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# Prospects for targeting the Bcl-2 family of proteins to develop novel cytotoxic drugs

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**Abstract**

Over the last decade the molecular mechanisms controlling programmed cell death (apoptosis) have become clearer. It appears that many physiological and damage signals activate the cell death machinery by inhibiting the pro-survival Bcl-2 proteins. Since many chemotherapeutic drugs used to treat cancers activate the cell death machinery indirectly, there is much interest in developing peptide and non-peptide mimics of the BH3-only proteins, a family of proteins that act as direct antagonists of Bcl-2, as novel anti-cancer agents. This commentary review current progress in our search for such drugs and discusses recent findings in light of our current understanding of the cell death signaling. The potential for discovering novel agents that may form a useful part of the treatment of malignant disease is enormous but we still lack critical understanding of precisely how Bcl-2 function. However, the frequency of mutations affecting proteins that (directly or indirectly) impinge on apoptosis suggests that the approach of targeting Bcl-2 might be a profitable one.


*Keywords:* Apoptosis; Cancer; Bcl-2; BH3; Drug design

## 1. Introduction

In the last decade, we have learnt much about the molecular control of programmed cell death (apoptosis), the evolutionarily conserved process of killing and removing excess, unwanted or damaged cells during development and in tissue homeostasis. Since the deregulation of apoptosis has been linked to a number of disease states, our understanding of how this process is controlled may allow novel ways to treat diseases, either by promoting or by inhibiting apoptosis [1]. For example, loss of myocardial tissues after acute myocardial infarcts may be limited by inhibiting apoptosis in the damaged tissues. Excessive apoptosis is also a feature of neurodegenerative conditions such as Alzheimer's disease, suggesting that drugs preserving neuronal integrity may have a role in delaying the loss of vital neurons. In contrast to excess cell death, insufficient apoptosis is a feature of malignant disease and autoimmunity [2]. In either condition, persistence of damaged or unwanted cells that should normally be removed can contribute to disease.

## 2. Deregulation of cell death in cancer and cancer therapy

In malignancies, mutations affecting cell death regulatory proteins or those that sense cellular damage have been described in various tumors. Bcl-2, the prototypic member of the Bcl-2 family of proteins, was cloned as the result of the t(14;18) chromosomal translocation in human follicular B cell lymphoma, which results in its overexpression [3,4]. Overexpression of Bcl-2, which functions to inhibit apoptosis [5], or its functional homologs have also been reported in other tumors. However, mutations to proteins involved in sensing DNA damage are much more common in tumors. It is estimated that over half of human cancers

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*Abbreviations:* Bcl, B-cell lymphoma; Caspase, cysteine proteases that cleave after aspartatic acids; TNF, Tumor Necrosis Factor; FLICE, FADD–like ICE; FADD, Fas associating death domain containing protein; MORT, 1 mediator of receptor-induced toxicity; TRAIL, TNF-related apoptosis inducing ligand; Apaf-1, Apoptosis protease activating factor 1; CED, cell death abnormal; BH, Bcl-2 homology; NMR, Nuclear Magnetic Resonance.

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have a mutation of the tumor suppressor protein, p53, or ones affecting this pathway [6]. p53 is necessary to elicit the appropriate cellular responses (growth arrest, apoptosis) to most forms of DNA damage. Interestingly, p53 kills cells mainly by a Bcl-2-dependent mechanism, since Bcl-2 overexpression can block most cell deaths induced by p53 [7,8]. Both clinical observations and experiments in mouse models suggest that inhibition of apoptosis (e.g. p53 mutation, Bcl-2 overexpression) [9,10] greatly promote oncogenic transformation caused by mutations that promote cellular proliferation alone (e.g. c-Myc overexpression, $p21^{ras}$ mutations). Thus, reversing the process of tumorigenesis by promoting cell death, such as by activating p53 function or by inhibiting Bcl-2 function, may allow novel ways to complement our current treatments for malignancies.

Furthermore, most of the cytotoxic treatments currently used to treat malignant diseases work partly by inducing the endogenous cell death machinery [11], although this has been disputed by some [12]. For example, radiotherapy and many chemotherapeutic drugs activate apoptotic machinery indirectly by inducing DNA damage. Since the majority of tumors have mutations affecting the p53 pathway, many forms of therapy are significantly blunted because of the frequent loss of p53 function. The resistance of tumor cells to conventional agents provides further impetus to discovering novel cytotoxic drugs that act directly on the cell death machinery.

## 3. Signaling pathways to cell death

The effectors of cell death are cysteine proteases of the caspase family that cleave vital cellular substrates after aspartate residues [13]. The caspases are synthesized as inactive zymogens and are only activated in response to cellular damage, thereby allowing exquisite control of apoptosis during normal tissue functioning in order to prevent inappropriate cell deaths. There are at least two distinct pathways to activate caspases in mammalian cells [14]. Binding of cognate ligands to the some members of the TNF receptor superfamily induce cell death by activating the initiator caspase, caspase-8/FLICE, which is recruited to form oligomers on the receptor by the adapter protein FADD/MORT1 [15]. Once activated, caspase-8 can cleave downstream effector caspases such as caspases-3, -6, and -7, thereby massively amplifying the process. Currently, there is much interest in harnessing the apparent selectivity of a TNF-related ligand, TRAIL, to kill tumor cells for treating malignancies [16,17].

A second pathway to caspase activation is that controlled by the Bcl-2 family of proteins (Figs. 1 and 2) [18]. Overexpression of Bcl-2 can block many forms of physiologically (e.g. developmentally programmed cell deaths, death due to growth factor deprivation) and experimentally applied damage signals (e.g. cellular stress, radiation, most chemotherapeutic drugs). Bcl-2 controls the activation of the initiator caspase, caspase-9, by the adapter protein Apaf-1, but this does not appear to be the critical or the sole molecule regulated by Bcl-2 [19–22]. In the nematode *C. elegans*, the Bcl-2 homolog CED-9 function by sequestering the activity of the adapter protein CED-4 which is required to activate the caspase CED-3 (Fig. 3A) [23–27]. However, a true mammalian homolog of CED-4 has not been discovered to date. The machinery is also more complex in mammals which express a number of structural and functional homologs of Bcl-2, namely Bcl-$x_L$, Bcl-w, Mcl-1, and A1 [28]. These proteins are functionally similar, promoting cell survival until antagonized by a family of distantly related proteins, the BH3-only proteins.

The BH3-only proteins are so-called because they share with each other, and with the other members of the Bcl-2 family of proteins, only the short BH3 domain (Fig. 1) [29]. This short domain forms an α-helical region, the hydrophobic face of which binds onto a hydrophobic surface cleft present on prosurvival Bcl-2 (Fig. 2) [30,31]. The BH3-only proteins probably function to sense cellular damage to critical cellular structures or metabolic processes, and are then unleashed to initiate cell death by binding to and neutralizing Bcl-2 [29,32]. Normally, the BH3-only proteins are kept inert by transcriptional or translational mechanisms, thereby preventing inappropriate cell deaths. Recently, two BH3-only proteins that are transcriptional targets of the tumor suppressor protein p53 have been described, namely Noxa [33] and Puma/Bbc3 [34–36]. These proteins are thus good candidates to mediate cell death induced by p53 activation [37]. Some other BH3-only proteins are controlled instead by post-translational mechanisms. In particular, two are sequestered to the cell's cytoskeletal network, Bim to the microtubules and Bmf to the actin cytoskeleton [38,39]. Damage signals that impinge upon these cytoskeletal structures will activate Bim or Bmf freeing them to bind to prosurvival Bcl-2 located on the cytoplasmic face of the outer mitochondrial membrane as well as those of the nucleus and endoplasmic reticulum. It is, however, unclear if the eight or so mammalian BH3-only proteins bind to all the prosurvival Bcl-2-related proteins, or whether there is some selectivity. It is also unclear how the binding of BH3-only proteins to Bcl-2 results in caspase activation [18].

## 4. How do BH3-only proteins kill?

Recently it has been shown that the killing by the BH3-only proteins is dependent on the activity of a third family of Bcl-2-related proteins, namely the Bax subfamily (Fig. 1A) [40,41]. Although, these proteins bear three of the four homology domains and are structurally very similar to the prosurvival proteins [42], Bax, Bak, and Bok/Mtd function instead to promote cell death. Biochemically, damage signals cause these proteins to aggregate and

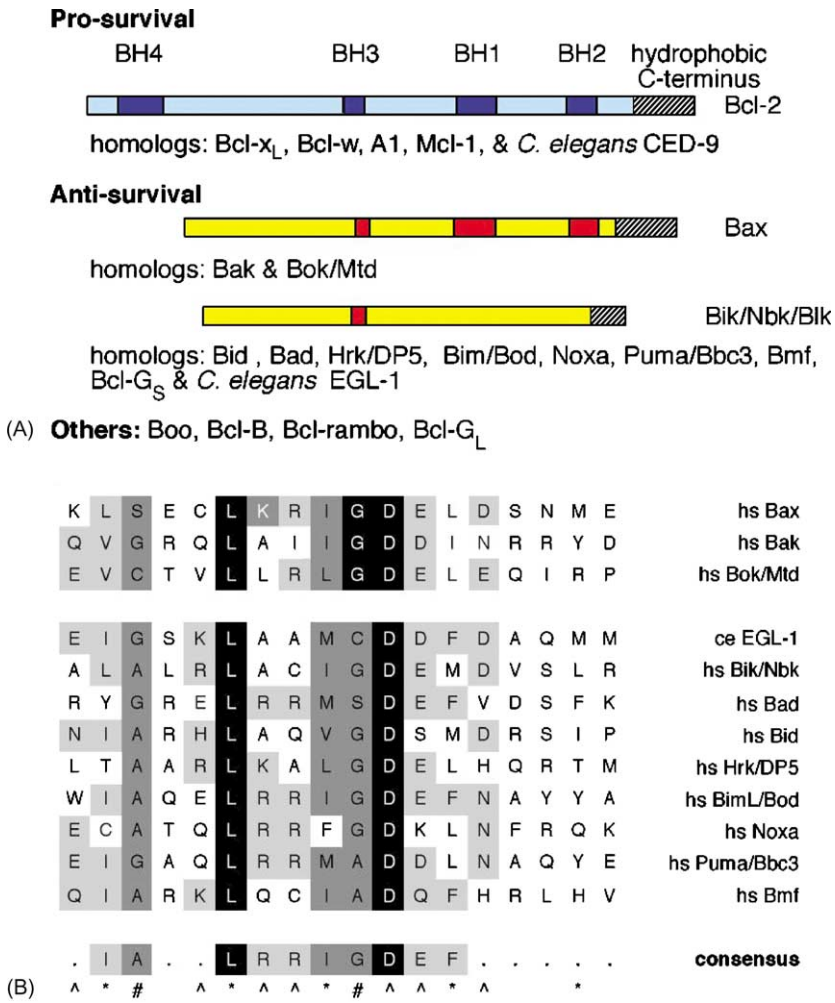



Fig. 1. The Bcl-2 family of proteins. (A) Schematic representation of some members of the Bcl-2 family of proteins. They are divided into those that promote cell survival and those that antagonize this. Bcl-2 and most of its prosurvival homologs share the Bcl-2 homology regions BH1–BH4. The proapoptotic proteins are further subdivided into those bearing the BH1-3 domains (multidomain Bax-like) and those that harbor only the short BH3 domain (BH3-only proteins). (B) Alignment of some BH3 domains. The BH3 domains from all known human Bax-like and BH3-only proteins, as well as *C. elegans* EGL-1, were lined up using GCG "PILEUP" program. Identical or very similar residues (K and R, D and E, V and I, and M and L) present in most of the proteins are shaded in black while less conserved residues are shaded in gray. (*) hydrophobic residues; (#) small residues; (^) charged residues.

such oligomers may function to cause damage to mitochondrial membranes [43–48], thereby causing the release of mitochondrial proapoptogenic factors such as Smac/Diablo [49,50] and cytochrome *c*, which is essential for the activation of caspase-9 by Apaf-1 [51–54]. Since killing by BH3-only proteins depend on Bax and Bak in fibroblasts, their physiological role may be to activate Bax and Bak [41,55]. In such a model, the prosurvival Bcl-2 proteins function merely to sequester the BH3-only proteins until such time as when there is insufficient capacity to do so (Fig. 3B). However, there are few reports of direct binding of the BH3-only proteins to Bax and Bak and even that in the case of the BH3-only protein, Bid appears weak [43,47,56]. To date, there are no experiments to directly compare the binding of BH3-only proteins to prosurvival Bcl-2 with that to proapoptotic Bax.

In addition to the tenuous biochemical evidence for the direct binding of BH3-only proteins to Bax-like proteins, careful analyses of the available genetic data also suggests that prosurvival Bcl-2 rather than proapoptotic Bax is the direct target of BH3-only proteins. In the nematode *C. elegans*, all the killing induced by the BH3-only protein, EGL-1, is dependent on the ability of EGL-1 to bind to and neutralize nematode Bcl-2, CED-9 [57,58]. The situation is somewhat more complex in mammals because of the functional redundancy in each class of proteins. Instead of a single BH3-only protein (EGL-1) and a single Bcl-2 homolog (CED-9), mammals express multiple proteins of each subclass making direct comparison with the nematode difficult. Furthermore, nematodes do not appear to express Bax-like proteins. However, if the Bcl-2-like proteins function merely to sequester BH3-only proteins, then the amount of prosurvival Bcl-2-like proteins in any cell type must be limiting. It is, therefore, surprising that mice lacking a single allele of the *bcl*-2 [59–61], *bcl*-x [62,63] or *bcl*-w [64,65] genes are normal whereas the homozygous

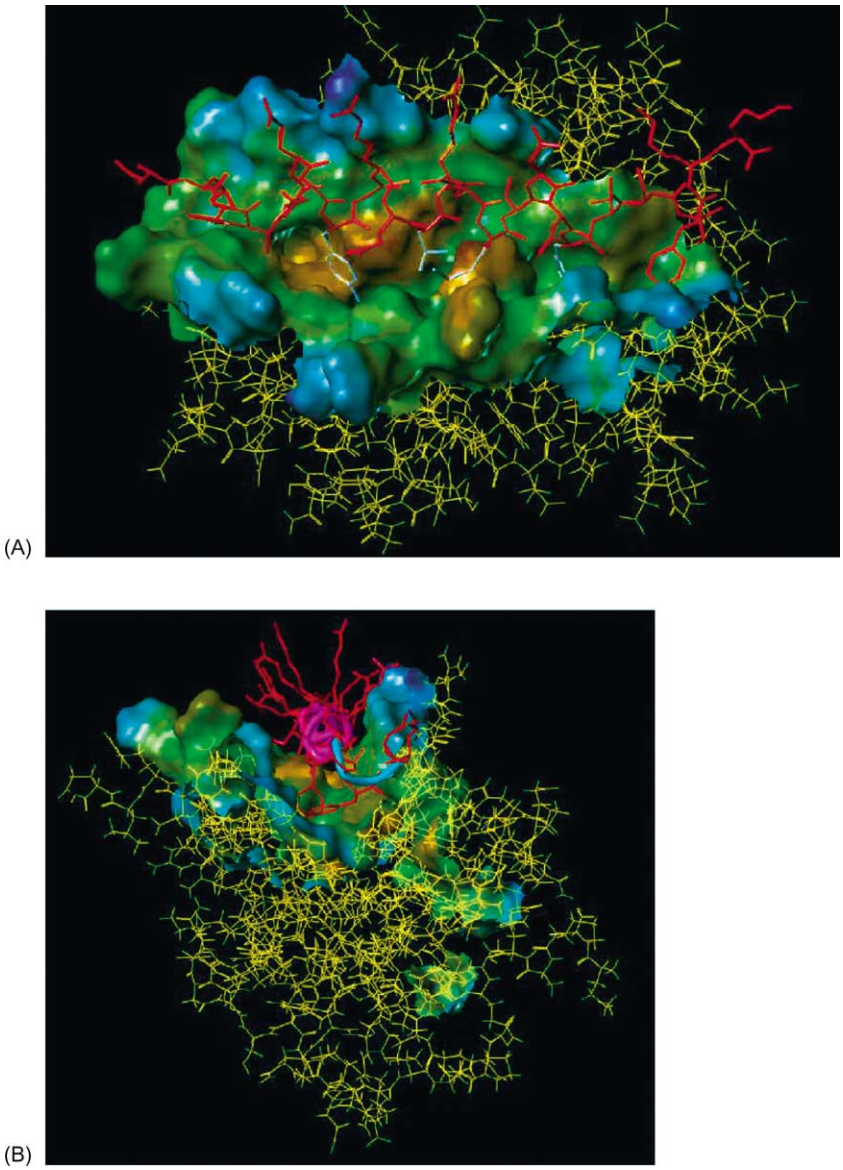



Fig. 2. Complex of the BH3 domain of Bad (red) bound to Bcl-$x_L$ (yellow) in two different views. The four particular hydrophobic residues in Bad, which project into the hydrophobic BH3-binding groove, are coloured in white. The molecular surface of Bcl-$x_L$ as shown was generated using MOLCAD within Sybyl6.7 (Tripos Associates, http://www.tripos.com) and is coloured according to hydrophobicity (most hydrophobic brown, medium hydrophobic green, hydrophilic blue). (A) The orientation is such that the N-terminus of the BH3 domain is on the left. (B) A different view of this complex, looking down the helical spiral.

knock-out mice all have striking phenotypes in the cell types where these genes play a crucial role. This suggests that the prosurvival Bcl-2-like proteins are not limiting; instead analysis of mice lacking the BH3-only protein, Bim, suggest that this class of proteins is limiting [32,66]. Taken together, the available data would suggest BH3-only proteins directly bind to Bcl-2 and it is by neutralizing Bcl-2 that BH3-only proteins can activate Bax-like proteins (Fig. 3C).

Thus, agents that directly mimic the BH3-only proteins would be predicted to induce cell death and may, therefore, be of value therapeutically. As Bcl-2 controls the critical point that determines a cell's fate, this class of proteins represent an attractive target for drug design. In particular, since many of the oncogenic mutations, such as those to p53 results in defects in sensing cellular damage that would normally result in cell death by a Bcl-2-dependent mechanism, directly targeting Bcl-2 and its homologs may circumvent such mutations. This may also permit an alternative route to overcome tumor resistance to current treatments.

## 5. Peptide-based BH3 mimetics

To date, the solution structures of Bcl-$x_L$ [67] and Bcl-2 [68] have been solved, and NMR structural analysis of Bcl-$x_L$ complexed with the BH3 domains of Bak [30] and Bad

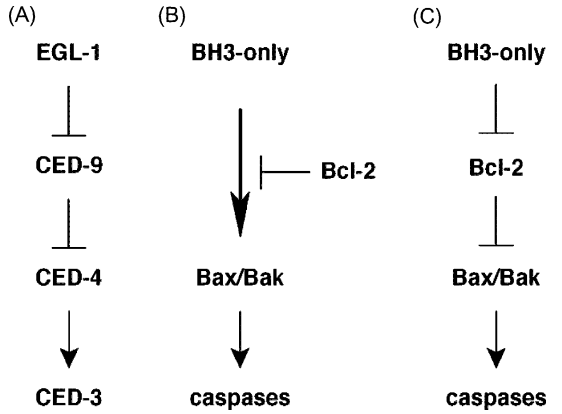



Fig. 3. Models of how BH3-only proteins might kill. (A) In the nematode *C. elegans*, the BH3-only protein directly bind to the Bcl-2 homolog, CED-9, freeing the adapter protein CED-4 to oligomerize and activate the caspase CED-3. (B) In mammals, the BH3-only proteins may function directly on Bax-like proteins. Prosurvival Bcl-2 functions solely as a "sink" until there is insufficient capacity to bind the BH3-only proteins. (C) Alternatively, Bcl-2 might function in a manner akin to *C. elegans* CED-9. It controls an activity required for the activation of Bax-like proteins until neutralized by the BH3-only proteins.

[31] shows that the BH1, BH2, and BH3 domains of Bcl-$x_L$ form a hydrophobic groove which envelops the α-helical BH3 domain of Bad or Bak (Fig. 2). Four hydrophobic residues in the amphipathic α-helical BH3 domain conserved in all the BH3-only proteins interact with the hydrophobic groove in the prosurvival protein. No significant conformational change in Bcl-$x_L$ upon binding of a BH3 peptide was detected, although it is important to note that prosurvival proteins used in these studies are not of the full-length proteins. Regardless of this, a possible approach to design BH3 mimetics is to design peptidomimetics of the BH3 peptides.

Even in the presence of structural information it is often difficult to develop peptidomimetics, since the interaction surface is often comprised of discontinuous binding elements [69]. In the case of the BH3-only proteins, this problem may be simplified because all the binding energy is derived from a short contiguous sequence in BH3 domains. The α-helix has been the subject of intense scrutiny because it is frequently associated with sequences that encode biological activity [70]. In the design of peptide-based mimetics of native sequences, a common aim is to restrict the peptides to include only those residues that are crucial for binding. In this way, it might be easier to fine tune the mimetics and the chances of embedding favorable therapeutic properties such as membrane permeability are enhanced. However, short α-helical peptides are often unstable thereby limiting the ability to maintain the biologically active conformation [71]. Incorporation of helix-stabilizing residues may help overcome this problem. For example, such as strategy has been shown to improve binding of an unstable 16-mer Bad BH3 to Bcl-$x_L$ manifold [31] (see peptides **13** and **14** in Table 1). A related strategy has been to obtain potent Bak BH3 mimetics by grafting potentially important $Bak_{72–87}$ residues (V74, R76, L78, I81, D83, and I85) onto a miniature α-helical protein scaffold, avian pancreatic polypeptide (aPP), and interrogate four positions (G22, A26, G29, D31) by phage display (see peptides **17** and **18** in Table 1) [72]. Peptides that bound to Bcl-2 with up to 100-fold higher affinity than $Bak_{72–87}$ were identified, although it appears that this was due largely to the α-helix-stabilizing effect of an N-terminal aPP 18-mer and not necessarily due to a phage-led optimization of the four variable residues. It would be interesting to have $Bak_{72–87}$ grafted to this N-terminal aPP 18-mer and measure its affinity for Bcl-2 as a control. Another problem with this approach from a therapeutic viewpoint is that it does not address the problem of shortening the binding sequence as much as possible.

Another way of stabilizing α-helices while keeping the peptide as short as possible is to covalently bond two residues using lactam bridges (Fig. 4A) [73]. Successfully applied lactam constraints range from the conceptually simple and well documented i(i+4) side chain lactamizations, involving one helical turn, to lesser known i(i+7) pentane-linked glutamate side chains [74–76], which involve two turns of the helix (Fig. 4A). The BH3 domains are an attractive set of peptides to try such a strategy for drug design and it may be possible to combine our current structural knowledge of the binding pocket together with molecular modeling and molecular dynamics [77] to rationally design constrained, short bioactive peptides.

Using a simpler approach of just introducing unmodified peptides into cells, it has been shown that some BH3 peptides can induce cell death [78]. However, even control peptides that would be predicted to have reduced or no binding to Bcl-2 are equipotent suggesting that the approach of using peptides may be hampered by lack of specificity [79]. Indeed, it has been noted that α-helices can often be toxic to cells and if this is the case with the BH3 peptides, their usefulness may be limited. Furthermore, the poor membrane permeability properties of peptides can make intracellular targets such as Bcl-2 virtually inaccessible. Although specialized attachments [80,81], such as that used successfully in truncated BH3 peptides [82], can overcome this problem, an alternative approach is to discover small molecule, nonpeptide antagonists.

## 6. Small molecule BH3 mimetics

Recently a number of groups have successfully reported the discovery of compounds that may act as BH3 mimetics from *in silico* or real screens [83–87]. Despite the difficulty of identifying agents that disrupt protein–protein interactions [88–90], the topography of the BH3 binding pocket of Bcl-$x_L$ seems to allow disruption by small molecules such as those listed in Table 1. Many of the compounds (**4**–**12**) were discovered *in silico* and validated by assaying purchased "hits". On the other hand, compounds **1** and **2** were

Table 1
Small molecule and peptide-based inhibitors of Bcl-$x_L$/Bcl-2, found through wet screening (**1**,**2**), serendipitously (**3**), *in silico* screening (**4**–**12**), or peptide-based design (**13**–**18**)

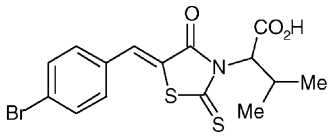
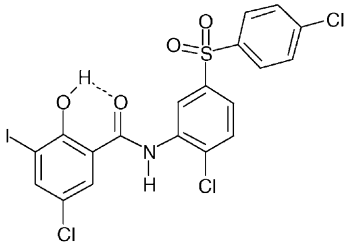
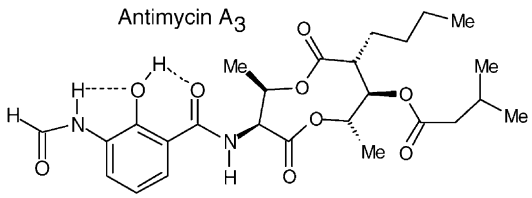
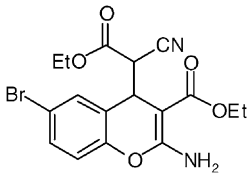
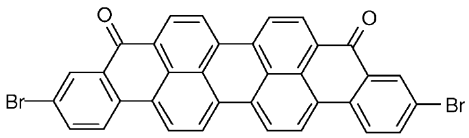
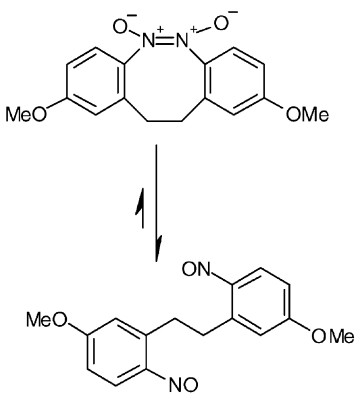
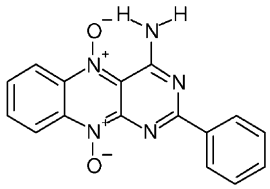
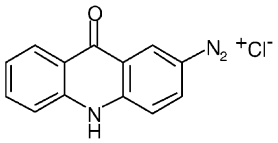
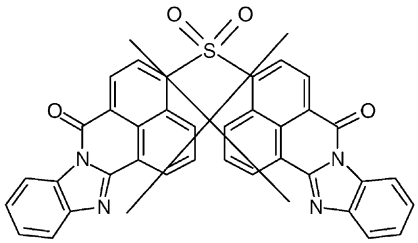
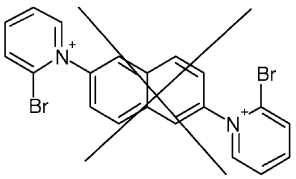
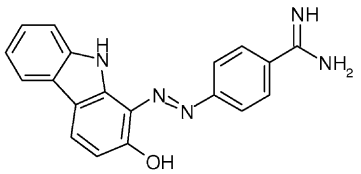
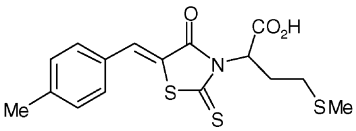
| Compound | Structure | Reported $IC_{50}$ (μM) (or $K_d$) |
|---|---|---|
| **1** |  | 7.8 ± 0.9 (Bcl-$x_L$) [84] |
| **2** |  | 3.3 ± 0.3 (Bcl-$x_L$) [84] |
| **3** |   | 2 ± 0.3 (Bcl-2) [85] |
| **4** |  | 9 (Bcl-2) [83] |
| **5** (NSC 7233) |  | 7.7 ± 4.5 (Bcl-2) [87] |
| **6a** (NSC 365400)<br>**6b** |  | 7 (Bcl-$x_L$)<br>10.4 ± 0.3 (Bcl-2) [87] |
| **7** (NSC 252041) |  | 1.6 ± 0.1 (Bcl-2) [87] |

Table 1 (*Continued*)

| Compound | Structure | Reported $IC_{50}$ (μM) (or $K_d$) |
|---|---|---|
| **8** (NSC 140067) |  | 10.4 ± 1.2 (Bcl-2) [87] |
| **9** |  | 14.0 ± 2.8 (Bcl-2) [87] |
| **10** |  | 11.7 ± 2.4 (Bcl-2) [87] |
| **11** (NSC 357777) |  | 5.8 ± 2.2 (Bcl-2) [87] |
| **12** |  | 8.5 ± 0.6 (Bcl-$x_L$) [86] |
| **13** | QRYGRELRRMSDEFVD (Bad 16-mer) | $K_d$ 50,000 nM [31] |
| **14** | **DDYA**RELR**MMA**DEFV**R** (stabilized Bad 16-mer) | $K_d$ 200 nM [31] |
| **15** | NLWAAQRYGRELRRMSDEFVDSFKK (Bad 25-mer) | $K_d$ 0.6 nM [31] |
| **16** | GQVGRQLAIIGDDINRRYDSEFQ ($Bak_{72–94}$ 23-mer) | $K_d$ 200 nM (Bcl-$x_L$) [30] |
| **17** | GQVGRQLAIIGDDINR ($Bak_{72–87}$ 16-mer) | $K_d$ 480 nM [31], 350 nM (Bcl-$x_L$), 5000 nM (Bcl-2) [72] |
| **18** | *GPSQPTYPGDDAPVEDLIRF*-VGRLLAYFGDTINR | $K_d$ 7 nM (Bcl-$x_L$), 52 nM (Bcl-2) [72] |

Compound **6a** exists in solution at room temperature as a mixture of two interconverting forms, **6a** and **6b**, although the latter is favored [87]. Compound **8** is listed as containing a methoxy group, but this compound is not present in the NCI database. The unsubstituted compound shown here is listed in the NCI database and is appears consistent with the MS and NMR data [87]. Compounds **9** and **10** are shown as the structures given in the NCI database, but actually have different, unknown structures, and so these have been crossed out. Stabilizing the helical form of Bad 16-mer (**13**) by changing certain residues (**14**, changed residues in bold) increases affinity for Bcl-$x_L$. Residues from Bak 16-mer (**17**) were grafted onto a helical miniature protein (italicized residues in **18**), and four positions (underscored) were interrogated by phage display.

discovered by wet screening a library of 16,320 compounds for ones that disrupted the complex between Bcl-$x_L$ and a Bak BH3 peptide [84], whereas antimycin A was discovered serendipitously [85].

The success of *in silico* screens using a modeled structure of Bcl-2 [83,87] provides some vindication for homology modeling as a viable basis for such screens. However, it is disconcerting that some of the compounds (**9** and **10**) were found to have molecular weights that do not correspond to their structures as listed in the NCI database. These structures must, therefore, be incorrect, as we indicate in Table 1. Furthermore, some of the other Bcl-2 inhibitors identified such as compound **5** (a large and hydrophobic molecular slab, calculated log P 11) and **9** are really not at all drug like, whereas the reactive diazonium salt **8** is highly unattractive as a lead. It would also be reassuring to see if **6a**, but not the thermodynamically favored form **6b**, binds to the BH3-binding domain as predicted [87].

Another issue to consider in such screens is to allow conformational flexibility of the target, and of the ligand. Enyedy *et al.* [87] point out that false negatives are likely to

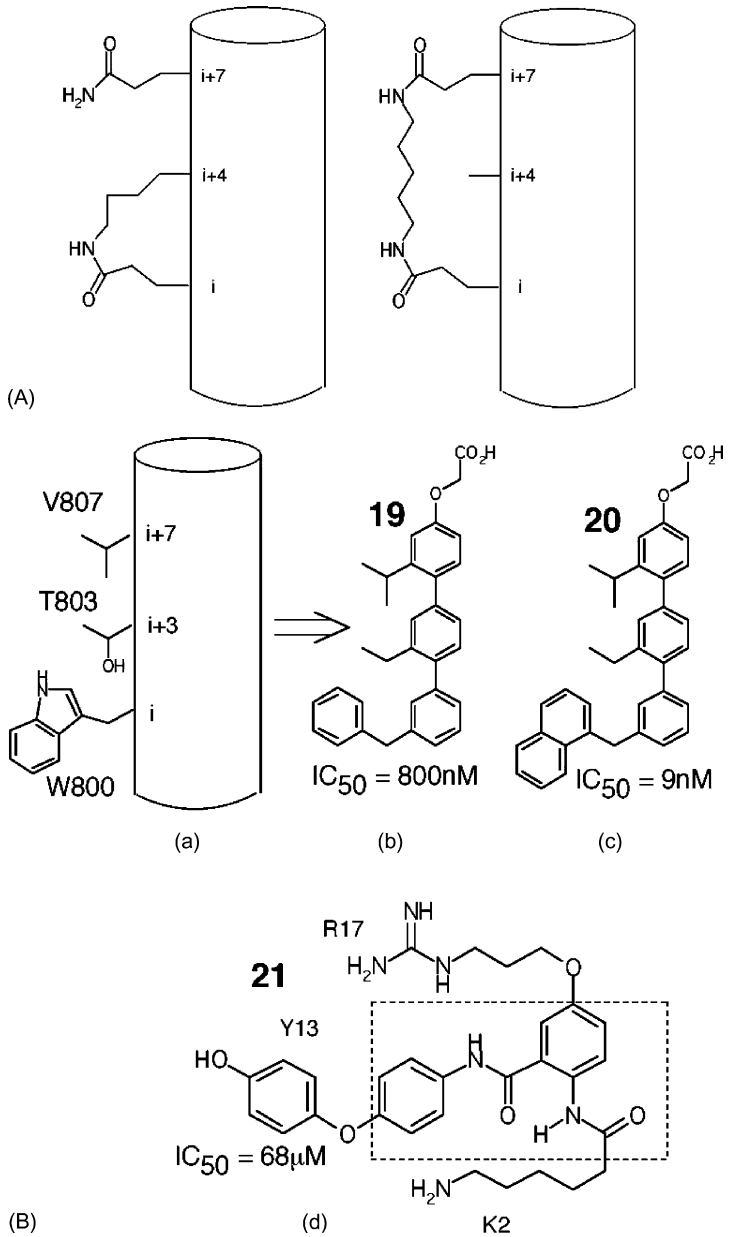



Fig. 4. Designing BH3 mimetics using rational approaches. (A) A schematic illustration of i(i+4) (left) and i(i+7) lactam (right) bridges which can be used to stabilize α-helices in peptides. An i(i+4) constraint covers one helical turn, whilst an i(i+7) constraint covers two turns of a helix. (B) The terphenyl scaffold designed as a helical mimetic [96] and used with success to generate a potent calmodulin antagonist **20** shown in (c), optimized from **19** in (b), based on a helical segment of myosin light chain kinase (a). Shown in (d) is a mimetic (**21**) of residues K2-Y13-R17 in the N-type calcium channel blocker omega-conotoxin GVIA containing a more drug-like scaffold. These two approaches to scaffold construction may need to be married to obtain potent, drug-like, rationally designed, small molecule BH3 mimetics.

arise by not being able to properly allow for flexibility in the Bcl-2 active site, and that better scoring functions are needed to reduce both the false negatives and the false positives although the latter probably represent the major problem for *in silico* screens [91]. Recently, Lugovskoy *et al*. [86] flexibly docked **1** and **2** onto a *flexible* Bcl-$x_L$ active site, reducing the size of the task by incorporating NMR-derived distance constraints to limit placement options for the ligand. They reported a good correlation between computed interaction energy and measured affinity. They used their method in a refined *in silico* screen, leading to the discovery of **12** (Table 1) as a novel Bcl-$x_L$ inhibitor, although it is clearly structurally related to **1**. Importantly, they were able to use their model to also predict certain close analogs of **12** to be inactive.

A potential limitation of *in silico* screens is that whilst large commercial manufacturers are usually a reliable source of compounds, other compound databases used for *in silico* screens may only have limited stocks of chemicals. For example, the NCI could only supply 35 compounds of 80 compounds ordered [87]. It is also debatable whether *in silico* screens for anticancer agents

using the NCI database of compounds, which may have already been tested for anticancer activity in cell-based assays, is likely to be profitable. However, such assays do not preclude the identification of promising lead compounds that could be modified to incorporate favorable features such as cell permeability. It is also unclear why a high throughput wet screens did not unearth a significant number of hits given the diversity of the structures identified *in silico* (**4**–**11**).

Where structural information is available, as it is for the BH3 domain and its binding groove, an attractive alternative for antagonist discovery is to rationally design scaffolds that project important side chain residues appropriately for binding. Such molecules would be more likely to be BH3 structural mimetics rather than compounds **1**–**12**, which could act allosterically. For discontinuous binding epitopes in particular, however, this is a deceptively difficult task and only a handful of attempts have been reported, and with varying degrees of success [92–102]. The problem lies mainly with the difficulty in combining synthetic, conformational, and physicochemical aspects of molecules during *in silico* construction. The problem of scaffold *synthesis* can be alleviated by computer-aided searching of databases of available compounds that *mimic* target pharmacophores (as opposed to the *in silico* screening methods reported earlier which seek compounds that *fit* into a defined active site). While this has met with some success in the discovery of nonpeptide mimetics of discontinuous pharmacophores [97], database diversity limits the generic potential of this approach. The problem of scaffold *conformation* is also suited to automation, particularly of the sort exemplified by Bartlett's CAVEAT vector matching program [103], which searches scaffold databases for matching bond vectors. However, the combined fields of diversity, synthesis, conformation, and physicochemistry again pose difficulties for the use of this technology in the efficient discovery of drug-like, nonpeptide mimetics. Rather, successes in the use of CAVEAT tend to be limited to inherently much simpler problems [104].

An exceptional result amongst the attempts of interactive design—and an example of particular relevance to the BH3 domain—is the use of a terphenyl scaffold, functionalized appropriately to mimic residues at positions i, i+3, and i+7 of the α-helix in myosin light chain kinase [96] in order to produce an inhibitor of calmodulin. As shown in Fig. 4B, mimetic side chains of Trp, Thr, and Val, respectively, were slightly modified for synthetic ease but still gave rise to a compound (**19**) with inhibitory potency ($IC_{50}$) of 800 nM. The carboxylic acid group was installed to encourage adequate water solubility. Optimization of the Trp mimetic side chain, involving the simple alteration of a phenyl ring to a naphthyl ring (**20**) led to an almost 100-fold increase in potency ($IC_{50} = 9$ nM). However, the terphenyl scaffold is not very drug-like and is excessively hydrophobic. We recently synthesized the anthranilamide derivative **21** shown in Fig. 4B as a mimetic of the discontinuous binding epitope in omega-conotoxin GVIA comprising the residues K2-Y13-R17 [94]. This compound blocked the N-type calcium channel with only modest affinity because the residues it targets in GVIA only bind weakly to this site, but the scaffold is much more drug-like than the terphenyl scaffold. The challenge will be to see if one can reproduce the conformation and potency of compounds of the type **20** with more drug-like scaffolds such as that in **21**.

## 7. Testing lead compounds for specificity and mechanism of action

Regardless of the source of the lead compound (high-throughput screens, *in silico* screens, and other structure-based design), it will be important to validate the molecular basis of their binding to Bcl-2 (or its homologs) and their mode of action *in vivo*. Because of the complexity and our uncertainties of the signaling pathways to cell death, this is probably one area that needs to be urgently addressed. To date, limited testing has been done of the compounds isolated for their ability to interact with prosurvival Bcl-2-like proteins. It is noteworthy that most of these compounds apparently bind with affinities that are significantly lower than the nanomolar binding reported for the binding of Bad to Bcl-$x_L$ [31]. Furthermore, most of the screens and validation have been carried out using truncated Bcl-$x_L$ or Bcl-2 molecules, or their modeled structures, which may not be the biological target *in vivo*. It is also unclear if the target surface hydrophobic groove is normally unoccupied as implied in these studies. Ideally, it will be important to compare the binding of any lead compound to Bcl-2-like proteins with binding of BH3-only proteins in the same assay system. Finally, some evidence that the lead compounds do bind into the surface hydrophobic groove of the prosurvival proteins rather than just modeled onto a known structure will be an important part of the process of characterizing these leads.

Extensive experimental data has apparently been gathered to show that these compounds do induce apoptosis by antagonizing Bcl-2 in cells. Given the complex nature of apoptosis, it will be important to demonstrate the specificity of the reagents used. Unfortunately, much of the data do not constitute convincing evidence for how the compounds might act. For example, the reported affinities of the compounds for Bcl-$x_L$ or Bcl-2 (micromolar range) are comparable to that observed with biologically inactive mutants of the BH3-containing proteins (Table 1). This raises doubts about the specificity of these compounds. Furthermore, protection against cell death induced by these compounds by Bcl-2 overexpression does not prove that the compounds act on the prosurvival proteins directly. As discussed, Bcl-2 overexpression, in a dose-dependent manner, inhibits cell death induced by a whole range of

cytotoxic agents, most of which are unlikely to act directly on Bcl-2. If a lead compound shows selectivity against one of the prosurvival proteins, then their killing activity should be abrogated in cells lacking that protein. Thus, the specificity of a Bcl-2 antagonist can be confirmed by comparing the activity of the compound in wild-type cells or those lacking Bcl-2. Such cells may be derived from knock-out mice or have the expression of Bcl-2 suppressed by antisense oligonucleotides, or by RNA interference. Since mammalian cells express multiple prosurvival Bcl-2 proteins and many of the compounds may not show target selectivity, it is currently an enormous challenge to develop robust cell-based assays in order to elucidate the mode of action of any new compound.

## 8. Perspectives

The importance of deregulated cell death in malignant transformation suggests that we might be able to exploit our current knowledge of the apoptotic machinery for therapeutic benefit. Furthermore, since many current therapies may work by indirectly activating the cell death machinery, the direct approach might be advantageous. The recent outstanding success of agents such as the inhibitor of the Abl tyrosine kinase STI571 (Glivec), based on a rational approach to treat chronic myelogenous leukemia, has encouraged many researchers in other areas of cancer biology. However, there are significant challenges ahead if we are to realize the dream of developing inhibitors of Bcl-2. Aside from issues such as bioavailability, pharmacokinetics, and the therapeutic index of any compound, our current knowledge of the core cell death machinery is still incomplete. For example, we do not know how Bcl-2 promotes cell survival [18] and whether it is the Bax-like proteins that are the target for the BH3-only proteins [41,105]. If the latter is the case, identifying BH3 mimetics based on the structure of prosurvival Bcl-2 may not be the best route to take.

There are also practical limitations to developing BH3 mimetics at the current time. To date, there are only limited biophysical studies on the interactions between the BH3-only proteins and prosurvival Bcl-2, and in order to design drugs efficiently, we will need a complete catalog of all the possible interactions and their affinities. The NMR structures of the complexes formed between Bcl-$x_L$ and the BH3 domains (of Bak and Bad) have illustrated ways that antagonist might be discovered. However, we will need other structures that may be more physiologically relevant. Finally, we will need improved assays for the *in vivo* activity of the compounds to show specificity and to confirm the mode of action.

In spite of these considerations, any reagents discovered will be invaluable reagents allowing us to experimentally probe the cell death machinery further. This will be an essential part of the reiterative process for mechanism-based drug discovery, a daunting challenge for identifying agents that modulate protein–protein interactions rather than blocking an enzymatic activity.

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