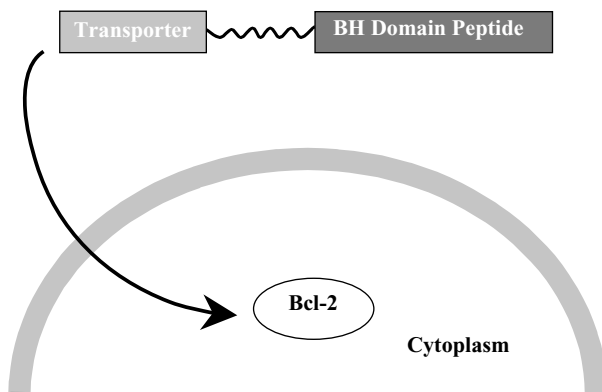
cytochrome c is involved in the activation of apoptosis, the activity of synthetic BH3 peptides in affecting cytochrome c release in isolated rat mitochondria was evaluated. Whereas a 16-amino acid peptide derived from Bax BH3 domain was shown to lack the activity in inducing cytochrome c release,⁴⁰ another study observed significant cytochrome c release after treatment with a 20-amino acid Bax BH3 peptide (residues 55–74) or a 15-amino acid Bak BH3 peptide (residues 73–87).⁴¹ Morgan *et al.* described in a conference report that synthetic peptides derived from the BH3 domain of Bak or Bax diminished the association of Bcl-2 with Bak in PC-3 cells.⁴² When introduced into prostate carcinoma PC-3 and DU145 cells by electroporation, these peptides caused apoptosis in these cells, which could be blocked by a broad-spectrum caspase inhibitor zVAD-fmk.

Besides the BH3 domain of pro-apoptotic members of the Bcl-2 family, synthetic peptides were used to study the biochemical role of the N-terminal BH4 domain conserved only among anti-apoptotic Bcl-2 family members (Figure 1). It has been suggested that anti-apoptotic Bcl-2 family members such as Bcl-x_L inhibit the activity of VDAC thus leading to the prevention of apoptotic cytochrome c release and changes in mitochondrial membrane permeability.²⁹ Shimizu *et al.* synthesized peptides derived from the BH4 domain of Bcl-2 and Bcl-x_L and found that they both inhibited the activity of VDAC whereas the corresponding peptide from Bak was inactive.⁴³ These results suggested that the BH4 domain of anti-apoptotic Bcl-2 family members is sufficient for the functional blockade of VDAC. The role of several conserved residues of the BH4 domain in inhibiting VDAC activity was also examined by synthetic peptide analogs. BH4 mutant peptides containing amino acid substitutions or deletion at Leu-8, Val-9, Val-10, Asp-11, Phe-12, Leu-13 and Leu-17 showed different degrees of decrease in VDAC inhibition, which correlated with reduced anti-apoptotic activity of some corresponding Bcl-x_L mutants as reported by Huang *et al.*⁴⁴ This was consistent with the argument that inhibition of VDAC activity by Bcl-x_L contributed to its anti-apoptotic function.

Modulation of apoptosis in living cells by cell permeable peptides

In addition to structure and function studies *in vitro* and in cell free systems as described above, synthetic peptides derived from the Bcl-2 family can be used to probe and modulate Bcl-2-regulated apoptotic pathways in living cells. To do this, one needs to modify these peptides to make them cell permeable because normally peptides have little ability to cross the cell membrane and arrive at their intracellular targets such as Bcl-2 family proteins. Several strategies have been reported in which synthetic peptides derived from Bcl-2 family proteins are linked

Figure 4. Transporter molecules used for the delivery into the cell of synthetic peptides derived from functional domains of Bcl-2 family proteins. The transporters that have been used for studying Bcl-2 family proteins include: (1) the internalization domain of the Antennapedia (Ant) protein;⁵² (2) the decanoic acid;⁵⁵ and (3) the HIV TAT domain.⁴³



Transporter:

- (1) RQIKIWFQNRRMKWKK—
- (2) CH₃(CH₂)₈CO—
- (3) RKKRRQRRR—

with transporter peptides or non-peptidic molecules that can deliver functional peptides into the cell (Figure 4).

The internalization domain of the Antennapedia (Ant) protein, RQIKIWFQNRRMKWKK has been used in a number of studies as the transporter to deliver functional peptides into cells.^{45–51} Holinger *et al.* synthesized a fusion Ant-BH3 peptide combining the 16-amino acid Ant internalization sequence and the Bak BH3 sequence (residues 71–89, Figure 3) and found that this fusion peptide caused the activation of caspases and triggered apoptosis in intact HeLa cells.⁵² While microinjection of recombinant Bcl-x_L into these cells suppressed programmed cell death induced by Fas, Ant-BH3 peptide was shown to antagonize the function of Bcl-x_L by inhibiting its ability to suppress Fas-induced apoptosis. It was suggested that the cell killing effect of Ant-BH3 peptide was due to its binding to Bcl-2 related death antagonists because a mutant Ant-BH3 peptide containing a single Ala substitution at Val-78 lost the Bcl-x_L binding activity and was incapable of inducing apoptosis. Interestingly, Ant-BH3 appeared to trigger apoptosis in a cytochrome c-independent manner. It did not cause the early loss of mitochondrial membrane potential or cytochrome c release from the mitochondria.

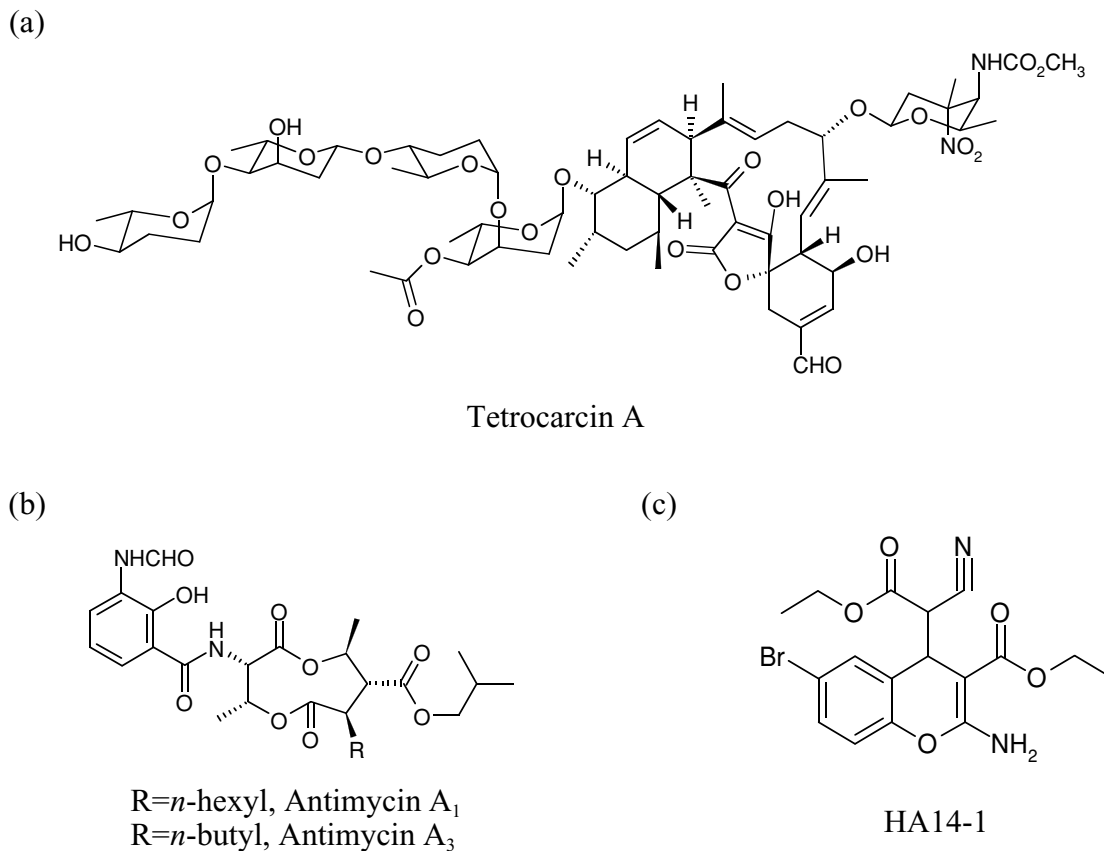
Fatty acids are a class of non-peptidic molecules that can help peptides cross the cell membrane. For examples, myristic acid and stearic acid have been used for the intracellular delivery of peptide inhibitors of protein kinase C⁵³ and protein-tyrosine phosphatase,⁵⁴ respectively. Using a smaller fatty acid, the decanoic acid as the cell permeable moiety (CPM), Wang *et al.* synthesized cell permeable

Bcl-2 binding peptides by attaching this CPM to the N-terminus of synthetic BH3 peptides (Figure 4).⁵⁵ One peptide termed CPM-1285 that contained the BH3 domain of mouse Bad protein (residues 140–165 of mBad, Figure 3) was shown to enter human myeloid leukemia HL-60 cells by confocal microscopy. The *in vitro* Bcl-2 binding assay suggested that CPM-1285 strongly competed with the binding of a fluorescein-labeled Bak BH3 peptide with an IC₅₀ of 130 nM. In intact HL-60 cells, CPM-1285 induced the activation of caspase-3 and triggered apoptosis characterized by DNA fragmentation and cleavage of PARP. The apoptosis induced by CPM-1285 was dependent of caspase activation since the addition of a caspase inhibitor zVAD-fmk completely blocked the effect of CPM-1285. Furthermore, CPM-1285 was shown to slow human myeloid leukemia growth in severe combined immunodeficient mice.

In addition to apoptosis induction, the cell permeable peptide approach was applied to inhibit apoptosis. Shimizu *et al.* showed that the BH4 domain of anti-apoptotic Bcl-2 or Bcl-x_L was required for inhibiting VDAC activity and apoptotic release of cytochrome c.⁴³ Using the protein transduction domain of human immunodeficiency virus (HIV) TAT protein that is known to facilitate the delivery of proteins into cells,^{56–58} they synthesized a fusion TAT-BH4 peptide that combined the HIV TAT domain (RKKRRQRRR) and Bcl-x_L BH4 sequence.⁴³ This TAT-BH4 peptide prevented apoptosis in HeLa cells whereas the TAT-only sequence did not show any effect. These results suggest that peptides mimicking the BH4 domain of anti-apoptotic Bcl-2 family proteins, when transferred into cells, act as inhibitors of apoptosis by suppressing VDAC activity and subsequently the release of cytochrome c through VDAC.

Natural products that induce apoptosis in cells overexpressing Bcl-2 or Bcl-x_L

Two natural products, tetrocarcin A and antimycin A have been reported to induce apoptosis in cell lines that overexpress anti-apoptotic Bcl-2 or Bcl-x_L protein. Tetrocarcins were originally discovered from random screening to have antibacterial activity against Gram-positive bacteria and antitumor activity against experimental murine tumors.⁵⁹ Tetrocarcin A is the major component and consists of a 13-membered macrocyclic ring containing tetronic acid, a nitro sugar and deoxy sugars (Figure 5a). In the recent study by Nakashima *et al.*,⁶⁰ the screening of a library of natural products using cells overexpressing Bcl-2 or Bcl-x_L led to the identification of Tetrocarcin A. It was found that Tetrocarcin A, when combined with anti-Fas antibody, tumor necrosis factor α , staurosporine, or Bax, induced apoptosis in these cells. In addition, Tetrocarcin A inhibited mitochondrial functions regulated by Bcl-2

Figure 5. The chemical structures of (a) tetrocarcin A,⁶⁰ (b) antimycin A⁶¹ and (c) HA14-1.⁶⁹

and thereby resulted in Fas-induced mitochondrial transmembrane potential loss and cytochrome c release. In contrast to apoptosis induction in cells overexpressing Bcl-2 or Bcl-x_L, tetrocarcin A did not cause apoptosis in cells overexpressing cytokine response modifier A (CrmA) or dominant-negative Fas-associated death domain (FADD). As to the biological target of tetrocarcin A, it is not clear whether it directly interacts with Bcl-2 or Bcl-x_L such as binding to the surface pocket formed by the BH1-3 domains or may actually target other proteins associated with the mitochondrial apoptotic pathway.

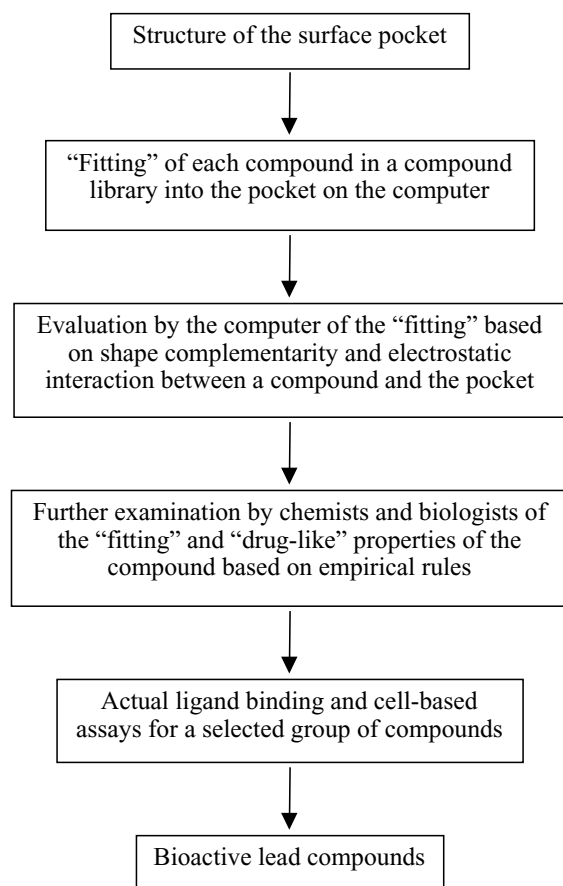
In a conference report,⁶¹ Tzung *et al.* described that antimycin A induced apoptosis in cell lines overexpressing Bcl-x_L. Antimycin A was originally discovered as an antibiotic active against a wide variety of fungi, yeasts, insects, and mammals.⁶² It consists of a 3-formamidosalicylic acid residue linked through an amide group to a dilactone ring containing an acyl side chain and an *n*-butyl or *n*-hexyl side chain (Figure 5b). Tzung *et al.* recently found that antimycin A inhibited mitochondrial function regulated by Bcl-x_L and blocked the channel activity of Bcl-x_L as measured by leakage of fluorescent calcein dye from synthetic lipid vesicles. It was suggested that antimycin A may bind the surface pocket of Bcl-x_L. Since antimycin A was also previously known to bind

cytochrome b and the structure-activity relationship of its inhibitory action was characterized,⁶³ it remains to be demonstrated as to the interaction and specificity of the compound with Bcl-2 family proteins.

Structure-based discovery of organic compounds that bind Bcl-2

The three-dimensional structures of Bcl-x_L and more recently Bcl-2 as determined by X-ray crystallography and/or NMR spectroscopy provides the structural basis for the discovery of new inhibitors of these anti-apoptotic proteins.^{7,8} These structures reveal a hydrophobic surface pocket formed by the BH1-3 domains. Residues at the BH1 and BH2 domains are essential for the anti-apoptotic function of Bcl-2 or Bcl-x_L as studies have shown that mutations at these sites abolished their biological function.⁶⁴ Death agonists, such as Bax, Bak and Bad, use their BH3 domains to bind to the surface pocket and promote apoptosis.⁶⁵⁻⁶⁷ Based on the high resolution three-dimensional structure of a targeted receptor protein, computer-aided techniques can be applied to search a large number of organic compounds for potential ligand molecules. This approach that exploits both new computational methods and the diversity of existing compound

Figure 6. The procedure for using computer screening of a non-peptidic compound library to discover small molecular ligands of a target protein.



databases for the identification of novel protein binding molecules has become a powerful tool for non-peptidic ligand discovery (Figure 6).⁶⁸

Using this computer screening strategy, Wang *et al.* recently reported a low molecular weight compound, HA14-1 identified from a large collection of more than 190,000 organic molecules (Figure 5c).⁶⁹ *In vitro* binding studies demonstrated the interaction of HA14-1 with the surface pocket of Bcl-2 ($IC_{50} = 9 \mu M$ in competing with the Bcl-2 binding of a fluorescein-labeled Bak BH3 peptide). HA14-1 induced apoptosis in HL-60 cells overexpressing Bcl-2 that was associated with the decrease in mitochondrial membrane potential and activation of caspase 9 followed by caspase 3. CrmA, a potent inhibitor of the death receptor Fas-mediated apoptotic pathway, did not block apoptosis induced by HA14-1. Whereas HA14-1 strongly induced the death of NIH3T3 (Apaf-1 +/+) cells, it had little apoptotic effect on Apaf-1-deficient (Apaf-1 -/-) mouse embryonic fibroblast cells. These findings were consistent with the notion that HA14-1 triggered the mitochondrial apoptotic pathway involving the activation of Apaf-1 and caspase 9 likely by

binding Bcl-2 and inhibiting its function. In conjunction with the discovery of HA14-1, Yu *et al.* developed an efficient synthetic method for the preparation of HA14-1 derivatives, which should facilitate efforts in further lead optimization to increase the binding potency and specificity of HA14-1 analogs for Bcl-2 protein.⁷⁰

Conclusion and future prospect

Synthetic peptides derived from Bcl-2 family proteins are useful models to study and understand the structure and function of the Bcl-2 family. Complementary to other biological and genetic experiments, studies of these peptides have provided new insights into the mechanism and role of different domains of Bcl-2 family proteins in mediating diverse biological functions and protein-protein interactions. So far studies have mainly focused on synthetic peptides derived from the BH3 domains of pro-apoptotic Bcl-2 members. Structure determination and functional characterization *in vitro* and in cell free systems have yielded valuable insights into the structural basis for the interactions of these pro-apoptotic BH3 domains with the surface pocket of Bcl-2 or Bcl-x_L and mechanism of BH3-mediated apoptosis. When chemically modified to link with peptide or non-peptidic transporter, these synthetic BH3 molecules can cross the membrane and induce apoptosis in tumor cells. These results demonstrate the potential use of BH3 peptides as triggers and probes of apoptotic signaling and templates for developing anti-cancer agents.

Many important questions regarding the mechanism of the BH3 domain remain to be further investigated. The BH3 domain is thought to mediate the affinity of different protein-protein interactions within the Bcl-2 family. Synthetic peptides derived from these regions showed interactions with Bcl-x_L corresponding to their native proteins.¹⁶ It remains to be further elucidated as to the amino acid sequences and structural factors that dictate the selectivity and hierarchy of associations between BH3 peptides or native BH3 domains in pro-apoptotic proteins with the surface pocket in anti-apoptotic Bcl-2 members. The experimentally determined structures of pro-apoptotic Bid and Bax show that their BH3 domain is buried in the core of the protein, implying that conformational changes may be necessary for the BH3 domain to interact with its anti-apoptotic partners.¹⁰⁻¹² More needs to be learned about the mechanism and kinetics of the conformational switch and refolding of BH3 domains during their binding to Bcl-2 or Bcl-x_L surface pocket. Finally, using BH3 peptides as probes, it is important to characterize specific residues within the surface pocket critical for peptide binding and investigate whether additional sites outside the pocket may also influence affinity and specificity. Information about these as well as other related issues should lead to better insights

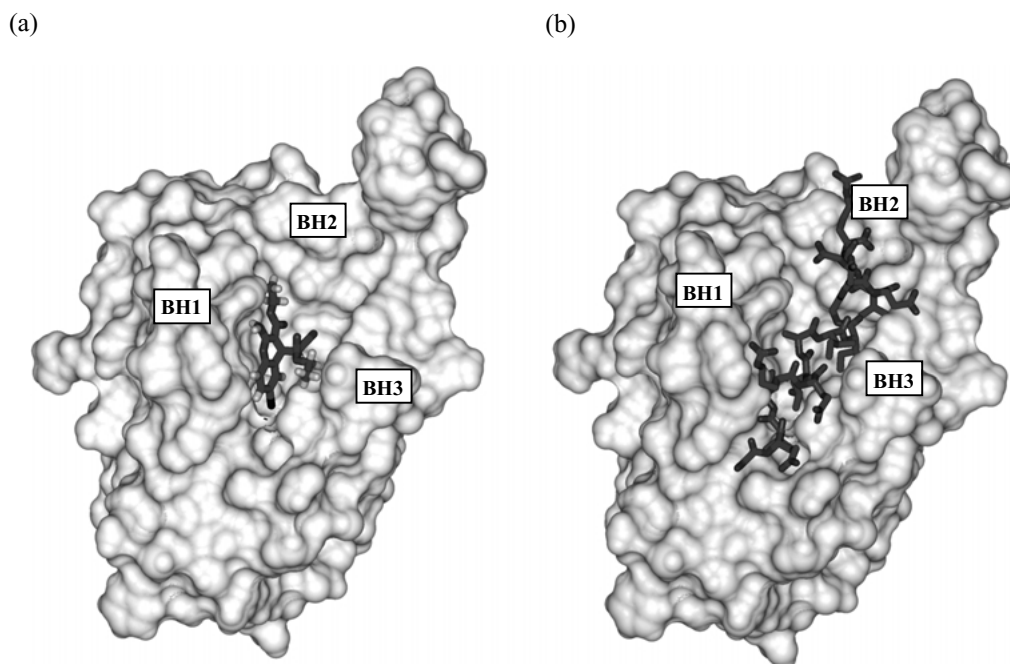
into the mechanism of the heterodimerization within the Bcl-2 family and foundation for designing highly potent and selective BH3-mimicking molecules.

Like the BH3 domain, the synthetic peptide approach may also find applications in structural and functional characterization of other BH domains and regions of heterogeneity in Bcl-2 family proteins. The BH1 and BH2 domains are involved in the function of anti-apoptotic Bcl-2 or Bcl-x_L and their interactions with pro-apoptotic Bcl-2 family members.^{64,71} In addition, parts of the BH1 and BH2 domains are suggested to mediate ion channel formation in some Bcl-2 family proteins.²³⁻²⁶ These ion channels display differences in ion-selectivity and conductance which are speculated to be due to the sequence variability in the BH1 and BH2 domains. It remains to be examined as to the structure and function of these different ion channels and their existence and activity *in vivo*. The BH4 domain is also involved in multiple activities. In addition to its inhibition of VDAC as studied by both synthetic peptides and deletion mutants,⁴³ the BH4 domain is shown to bind other apoptosis regulating proteins including calcineurin,⁷² Raf-1,⁷³ and Ced-4.⁴⁴ Finally, as to other regions lack of sequence homology, it has been speculated that these heterogeneous sites present in the growing sub-family of BH3-only proteins may play a role in receiving different death signals. In conjunction with studies using genetic and molecular and cellular biological techniques, synthetic peptides derived from these homologous or heterogeneous domains may provide sim-

plified models to dissect their complex functional roles and activities.

Non-peptidic molecules discovered by either random screening or structural-based and computer-assisted techniques provide another class of small molecular probes of the biological function of the Bcl-2 family. Such low molecular weight compounds usually have high membrane permeability and thus can be used to study and control intracellular apoptotic signaling pathways. Like synthetic peptides, these compounds can disrupt the function and interaction of interesting sites of a Bcl-2 family member. One of such sites is the surface pocket of anti-apoptotic Bcl-2 or Bcl-x_L which has been exploited for the discovery of organic ligands such as HA14-1 (Figure 7).⁶⁹ The delineation of detailed binding modes of these molecules with Bcl-2 or Bcl-x_L surface pocket from further structure-function analyses should allow the design of new analogs with higher affinity for the surface pocket. A similar approach may be extended to explore other surface sites that are potentially involved in other protein-protein interactions and important for regulating apoptosis. Finally, with a wealth of information about proteins besides the Bcl-2 family that have been shown to regulate apoptotic pathways, the strategies and methods in using synthetic peptides and non-peptidic compounds targeted to the Bcl-2 family as reviewed here may be applicable to the study of other apoptosis-related macromolecules.

Figure 7. The structure as predicted by molecular modeling techniques of HA14-1 bound to the surface pocket of Bcl-2 (a). For comparison, the experimentally determined structure¹⁶ of a Bak BH3 peptide bound to the similar surface pocket of Bcl-x_L is also shown (b). The BH1-3 domains that form this surface pocket on Bcl-2 and Bcl-x_L are indicated.



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Notes in proof:

After the completion of this article, we noted two recent publications of small molecule inhibitors of Bcl-2 family. Degterev, A. *et al.* reported the identification of non-peptidic BH3 inhibitors from the screening of a compound library (Nature Cell Biology, 2001; 3: 173–182). Tzung, S.P. *et al.* described more detailed studies of antimycin A that was originally reported in ref. 61 (Nature Cell Biology, 2001; 3: 183–191).

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