A view to a kill: ligands for Bcl-2 family proteins

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Apoptosis is the essential process of programmed cell death that, in multicellular organisms, regulates development and maintains homeostasis. Defects in the apoptotic molecular machinery that result in either excessive or insufficient apoptosis are observed in a remarkably wide range of human disease, prompting intense interest in pro- and anti-apoptotic proteins as therapeutic targets. A number of recent reports have described the discovery of ligands for anti-apoptotic Bcl-2 family proteins by a variety of approaches, including computational, combinatorial and evolutionary strategies. Both the design of ligands and the exploration of their mechanisms of action have been greatly enhanced by recent high-resolution structure determinations of proteins from this family. Several of the newly discovered ligands promote apoptosis, and some do so even in the face of overexpressed anti-apoptotic Bcl-2 proteins. Ligands that overcome the protective effects associated with up-regulation of anti-apoptotic Bcl-2 proteins represent especially promising therapeutic leads.

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Abbreviations

Bak	B-cell homologous antagonist/killer
Bax	Bcl-2 associated protein X
Bcl-2	B-cell lymphocyte/leukemia-2
BH	Bcl-2 homology domain
KSHV	Kaposi sarcoma-associated herpesvirus
SELDI	surface-enhanced laser desorption/ionization

Introduction

Can't live with it, can't live without it. Such is the paradox of apoptosis, the tightly regulated process of programmed cell death [1]. Apoptosis is essential in multicellular organisms, where it regulates normal development and the maintenance of tissue homeostasis. Apoptosis also provides a self-destruct button for cells damaged by the assaults of genomic instability, cytotoxic stress and/or damaging radiation or chemotherapy. The balance between these processes is delicate, and it is perhaps not surprising that apoptotic defects are observed in a remarkably wide range of human disease [2]. Excessive apoptosis is implicated in non-proliferative diseases including spinal muscular atrophy, AIDS, Alzheimer's disease, myocardial infarction and stroke. Inhibition of apoptosis is implicated in virtually every known human malignancy [3]. This inhibition provides malignant cells with a selective growth advantage [4,5], allowing proliferation in the face of radiation or chemotherapy [6]. The current intense interest in both the molecular details of apoptosis and the discovery of molecules that mitigate apoptotic defects [7–10] is thus motivated by the needs of a large and diverse clinical population.

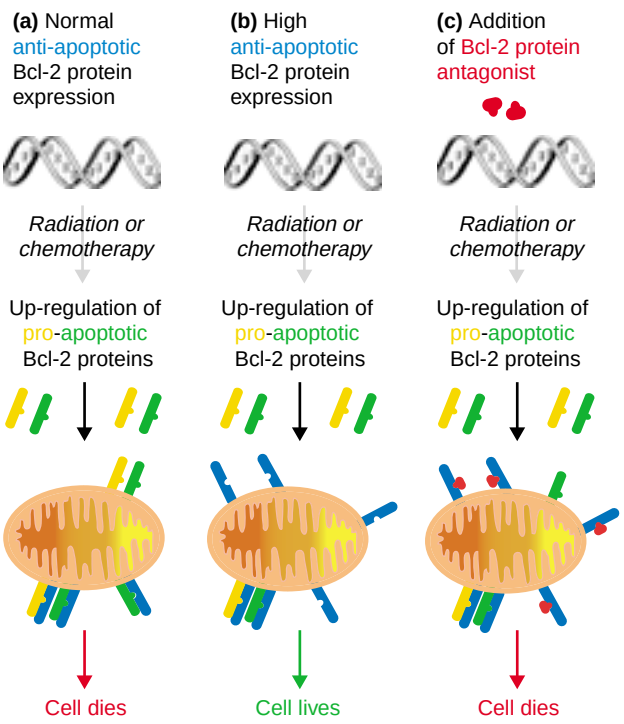
Live and let die: Bcl-2 family proteins regulate the intrinsic apoptotic pathway

Proteins in the Bcl-*2* (B-cell lymphocyte/leukemia-*2*) family are critical components of the trigger and the safety that regulate the firing of the intrinsic apoptotic pathway [11,12], one of two multistep biochemical cascades leading to programmed cell death [1,13]. At least 16 Bcl-*2* homologs, as defined by sequence similarity to some or all of the four Bcl-*2* homology (BH) domains in Bcl-*2*, are found in humans. These homologs include Bcl-X_L, Bcl-w, and Mcl-1, which, like Bcl-*2*, inhibit apoptosis, and Bax, Bak, Bok, Bad, Bid, Bim and Noxa, which promote apoptosis. Pro-apoptotic Bcl-*2* proteins [14] subdivide further into multi-BH domain proteins (Bax, Bak, Bok), which contain BH1-3 domains, and 'BH3-only' proteins [15] (Bad, Bim, Bid, Noxa), which share sequence homology only within the BH3 domain.

The BH3 domains of pro-apoptotic Bcl-*2* proteins represent the triggers of the intrinsic apoptotic pathway. Although the triggering mechanism remains controversial, even short peptides comprising the BH3 domain of Bak induce apoptosis in HeLa cells [16] and BH3 peptides from Bak, Bax and Bid induce apoptosis in *Xenopus* extracts [17]. In contrast, anti-apoptotic Bcl-*2* family members act as apoptotic safeties by rendering cells refractory to apoptosis that is otherwise induced by a variety of stimuli [11,18,19]. The BH3 domains of pro-apoptotic Bcl-*2* proteins form heterodimers, both *in vitro* and *in vivo*, with anti-apoptotic Bcl-*2* proteins. These direct protein–protein interactions are believed to neutralize the anti-apoptotic functions of Bcl-*2* and its pro-survival homologs, leading directly or indirectly to apoptosis [11,16,17]. Uncovering details of the apoptotic switch along the intrinsic pathway represents important work for the future. Nevertheless, despite the currently limited understanding of this switch, disruption of the interaction between pro- and anti-apoptotic Bcl-*2* family members by molecules that mimic pro-apoptotic BH3 domains represents a direct approach to overcoming the protective effects of Bcl-*2* and Bcl-X_L [20••].

In view of their regulatory role in the intrinsic apoptotic pathway, it is not surprising that anti-apoptotic Bcl-*2* proteins are overexpressed in many human cancers [21]. Bcl-*2* is overexpressed in 80% of B-cell lymphomas, 30–60% of prostate cancers, 90% of colorectal adenocarcinomas and a wide variety of other cancers, and Bcl-X_L is overexpressed in many breast [22] and lung cancers [23].

Figure 1

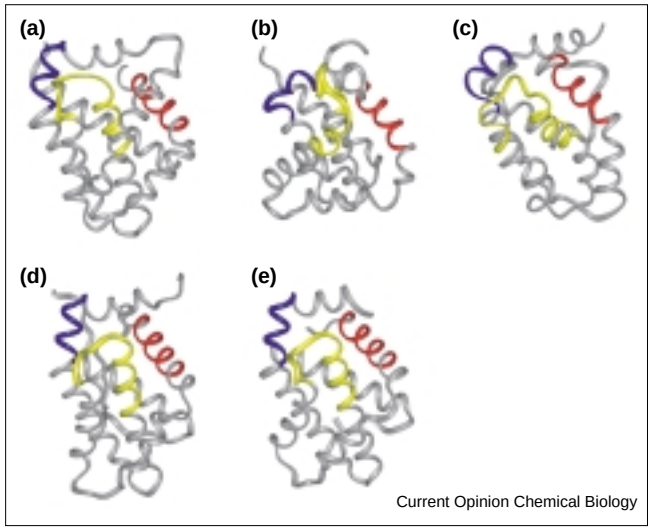


Relationship between the induction of apoptosis and the relative levels of pro- and anti-apoptotic Bcl-2 family proteins and their ligands. **(a)** In cells with normal levels of anti-apoptotic proteins, radiation or chemotherapy up-regulates the expression of pro-apoptotic Bcl-2 proteins and induces apoptosis. **(b)** In cancer cells, the up-regulated expression of pro-apoptotic Bcl-2 proteins is mitigated by high levels of anti-apoptotic proteins. **(c)** In theory, combination of radiation or chemotherapy with non-natural ligands for anti-apoptotic Bcl-2 proteins would overcome the effects of high anti-apoptotic Bcl-2 protein levels.

The upregulation of anti-apoptotic Bcl-2 family members is often associated with resistance to traditional chemotherapy and radiation, therapeutic strategies that rely on the ability to induce apoptosis [6]. Thus, optimized strategies for treatment of these cancers might combine traditional chemotherapeutics with molecules that neutralize the effects of the relevant anti-apoptotic Bcl-2 family proteins (Figure 1).

The remainder of this review focuses on recent reports of the discovery of ligands for anti-apoptotic Bcl-2 proteins. These ligands are diverse in structure and have been identified by computational, combinatorial and evolutionary methods. Several of the ligands identified promote apoptosis, and some do so even in cells that overexpress anti-apoptotic Bcl-2 family members. Those ligands that overcome the protective effects associated with up-regulation of anti-apoptotic proteins are promising therapeutic leads. Development of these ligands and exploration of their mechanisms of action have been guided by high-resolution NMR and X-ray crystallographic studies of

Figure 2



Bcl-2 family members display significant structural homology despite limited sequence similarity. Shown are ribbon diagrams of Bcl-2 proteins **(a)** Bax [24]; **(b)** Bid [25,26]; **(c)** KSHV Bcl-2 [30••]; **(d)** Bcl-2 [28••]; and **(e)** Bcl-X_L [27]. BH domains (or regions of structural homology in the cases of Bid and KSHV Bcl-2) are color-coded as follows: BH1 domains are in yellow, BH2 domains are in blue and BH3 domains are in red.

Bcl-2 family proteins. The structural features of these proteins are described below.

A conserved death valley: structural studies on cellular and viral Bcl-2 family members

Five Bcl-2 family proteins have been characterized structurally at high resolution, including at least one representative from each functional subfamily. These proteins are the pro-apoptotic, multi-BH domain protein Bax [24], the pro-apoptotic 'BH3-only' protein Bid [25,26], and the anti-apoptotic proteins Bcl-X_L [27], Bcl-2 [28••] and a Bcl-2 homolog from Kaposi sarcoma-associated herpesvirus [30••]. In terms of global topology, these five proteins display remarkable conservation despite limited overall sequence homology. All contain a central hydrophobic α-helix (α5) surrounded by five amphipathic α-helices (α1–α4 and α6) (Figure 2).

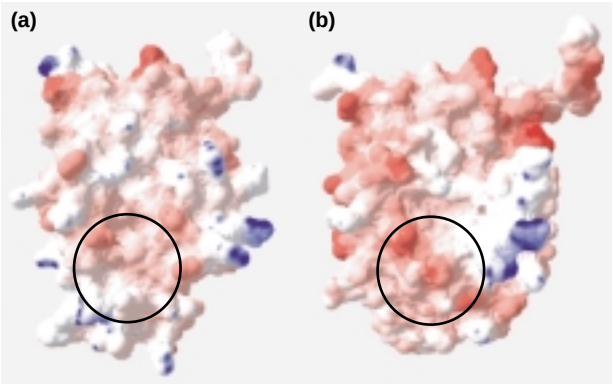
The first details of the interactions between pro- and anti-apoptotic Bcl-2 family proteins were revealed in 1997 by the NMR structure of a complex between Bcl-X_L and a short peptide containing the Bak BH3 domain ($Bak_{72–87}$) that induces apoptosis in HeLa cells [31]. The structure shows the $Bak_{72–87}$ peptide, which is unstructured in the absence of Bcl-X_L, bound as an amphipathic α-helix within a deep hydrophobic groove — an 'open cleft' — formed by the BH1-3 domains of Bcl-X_L. More recent structures of two Bcl-2 isoforms reveal that, despite the global conservation of fold, the molecular landscapes of the Bcl-2 and Bcl-X_L hydrophobic grooves are significantly different from one another (Figure 3) [28••]. In particular, three

amino acid differences between Bcl-2 and Bcl-X_L (Asp vs. Ala in position 104, Met vs. Leu in position 108, and Arg vs. Ser in position 122; Bcl-X_L numbering) translate into significantly increased negative electrostatic potential at one end of the BH3-binding groove of Bcl-X_L relative to the corresponding groove of Bcl-2. This end of the binding groove interacts with electropositive residues near the N-termini of the BH3 domains of Bad [32] and Bak [31], providing a rationalization for the greater affinity of the corresponding peptides for Bcl-X_L (Bad, 0.6 nM; Bak, 480 nM) than for Bcl-2 (Bad, 8 nM; Bak, 1600 nM) [28••]. Taken together, these studies provide important insight into the structural features governing the specificity of Bcl-2 family heterodimerization events, which could be used to guide design of highly specific Bcl-X_L or Bcl-2 ligands.

The recently reported NMR structure of a Bcl-2 homolog from Kaposi sarcoma-associated herpesvirus (KSHV) [30••] provides the first look at a viral homolog of Bcl-2 (Figure 2e). Gammaherpesviral Bcl-2 is important for viral reactivation from latency and is essential for persistent viral replication in immunocompromised hosts [33]. Furthermore, it appears to play a key role in tumorigenesis [34]. Although the precise role of KSHV Bcl-2 is not yet clearly understood, it can prevent apoptosis induced by the KSHV cyclin [35] and may also prevent host-induced apoptosis [29]. The structure of KSHV Bcl-2 reveals the same overall fold found in mammalian Bcl-2 family members, despite the fact that sequence homology between the mammalian and viral proteins is limited to the BH1 and BH2 domains.

Fluorescence polarization experiments indicate that the heterodimerization specificity of KSHV Bcl-2 is significantly different from that of its well-characterized cellular homologs. Whereas Bcl-2 and Bcl-X_L bind with high affinity to a BH3 domain peptide from the 'BH3-only' protein Bad (K_d values between 0.6 and 15 nM) [31,32], KHSV binds this peptide poorly (K_d = 3.9 μM) [30••]. Conversely, Bcl-2 binds the BH3 peptide from the multi-BH domain pro-apoptotic protein Bak poorly (K_d = 12.7 μM) [31], whereas KHSV Bcl-2 binds this peptide well (K_d < 50 nM) [30••]. It will be interesting to see if this apparent pattern of specificity holds across a larger sample of pro-apoptotic BH3 peptides for at least two reasons. First, it is emerging that the cellular role of the multi-BH domain proteins Bak and Bax is different from that of 'BH3-only' proteins such as Bad [14]. Thus, *in vitro* specificity data may reflect functional differences between viral Bcl-2 and cellular Bcl-2 and Bcl-X_L *in vivo*. Second, the specificity data, in combination with preliminary structural characterization of the viral protein bound to BH3 peptides from Bak or Bax [30••], provide a thermodynamic and molecular basis for the design of chemical knockouts [36] of viral Bcl-2 but not cellular anti-apoptotic Bcl-2 proteins. Given the importance of viral Bcl-2 to the stages of viral infection associated with human disease [33], KSHV Bcl-2 is a relevant and intriguing new therapeutic target.

Figure 3



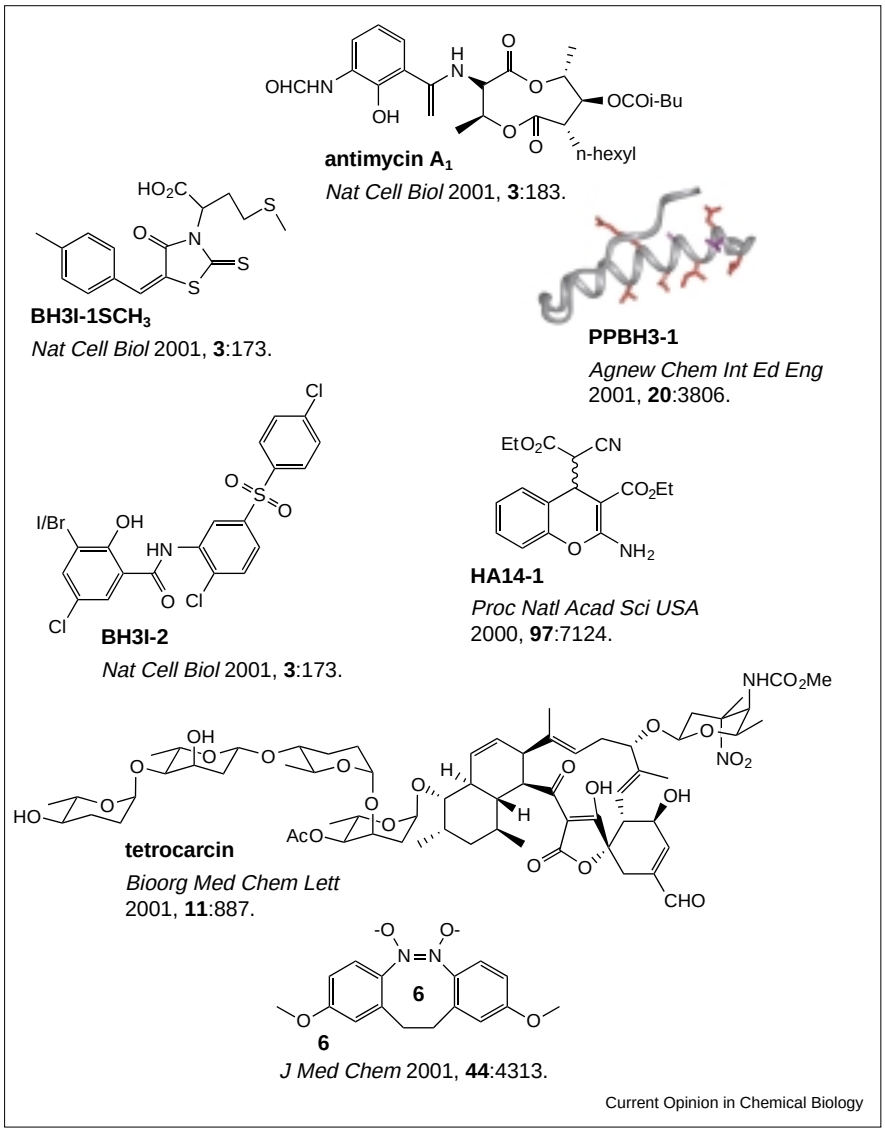
The molecular landscapes of the Bcl-2 and Bcl-X_L hydrophobic grooves differ significantly. Shown are surface electrostatic potential diagrams of **(a)** Bcl-2 and **(b)** Bcl-X_L; hydrophobic regions are colored white, whereas regions with negative or positive electrostatic potential are color-coded red or blue, respectively. The circles denote the locations within the binding grooves where the negative electrostatic potentials differ significantly. Proteins are oriented as shown in Figure 2.

License to kill: small-molecule ligands for Bcl-2 and Bcl-X_L

The relatively flat surfaces found at protein–protein interfaces are notoriously difficult targets for small-molecule ligands [37]. However, the Bak•Bcl-X_L interface is not flat — the Bak peptide nestles into a deep hydrophobic groove in the surface of Bcl-X_L. This feature, coupled with the therapeutic relevance of this target, has motivated considerable efforts to develop small-molecule ligands for Bcl-2 and its anti-apoptotic homologs. A variety of methods, including computational analysis, natural-product screening and screening of synthetic libraries, have been used to identify natural and non-natural small molecules capable of binding to Bcl-2 and/or Bcl-X_L, inhibiting their anti-apoptotic function and thereby promoting apoptosis.

The first small-molecule Bcl-2 inhibitor (HA14-1, Figure 4) was reported by Huang and co-workers in 2000. HA14-1 was identified by a computational screen of ~190 000 compounds from the MDL/ACD 3D database using a homology model of Bcl-2 as a target [38]. Compounds were scored on the basis of favorable shape complementarity, high virtual affinity and potential for hydrogen-bond formation with Bcl-2. The experimental potencies of 28 high-scoring, drug-like compounds were then examined *in vitro* and *in vivo*. In a competition fluorescence polarization assay, HA14-1 (as a mixture of diastereomers) displaced the $Bak_{72–87}$ peptide from Bcl-2 with an IC_{50} of 9 μM. Synthetic analogs related to HA14-1 possessed widely varying affinities for Bcl-2, suggesting a reasonably specific binding interaction. More importantly, despite having only moderate affinity for Bcl-2, HA14-1 induced apoptosis in 90% of cells from the etoposide-resistant

Figure 4



Ligands for Bcl-2 and/or Bcl-X_L.

human myeloid leukemia cell line HL-60 when present at 50 μM concentration (IC_{50} ~ 17 μM). It remains to be determined whether HA14-1 acts only through Bcl-2, or through other anti-apoptotic family members such as Bcl-X_L. Additional synthetic analogs of HA14-1 have been reported [39], though their potential as Bcl-2 inhibitors remains to be determined.

A similar computational screen by Wang and co-workers evaluated ~206 000 compounds from the National Cancer Institute database and identified a second class of small-molecule Bcl-2 inhibitors [40]. The affinities of 35 high-scoring, drug-like compounds for Bcl-2 were measured using a competition fluorescence polarization assay. Seven compounds with IC_{50} values between 1.6 and 14 μM were screened for anti-proliferative activity in HL-60 cells; the most active compound, **6** in Figure 4, displayed an IC_{50} of 4 μM. The activity of this compound correlated directly with Bcl-2 expression level; the compound induced apoptosis in a dose-dependent manner in cell lines with high Bcl-2 levels, but exhibited low or no activity in cell lines with low levels of Bcl-2. NMR experiments with Bcl-X_L suggest that the compound binds in the BH3-binding site of Bcl-X_L. Interestingly, binding of the compound altered the chemical shifts only of residues located near the portion of the binding groove that contacts the Bak BH3 N-terminus. As discussed earlier, the electrostatic potential of this portion of the BH3-binding groove differs significantly between Bcl-2 and Bcl-X_L [28••]. Thus, it would be interesting to investigate the relative activity of this compound in cell lines that selectively overexpress Bcl-X_L or Bcl-2.

Two distinct classes of Bcl-X_L inhibitors (BH3I-1s and BH3I-2s) were discovered by Degterev and co-workers in a high-throughput fluorescence polarization screen of a

16 320-member small-molecule library [20••]. The most potent molecules inhibited formation of the Bak BH3•Bcl-X_L complex with inhibitory potencies (as measured by K_i) in the low-micromolar range, as judged by fluorescence polarization, SELDI (surface-enhanced laser desorption/ionization) assays, and NMR titrations. SELDI assays also showed that all BH3Is inhibit the formation of Bak BH3•Bcl-2 complexes, although no K_i values were reported. The BH3Is did not bind to the closely related protein Bid, or several unrelated proteins, in any assay used, suggesting an interesting level of specificity. BH3Is induced apoptosis in JK cells in a dose-dependent manner that correlated with their *in vitro* inhibitory potencies, suggesting that disruption of pro-apoptotic BH3 domain•anti-apoptotic Bcl-2 family protein interactions is crucial for BH3I-induced apoptosis. This hypothesis is supported by *in vivo* fluorescence resonance energy transfer (FRET) experiments monitoring Bax•Bcl-2, Bad•Bcl-2 or Bad•Bcl-X_L heterodimerization, which indicated that *in vivo* complex inhibitory potencies of BH3Is closely correlated with K_i values measured *in vitro*, as well as with their cytotoxicities. Overall, the experimental evidence suggests that BH3Is function as true mimics for the functional epitope of the Bak BH3 domain.

Based on the results of a novel ligand validation molecular-modeling method [41], BH3Is are believed to bind and stabilize an 'open cleft' conformation of Bcl-X_L, in a manner reminiscent of the Bak BH3 peptide. The computed interaction energies of the BH3Is closely correlated with their experimentally determined *in vitro* binding affinities. A computational screen of 93 compounds closely related to the BH3Is identified a novel analog (BH3I-ISCH$_3$) predicted to have similar affinity to BH3I-I; *in vitro* fluorescence polarization and NMR titration experiments were used to confirm the Bcl-X_L-binding affinity of the new analog (K_i = 8.5 μM). Molecular modeling suggests that both classes of BH3Is target the top portion of the BH3-binding groove, with the BH3I-2s targeting a slightly extended portion relative to the BH3I-1s. Interestingly, the BH3Is do not appear to interact with the portion of the BH3-binding groove suggested to differ in electrostatic potential between Bcl-X_L and Bcl-2. Although not intended for the discovery of novel classes of Bcl-2 ligands, this modeling method may be generally useful for identification of closely related analogs of known Bcl-2 ligands. As the affinity of the newest BH3I for Bcl-X_L is similar to those of the BH3Is previously reported, it remains to be seen if the method will be useful for identification of analogs of known Bcl-2/Bcl-X_L ligands that exhibit significantly improved affinity.

In addition to the discovery of the non-natural molecules described above, this year also witnessed the identification of natural-product inhibitors of Bcl-2 and Bcl-X_L. A team at the Fred Hutchinson Cancer Center screened known inhibitors of mitochondrial respiration for their ability to induce apoptosis and discovered that the cytochrome *b* inhibitor antimycin A is also a Bcl-X_L/Bcl-2 inhibitor [42••,43]. A competition fluorescence assay confirmed that antimycin A displaces the $Bak_{72–87}$ peptide from both Bcl-2 and Bcl-X_L *in vitro*. The K_d for the Bcl-2•antimycin A complex was determined to be 0.82 μM by isothermal titration calorimetry; the affinity of antimycin A for Bcl-X_L was not reported. A 2-methoxy derivative of antimycin A retained the ability to bind Bcl-2 and induced Bcl-X_L-dependent apoptosis without the general mitochondrial toxicity of antimycin A. This derivative might be a useful lead for the development of therapeutics to target cancers otherwise resistant to chemotherapeutic agents because of Bcl-X_L or Bcl-2 overexpression.

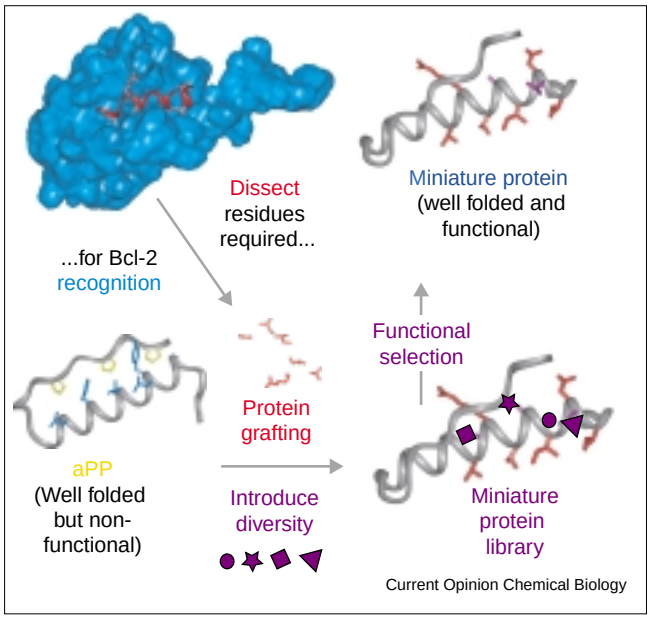
In addition to antimycin A, the natural product tetrocarcin A has been demonstrated to inhibit the anti-apoptotic function of Bcl-2 [44], although the molecular target is unknown. Kaneko and co-workers prepared several tetrocarcin analogs in an attempt to identify more selective inhibitors of Bcl-2 function. 21-Acetoxy-9-glycosyloxy tetrocarcin derivatives act as selective inhibitors of Bcl-2 function with potencies comparable to that of tetrocarcin A [45].

Miniature protein ligands for Bcl-2 and Bcl-X_L

An alternative strategy for the design of Bcl-2 ligands builds on the observation that folded proteins routinely recognize other proteins with nanomolar affinities and discriminate effectively among the myriad protein surfaces present inside or outside the cell. This feat is accomplished, at least in part, through the formation of extended surfaces of complementary shape and electrostatic potential [46,47]. The BH3 domains of pro-apoptotic Bcl-2 family proteins and the hydrophobic grooves of anti-apoptotic Bcl-2 family members form just such complementary surfaces [31,32]. However, peptides corresponding to the BH3 domains of pro-apoptotic Bcl-2 family members are largely unstructured in the absence of anti-apoptotic Bcl-2 proteins [31,32]. To the extent that this lack of structure persists *in vivo*, there is a significant entropic penalty associated with the folding transition necessary for these BH3 peptides to bind Bcl-2. This penalty limits their affinity and thus, their utility as ligands. An ideal ligand for Bcl-2 might be defined as one that presents the pre-organized functional epitope of a pro-apoptotic BH3 domain. Such a ligand would be expected to exhibit higher affinity and perhaps higher specificity than an unstructured peptide. In support of this idea, the greater affinity of the Bad BH3 peptide compared with that of the Bak BH3 peptide for Bcl-2 and Bcl-X_L has been attributed to a greater helical propensity, and thus a greater pre-organization, of the Bad peptide [32].

Miniature protein ligands for anti-apoptotic Bcl-2 proteins that possess these properties *in vitro* have been described by Chin and Schepartz [48••]. These molecules were developed using protein grafting, a strategy in which a protein-binding epitope is presented on the avian pancreatic polypeptide scaffold (Figure 5). Miniature Bcl-2-binding

Figure 5



Protein grafting and evolution of high affinity miniature protein ligands for Bcl-2 and Bcl-X_L [48••].

proteins, called PPBH3s, are well folded on their own (PPBH3-1 melts cooperatively with $T_m = 65°C$) and bind Bcl-2 100 times more tightly than the BH3 domain of Bak. Further, they discriminate effectively against a range of non-specific proteins (including protein kinase A, carbonic anhydrase and calmodulin) and recapitulate the specificity of Bak in discriminating between Bcl-2 and Bcl-X_L. It will be of interest to determine whether PPBH3s induce apoptosis in cell culture and to improve the specificity of PPBH3s for one Bcl-2 family member over others.

Die another day: challenges for the future

Several promising strategies exist for the rational manipulation of apoptosis via Bcl-2 family members. Each of the approaches described is faced with a unique set of challenges. Although presumably cell-permeable and 'drug-like', the small molecules identified so far have only moderate affinity and, in most cases, low specificity. The miniature proteins described may possess high affinity and specificity, but are decidedly not 'drug-like' and, if not encoded genetically, require sophisticated means to gain access into a cell [49]. Finally, although much has been learned about Bcl-2-family-mediated apoptosis, there is clearly more to discover. What is the mechanism by which heterodimerization mediates inactivation of anti-apoptotic family members? By what heterodimerization-independent mechanisms do pro-apoptotic Bcl-2 proteins signal initiation of the apoptotic cascade? How, if at all, do subtle structural differences among anti-apoptotic Bcl-2 proteins translate into functional differences? A more complete molecular understanding of the subtleties of apoptosis will undoubtedly provide more therapeutic opportunities and facilitate the drug-discovery process.

Acknowledgements

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