

# Modulating Mitochondria-Mediated Apoptotic Cell Death through Targeting of Bcl-2 Family Proteins

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Received: September 7, 2006; Accepted: September 28, 2006; Revised: October 4, 2006

**Abstract:** Research demonstrated that the function of mitochondria extends well beyond that of being cell powerhouses and revealed that these organelles fulfil a dual role in both cellular life and death. In most vertebrates, execution of the mitochondrial pathway of apoptosis requires permeabilization of the mitochondrial outer membrane, an event which allows for the release of a variety of intramembrane space proteins, leading to the activation of caspases and ultimately cell demise. Bcl-2 family proteins, which include pro- and antiapoptotic members, positively or negatively regulate mitochondrial outer membrane permeabilization, i.e. a barrier to apoptosis induction. Over-expression of Bcl-2 and Bcl-x<sub>L</sub> is associated with tumor progression and may be responsible for drug resistance, making pro-survival Bcl-2 family members important targets for the development of anticancer agents. Pharmacological apoptosis modulation by manipulation of pro-apoptotic Bcl-2 family proteins, with the goal to treat disorders associated with uncontrolled cell death or to kill unwanted cells, is likely to represent an additional research focus in the coming years. The purpose of this review is to describe, with examples taken from recent patents, novel strategies for targeting the Bcl-2 family of apoptotic regulators through peptide-based approaches and selective delivery of functional nucleic acids.

**Keywords:** Mitochondrial outer mitochondrial membrane permeability, cancer therapy, apoptosis, programmed cell death, Bcl-2 family.

## I-INTRODUCTION

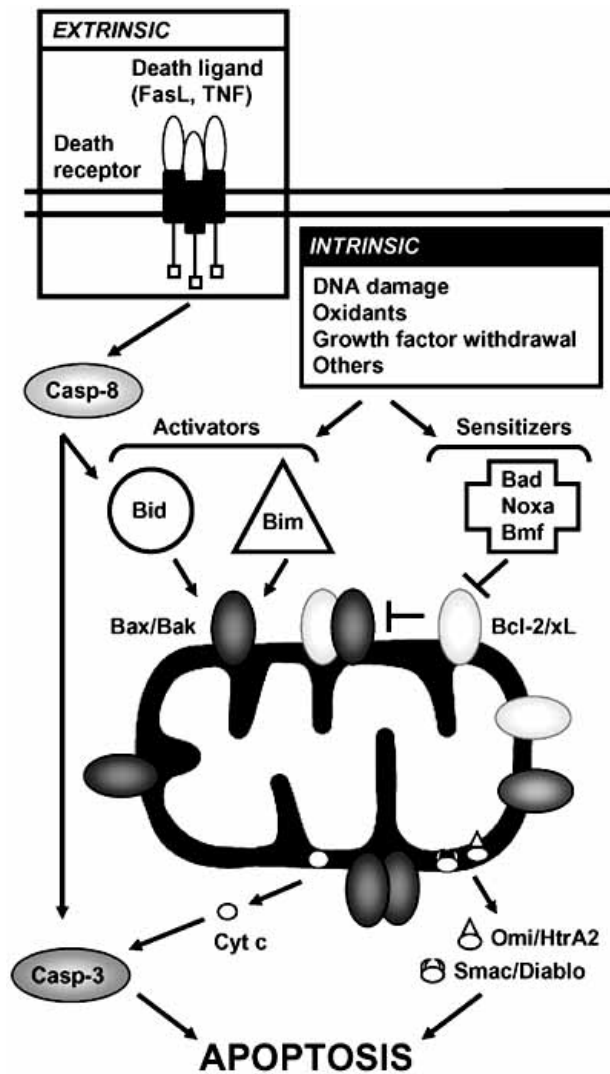
Apoptosis (programmed cell death) has evolved to fulfil a vast range of functions in metazoans, such as regulating tissue homeostasis and eliminating unnecessary or potentially harmful cells. Since the beginning of the 1990s, many human diseases have been associated with too much or too little apoptosis, such as degenerative diseases and cancer, respectively [1]. In their influential paper, Hanahan and Weinberg defined the ability to evade programmed cell death as a hallmark of most human cancers and pointed to the fundamental interplay between apoptosis and cell proliferation [2]. The expression of antiapoptotic proteins or the inactivation of proapoptotic proteins can disturb this delicate balance, resulting in failure of apoptosis, and are thus important factors involved in tumor progression and resistance to chemotherapy. Academic and pharmaceutical research aim at developing effective drugs intended to restore physiological levels of apoptosis and, ideally, induce cell death in cancer cells without damaging normal cells. In addition to cancer, our better understanding of the apoptosis machinery opens up the way to novel therapeutic targets for the treatment of diseases of excessive cell death, i.e. neurodegenerative disorders, stroke, or brain injury. This paper will summarize recent advances in apoptosis-targeting approaches, with special emphasis on modulation of Bcl-2 family proteins. A selection of original patent applications claiming the use of nucleic acid-based strategies (including antisense oligonucleotides and delivery of functional plasmid

DNA) or peptidyl agents (such as synthetic peptides and related peptidomimetics) is reviewed.

## II-APOPTOSIS SIGNALING: THE MITOCHONDRIA-DEPENDENT PATHWAY

In mammalian cells, at least two main pathways leading to apoptosis induction have been described: the intrinsic mitochondrial pathway and the extrinsic death receptor pathway [3], Fig. (1). The extrinsic pathway is activated by the ligation of death receptors of the TNF receptor superfamily (e.g. Fas or TRAIL receptors) by their cognate ligands. In the intrinsic pathway, various noxious stimuli including growth factor withdrawal, DNA damage, or hypoxia, cause the translocation of apoptogenic proteins such as cytochrome C [4] and Smac/DIABLO [5,6] from the mitochondrial intermembrane space into the cytosol [7]. There is now compelling evidence that permeabilization of the mitochondrial outer membrane, and the subsequent release of mitochondrial intermembrane space proteins, have a decisive impact on cell fate. Once in the cytosol, cytochrome c together with dATP binds to the adaptor molecule Apaf-1, which recruits and activates pro-caspase-9 in a large multimeric structure termed the apoptosome. This macromolecular complex activates downstream caspases such as caspase-3 and -7 that cleave important regulatory and structural proteins, resulting in cell death. The mitochondrially released Smac/DIABLO and possibly the mitochondrial serine protease Omi/HtrA2 [8] induce apoptosis by binding to and inactivating inhibitor of apoptosis proteins (IAPs), which are caspase inhibitors. Other mitochondrial factors (e.g. AIF and EndoG) are released into the cytosol during apoptosis and may contribute

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**Fig. (1).** The extrinsic and intrinsic pathways of apoptosis.

There are two major apoptotic pathways involved in programmed cell death: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. In the mitochondrial pathway, apoptotic signals are relayed by pro-apoptotic members of the Bcl-2 family to mitochondria, where release of apoptogenic factors (e.g. cytochrome c, Smac/Diablo, Omi/HtrA2) is induced. Anti-apoptotic members such as Bcl-2 and Bcl-xL inhibit and pro-apoptotic members such as Bax and Bak promote cytochrome C release. BH3-only proteins act as upstream sentinels for cellular stress or damage and either activate Bax/Bak ("activators") or inactivate Bcl-2/xL ("sensitizers"). Once released, cytochrome c interacts with Apaf-1 in the presence of dATP to form the apoptosome, which binds and activates procaspase-9. Caspase-9 in turn activates downstream caspases such as caspase-3, -6 and -7, which cleave a variety of substrates and the cell undergoes apoptosis. In contrast, the death receptor apoptotic pathway is initiated by the binding of death ligands (i.e. TNF, FasL, TRAIL) to their cognate transmembrane death receptors. Upon interaction, caspase-8 is activated and, similar to caspase-9 in the intrinsic pathway, processes downstream caspases. Caspase-8 also cleaves cytosolic Bid generating a truncated Bid (tBid) that translocates to the mitochondria and promotes the release of cytochrome c in concert with Bax/Bak.

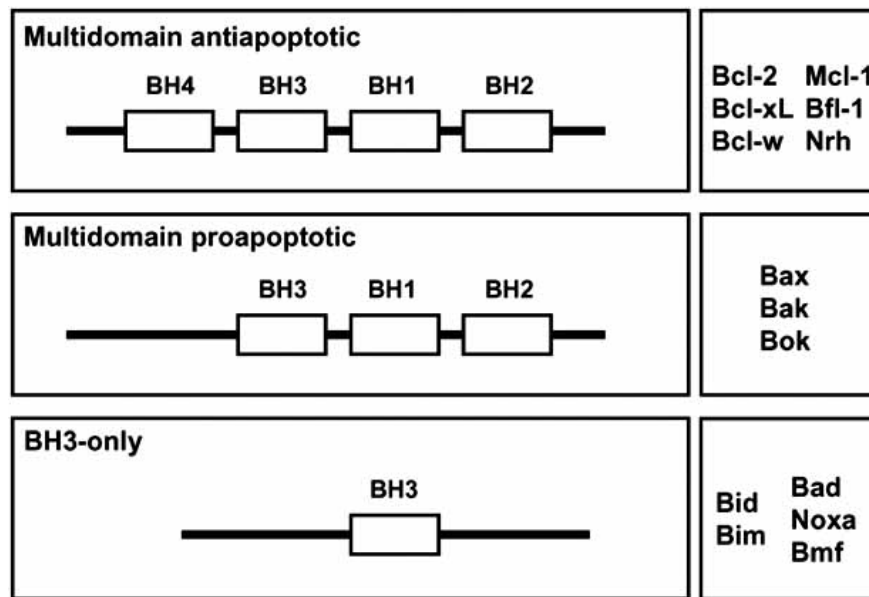
to caspase-independent cell death. Thus, disruption of the outer mitochondrial membrane plays a key role in the intrinsic death pathway, and is considered to determine a point-of-no-return in many cascades leading to apoptosis.

### III-THE BCL-2 FAMILY: KEY REGULATORS OF MITOCHONDRIAL APOPTOSIS

#### III-1-Structure and Function

The integrity of the mitochondrial outer membrane is regulated by an evolutionary conserved group of proteins known as the Bcl-2 family [9,10]. Bcl-2 family proteins have been defined both by their function (either pro- or antiapoptotic) and overall conservation of sequence motifs known as Bcl-2 homology (BH) domains, Fig. (2). Anti-apoptotic multidomain members generally share sequence similarity in four conserved BH domains (BH1-4), while proapoptotic multidomain members contain only two or three BH domains (BH1-3). To date, the human repertoire of multidomain members comprises six antiapoptotic members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Nrh/Bcl2l10/Bcl-B and Bfl-1) and up to seven pro-apoptotic members (Bax, Bak, Bok, Bcl-G, Bcl-rambo, Bfk and also probably BPR). Among the proapoptotic Bcl-2 family members is a group of death proteins collectively termed the "BH3-only" subfamily (including Bid, Bad, Bik, Bim, Bmf, Noxa, Puma and Hrk in humans) that display sequence similarity only in the BH3 domain. Most multidomain members and several BH3-only proteins contain a hydrophobic segment at their C-termini and, at least to some members (e.g. Bcl-2) this transmembrane domain (TM) is important for subcellular localization and/or activity. Some Bcl-2 family members such as Bcl-2 and Bak are constitutively localized to the mitochondrial membrane, whereas others such as Bax and Bid reside in the cytosol and translocate to the mitochondria during apoptosis. It is worth stressing that Bcl-2 family members have been found in other organelles such as the ER and the nuclear envelope in addition to their localization in mitochondria.

Despite opposite biological functions and wide differences in amino acid sequences, experimentally-determined three-dimensional (3D) structures and secondary structure predictions suggested that all multidomain members share a similar helical bundle structural fold, resembling the pore-forming domains of bacterial toxins [11-16]. Based on this structural analogy, at least four members of the Bcl-2 family have been shown to produce ion-conducting pores in synthetic lipid membranes *in vitro* [17-20]. BH3-only proteins (with the notable exception of Bid) have unrelated predicted secondary structures and probably do not share structural similarity with the pore-forming helical bundle class. Consistently, a recent structural study revealed that Bim, Bmf and Bad belong to the intrinsically unstructured class of proteins [21]. The Bim protein was found to be disordered in absence of interaction partners and to experiment a conformational change in its BH3 region upon binding to a prosurvival member. In contrast, Bid appears unique among the BH3-only proteins because its experimentally determined structure is similar to that of Bcl-2, Bcl-xL and Bax, i.e. multidomain members of the Bcl-2 family [12,22]. Different from other proapoptotic Bcl-2 family members, Bid has evolved unique functions both in apoptosis signalling (in interconnecting the death receptor



**Fig. (2).** Schematic representation of the three types of Bcl-2 family members.

The Bcl-2 family currently includes ~20 proteins (see Table 1), many of which are still awaiting for precise structure-function characterization (e.g. BPR, Bfk and Bcl-G). Multidomain members can be divided into antiapoptotic proteins that possess Bcl-2 homology (BH) domains BH1, BH2, BH3 and BH4, and proapoptotic proteins with three homology domains (BH1-BH3). BH3-only members display sequence conservation only in the third BH domain. Bid and Bim are able to bind Bax or Bak and are termed “activator” BH3-only proteins. The other BH3-only proteins such as Bad, Noxa and Bmf only bind to the antiapoptotic members and are called “sensitizers”.

pathway to the mitochondrial intrinsic pathway) [12,22], and in the control of cell cycle progression [23,24].

### III-2-Mechanisms of Apoptosis Regulation by the Bcl-2 Family

The mitochondrial cell death pathway is thought to be controlled by the relative balance of opposing Bcl-2 family members and their mutual interactions. However, despite extensive investigation, the precise biochemical mechanisms by which the three subgroups of Bcl-2 family members (multidomain antiapoptotic, multidomain proapoptotic and BH3-only proteins) control mitochondrial apoptosis are still unclear. BH3-only proteins function upstream of the multidomain proteins and act as sensors connecting multiple cytotoxic signals to the core apoptotic pathway. Because cells from mice lacking both Bax and Bak do not undergo mitochondrial outer membrane permeabilization in response to apoptosis induction and are resistant to a wide range of cytotoxic stimuli [25,26], these proapoptotic multidomain members appear to be crucial players in intrinsic apoptosis signalling. Mechanistically, Bax and Bak are thought to homo-oligomerize into pores in the mitochondrial outer membrane, thereby causing (either alone or in cooperation with other factors) the leakage of cytochrome C and other proteins sequestered in the mitochondrial intermembrane space. The BH3-only proteins Bid and Bim can directly promote Bax and Bak activation and are therefore called “activators” [27,28]. Other BH3-only proteins such as Bad, Puma and Noxa (termed “sensitizers”) may instead induce apoptosis by releasing Bid and Bim from prosurvival members (e.g. Bcl-2 or Bcl-x<sub>L</sub>). It is likely that antiapoptotic Bcl-2 family members regulate mitochondrial apoptosis not

only via interaction with BH3-only proteins, but also through heterodimerization with Bax and Bak and prevention of their insertion and/or oligomerization within the mitochondrial outer membrane. At the structural level, a hydrophobic groove formed on the surface of prosurvival Bcl-2 proteins by the combination of their BH1, BH2, and BH3 regions is the binding site for the amphipathic,  $\alpha$ -helical BH3 domain of the proapoptotic family members [29,30]. Structure of the Bax protein revealed that the C-terminal hydrophobic helix occupies the BH3-binding pocket [13]. Therefore, displacement of the C-terminal tail from the hydrophobic groove is probably a key event involved in the activation and targeting of Bax to the mitochondrial membrane upon apoptosis induction. Intriguingly, in members such as Bcl-w, Bcl-x<sub>L</sub> and Mcl-1, hydrophobic residues from the C-terminal region also obstruct the hydrophobic BH3 binding groove [16, 31,32]. Altogether, these data suggest that conformational changes of multidomain Bcl-2 family members may be a general mechanism of regulation, functioning aside or in addition to functional homo- and heterodimerizations [33,35].

As a further layer of complexity, proteins from outside the Bcl-2 family have been reported to interact with and modulate the function of all three subfamilies of Bcl-2 proteins. Antiapoptotic multidomain members interact with proteins such as calcineurin, Aven and Raf-1 [36,38], Bax has been shown to bind Ku70, Humanin and ASC [39-41], Bak was found complexed with VDAC2 [42] and the BH3-only proteins Bim and Bmf associate with motor complexes that interact with the cytoskeleton [43-45]. Last, post-translational modifications of Bcl-2 family proteins through proteolysis [46,47] or phosphorylation [48,49] can be

important for their activity, while alternative splicing, which increases functional diversity, is a feature of many Bcl-2-related genes [50].

#### IV-TARGETING BCL-2 FAMILY MEMBERS

##### IV-1-The Bcl-2 Family in Gene- and Protein-Based Drug Development

The *bcl-2* gene, isolated from follicular B-cell lymphoma cells bearing the t14;18 translocation, was one of the first oncogenes to be identified and the first found to function through apoptosis inhibition [51,52]. At present, prosurvival members of the Bcl-2 family are known to be widely expressed in human cancers and are thought to play a significant role in the pathogenesis of cell proliferative diseases and resistance to chemotherapy [9]. It is therefore believed that an effective therapy for cancer may be provided by blocking Bcl-2 or Bcl-x<sub>L</sub>. Additionally, because of the role apoptosis plays in degenerative diseases, modulation of pro-apoptotic members of the Bcl-2 family (mainly Bax and Bid) is becoming therapeutically relevant. A number of breakthrough studies and related patents exemplified the interest in targeting Bcl-2 family genes at the level of gene expression, e.g. through antisense oligonucleotides (part IV-2). Moreover, X-ray/NMR structure determination and biochemical characterization of a number of Bcl-2 family proteins prompted the design of active peptides and their use as tools to interfere with the apoptotic cascades (part IV-3). Synthetic small-molecules inhibitor drugs that bind the BH3 pocket of Bcl-2 proteins, such as Gemin X Pan-Bcl-2 Inhibitor GX15-070, gossypol derivatives, or Abbott's ABT-737, also offer promising new possibilities for cancer treatment, but lie out of the scope of the present paper (see references [53-55] for excellent recent reviews).

As shown in Table 1 [56-76], investigators have been patenting Bcl-2-related gene sequences and corresponding proteins for more than 10 years. Will this trend continue? As many as ~ 2000 proteins are retrieved when a rather conservative BH3 domain signature ([L-(X)<sub>3</sub>-G-D-D/E]) is used to search Uniprot [10], suggesting that additional BH3-only gene sequences may be patented for their technical use and function in the near future. In contrast, according to our predictions, subfamily size for multidomain members is not likely to vary much. This assumption is based on a survey for genes containing a conserved intron within the BH2 domain (i.e. the "GGW\_X" motif characteristic of all multidomain Bcl-2 family members). This computational search based on nucleotides profiles and intron location failed to detect novel Bcl-2 homologues in the human genome (our unpublished results); however, it is possible that distant homologues may be identified using structure prediction tools. Even though no other homologues were retrieved, novel Bcl-2-interacting partners may be identified from functional genomics studies, such as Beclin or scramblase-3 (see WO011256 [77] and WO04081200 [78]; these patents and others are referenced in Table 2) [79-118].

##### IV-2-Nucleic Acid-Based Strategies for Modulation of Mitochondrial Apoptosis

The feasibility of using strategies targeting Bcl-2 was first exposed in a patent owned by John C. Reed more than

ten years ago (WO9427426A1) [79]. This original patent described two opposite situations in which Bcl-2 levels could be modulated. First, in some diseases or pathological conditions resulting in accelerated apoptotic cell death, e.g. stroke or neurodegenerative diseases, it was regarded as desirable to reinforce Bcl-2 protein levels. This strategy was also predicted to be useful in prolonging the *in vivo* survival of transplanted cells. Conversely, decreasing the expression of Bcl-2 in a malignant or virus-infected cell was supposed to enhance sensitivity to therapy (anti-cancer or antiviral treatment). This latter approach has been documented in hundreds of publications and several patents, mainly through two major technologies: antisense oligonucleotides and RNA interference.

##### IV-2-1-Antisense Strategies to Downregulate Bcl-2 Protein Levels

The antisense strategies rely on either introducing a reverse complementary nucleic acid sequence into the target cell, or on expressing a reverse complementary sequence in the target cell from a transfected viral or plasmid vector [119]. Antisense compounds may elicit their therapeutic action *via* a variety of mechanisms, and may be able to combine different biological activities in order to achieve efficient inhibition of mRNA expression. In theory, if hybridization between the target mRNA and the exogenous nucleotide occurs, a duplex is created which disables the translation process by creating a physical block to the ribosomal complex, resulting in translational arrest. The double-stranded RNA is then targeted for destruction inside the cells (this is believed to be a natural antiviral defence, since many viruses contain double-stranded RNA). Alternatively, association of the oligonucleotide to its target (pre-mRNA or RNA) can occur in a region essential for binding of trans-acting factors involved in splicing, or in sites important for polyadenylation, cellular transport, post-transcriptional modifications of nucleosides, capping of the 5'-end, etc., thereby inhibiting translation, maturation or stability of the sense mRNA. In addition, oligonucleotides also support the binding of RNase H at sites of RNA-DNA duplex formation. Such binding is an important effector of antisense action because once bound, RNase H functions as an endonuclease that cleaves the RNA in the duplex. Also of significant interest is that the DNA is undamaged during the enzymatic attack, and free to hybridize successively with multiple RNA molecules. Nevertheless, within the cells, RNA-RNA as well as DNA-RNA duplexes can be unwound by a variety of repair/editing enzymes such as RNA unwinding and helicases. A number of chemical modifications or analogues of anticodon oligomers have been proposed to enhance their *in vitro* and *in vivo* stability. Among them, phosphorothioates [120], methylphosphonates [121], 2'-O-methyl-ribonucleotides [122], N<sup>3</sup>P<sup>5</sup> phosphoramidates [123] and LNA (locked nucleic acids, for a review see [124]) are the more used. While some of these modifications do not alter the potential to stimulate RNase H, many second-generation oligonucleotides fail to activate the enzyme and therefore abrogate this important pathway to antisense action [125].

In 1990, *in vitro* cellular studies were published showing that *bcl-2* expression can be inhibited by antisense oligo-

Table 1. List of Patented Bcl-2 Family Members. NF = Not Found

Gene	Homology Boxes (protein)	Activity	Patent	Year	Title	Applicant/Inventors	
<b>Multidomain</b>							
Bcl-2	BH4, BH3, BH1, BH2, TM	Anti	US5015568 US5202429	1991 1993	Diagnostic Methods For Detecting Lymphomas in Humans; DNA Molecules having Human Bcl-2 Gene Sequences	<i>Tsujimoto, Y. and Croce, C A.</i> <i>Tsujimoto, Y. and Croce, C A.</i>	[56]
Bcl-X, Bcl211	BH4, BH3, BH1, BH2, TM	Anti	WO9500642	1995	Vertebrate Apoptosis Gene: Compositions and Methods	Arch Development Corporation The Regent of the University of Michigan; <i>Thompson, CB., Boise, LH., Nunez, G.</i>	[57]
Bcl-W, Bcl212	BH4, BH3, BH1, BH2, TM	Anti	WO9735971	1997	A Novel Mammalian Gene, Bcl-W, belongs to the Bcl-2 family of Apoptosis-Controlling Genes	Amrad Operations Pty. Ltd.; <i>Cory, S., Adams, JM., Gibson, LM., Holmgreen, SP.</i>	[58]
Bfl-1, Bcl215	BH4, BH3, BH1, BH2, (TM)	Anti	WO9630513	1996	Apoptosis Regulating Gene	Korea Green Cross Corporation Postech Foundation; <i>Shin, HS., Sung, YC., Hong, SI., Choi, SS., Yun, JW., Choi, EK., Park, IC.</i>	[59]
Mcl-1	(BH4), BH3, BH1, BH2, TM	Anti	WO9429330	1994	Myeloid Cell Leukemia Associated Gene MCL-1	The Johns Hopkins University School of Medecine; <i>Craig, RW.</i>	[60]
Nrh, Bcl-B, Bcl2110	(BH4), BH3, BH1, BH2, TM	Anti (Pro)	WO0157060 WO02072601	2001; 2002	Bcl-2-Like Polynucleotides, Polypeptides, and Antibodies; Apoptosis Modulator Bcl-B and Methods for Making and using same	Human Genome Sciences, Inc.; <i>Ruben, SM., Duan, DR., Ni, J.,</i> The Burnham Institute; <i>Reed, JC., Ke, N., Godzik, A.</i>	[61] [62]
Bak1, Bcl217	(BH4), BH3, BH1, BH2, TM	Pro (Anti)	US5656725	1997	Peptide and Compositions which Modulate Apoptosis	<i>Chittenden, TD., Lutz, RJ.</i>	[63]
Bax	(BH4), BH3, BH1, BH2, TM	Pro	US5691179	1997	Cell Death Regulators	<i>Korsmeyer, SJ.</i>	[64]
Bok, Bcl219	(BH4), BH3, BH1, BH2, TM	Pro	WO9924453 WO0157060	1999 2001	Mammalian pro-Apoptotic BOK genes and their uses;  Bcl-2-like Polynucleotides, Polypeptides, and Antibodies	The Board of Trustees of the Leland Stanford Junior University; <i>Hsueh, AJW., Hsu, SY.;</i> Human Genome Sciences, Inc.; <i>Ruben, SM., Duan, DR., NI, J.</i>	[65] [61]
Bcl-G, Bcl2114	BH3, BH2	Pro	WO0157213	2001	Human Bcl-X-Like Proteins and Polynucleotides Encoding the same	Lexicon Genetics Incorporated; <i>Donoho, G., Hilbun, E., Turner, CA., Friedrich, G., Abuin, A., Zambrowicz, B., Sands, AT.</i>	[66]
Bcl-rambo, Bcl2113	(BH3), BH1, BH2, (BHNo), TM	Pro	WO0248353	2002	DNA-Sequences, Which Code for an Apoptosis Signal Transduction Protein	Apoxis SA; <i>Tschopp, J., Hofmann, K.</i>	[67]

(Table 1) Contd....

Gene	Homology Boxes (protein)	Activity	Patent	Year	Title	Applicant/Inventors	
Bfk	BH3, BH2	Pro	WO04037857	2004	BFK Protein As Therapeutic Molecules	The Walter And Eliza Hall Institute Of Medical Research <i>Coultas, L., Strasser, A.</i>	[68]
BPR, Bcl2l12	(BH3), BH2	ND (Pro?)	WO04096991	2004	BCL2L12 Polypeptide Activators And Inhibitors	Dana-Farber Cancer Institute, Inc. <i>Stegh; A., Kim, H., Depinho, RA., Chin, L.</i>	[69]
<b>BH3-only</b>							
Bid	BH3	Pro	WO9809980 WO0157060	1998 2001	BH3 Interacting Domain Death Agonist; BCL-2-Like Polynucleotides, Polypeptides, And Antibodies	Washington University <i>Korsmeyer, S.J.; Human Genome Sciences, Inc.; Ruben, SM., Duan, DR., NI, J.</i>	[70] [61]
Bad, Bcl2l8	BH3	Pro	WO9613614; WO9812328	1996; 1998	Bcl-X/Bcl-2 Associated Cell Death Regulator Human Bad Polypeptides, Encoding Nucleic Acids And Methods Of Use	Washington University; <i>Korsmeyer, S.J.; Idun Pharmaceuticals, Incorporated Horne, WA., Oltersdorf, T.</i>	[71] [72]
Bik	BH3, TM	Pro	WO9950414	1999	BLK Genes, Gene Products And Uses Thereof In Apoptosis	Thomas Jefferson University <i>Alnemri, ES.</i>	[73]
Bim, Bcl2l11	BH3, TM	Pro	WO9914321	1999	Novel Therapeutic Molecules	The Walter And Eliza Hall Institute Of Medical Research; <i>Cory, S., Adams, J., Huang, DCS., O'Connor, L., Strasser, A., Puthalakath, H., O'Reilly, L.</i>	[74]
Bmf	BH3	Pro	WO02097094	2002	Bcl-2-Modifying Factor (BMF) Sequences And Their Use In Modulating Apoptosis	The Walter And Eliza Hall Institute Of Medical Research; <i>Strasser, A., Puthalakath, H., Villunger, A., Coultas, L., Beaumont, J., O'Reilly, LA., Huang, DCS.</i>	[75]
Noxa	BH3	Pro	NF				
Puma	BH3	Pro	NF				
Hrk	BH3, TM	Pro	WO9830101	1998	Gene Therapy Approaches to Target Cell Death for Cancer Treatment: Compositions and Methods	The Regents of the University of Michigan; <i>Clarke, MF., Wicha, M., Nunez, G., Han, J., Inohara, N.</i>	[76]

nucleotides and that phosphorothioates were 10-fold more potent than phosphodiester [126]. In patent (WO 9508350A1) [80] issued five years later, 18 oligomers were described and tested to abrogate *bcl-2* expression. Since then, expression of the *bcl-2* oncogene has been linked to resistance to chemotherapy in a range of cancers [127,128], and it has been demonstrated that administration of a *bcl-2* antisense oligomer could selectively reduce Bcl-2 protein

levels in tumor xenografts and make these tumors more susceptible to chemotherapeutic agents [129]. Some of the oligomers originally described in patent (WO9508350A1) [80] straddle the translation-initiation site of the mRNA coding strand (i.e. have complementary sequences encompassing the ATG initiation codon), while another one (oligo 17 of the patent) corresponds to the 6 first codons of *bcl-2* (18 nucleotides). This latter 18-mer phosphorothioate *bcl-2*

antisense oligonucleotide is now largely known as Genasense® or oblimersen sodium (formerly G3139). Potential use of Genasense® in human therapy has been patented by Genta Incorporated (WO0217852A2 and WO0217852A3 [81]; WO2004056971 [82]). This oligonucleotide has been shown to have antitumor efficacy against human B-cell lymphoma cells bearing the t(14;18) translocation treated *in vitro* prior to inoculation into SCID mice [130]. Clinical trials established single-agent activity of Genasense® in patients with various hematologic malignancies [131]. Moreover, downregulation of Bcl-2 by Genasense® has been reported to enhance the cytotoxicity of several conventional chemotherapeutic agents [132, 133]. A range of clinical programs are now ongoing or have completed accrual to their trials evaluating the effects of Genasense® in addition to standard therapies in both hematologic and solid malignancies (www.genta.com). Genta has submitted a New Drug Application to the Food and Drug Administration for the use of Genasense® plus fludarabine and cyclophosphamide for treatment of patients with relapsed or refractory chronic lymphocytic leukemia. Genta has also completed a Marketing Authorization Application to the European Medicines Agency for use of Genasense® plus dacarbazine for treatment of patients with advanced melanoma. Excitingly, a recent patent by Genta (WO2004046327) [83] described a series of new inhibitory oligonucleotides targeting the *bcl-2* mRNA in 2 additional regions of the coding sequence. Compared to Genasense®, some of these antisense oligonucleotides showed very promising results. For example, so-called “SEQ ID NO: 32” resulted in 72% of tumor growth inhibition after 9 days (compared to 24% for G3139) in an *in vivo* assay with nude mice implanted with human ovarian tumor cells. Using a similar antisense strategy, Tormo *et al.* (WO9814172A1) [84] designed another oligodeoxynucleotide targeting the translation initiation region of *bcl-2* and reported that they were able to effectively treat certain follicular lymphomas by a polynucleotide/phospholipid mix.

As a slightly different approach, the invention of oligomers overlapping the acceptor (SA-AS oligonucleotide) or the donor (SD-AS) splicing regions of the human *bcl-2* mRNA was also introduced (first in the previously mentioned patent WO9508350A1 [80]). Indeed, the human *bcl-2* gene gives rise to several transcripts through alternative splice site selection [134]. The majority of the transcripts are spliced and encode a 26kDa Bcl-2 protein (Bcl-2<sub>β</sub>). One minor transcript, however, does not undergo splicing and consequently encodes a 22kDa protein, Bcl-2<sub>α</sub>, which differs at the C-terminal hydrophobic region. The SD-AS oligodeoxynucleotide can thus potentially block maturation of Bcl-2<sub>α</sub>, which requires splicing, but not of Bcl-2<sub>β</sub>, which is derived from unspliced mRNA. This may be important under physiological conditions where *bcl-2* beta is specifically upregulated, e.g. in mature B cells [135]. While Bcl-2<sub>α</sub> and Bcl-2<sub>β</sub> represent normal alternatively spliced variants of the *bcl-2* gene, exon skipping can occur by disruption of “exonic splicing enhancers”, i.e. sequences present within exons that stimulate messenger RNA splicing. Alternatively, internal structures within the RNA transcript, e.g. stem loop and pseudo knots, can also affect the information flow from transcript to translated protein product.

Abnormal splice variants of *bcl-2* have been recently detected in colorectal carcinomas using RT-PCR by Ming Jiang and Jo Milner (WO2005012357) [85]. The shorter *bcl-2* cDNAs that were amplified did not shift the normal triplet reading frame and all known functional domains of the Bcl-2 protein were maintained. The corresponding proteins were also functional in the suppression of apoptosis. The possible application of this discovery in treatment of cancers has been patented (WO2005012357) [85] with the justification that alternative abnormal variants of Bcl-2 could represent a tumor-related abnormality. Such variants might be suppressed or inhibited through selective binding molecules targeting abnormal RNA structures or differences in protein structure created as a result of abnormal splicing. Like *bcl-2*, the *bcl-x* gene encodes multiple isoforms: a long, anti-apoptotic protein termed Bcl-x<sub>L</sub> and a pro-apoptotic shorter product, Bcl-x<sub>S</sub>. Inhibitors of Bcl-x<sub>L</sub> are potential inducers of apoptosis while inhibitors of Bcl-x<sub>S</sub> should promote apoptosis. Isis Pharmaceuticals patented in 2000 the use of anti-*bcl-x* antisense oligonucleotides to sensitize cancer cells to apoptotic agents (WO0020432A1) [86]. The inventors describe a series of phosphorothioates targeting the 3′- and 5′-UTRs, coding exons 1 and 2, and the translation initiation site, several of them inhibiting *bcl-x* mRNA levels by greater than 85% in cultured cells. Interestingly, some of the antisense oligonucleotides tested were also able to alter the *bcl-x<sub>S</sub>/bcl-x<sub>L</sub>* transcript ratio. Based on these encouraging data, the authors developed chimeric phosphorothioates (“gapmers”) composed of a central region consisting of ten 2′-deoxynucleotides flanked on both sides (5′ and 3′ directions) by five 2′-O-methoxyethyl nucleotides. Because of the strong homology between Bcl-x<sub>L</sub> and Bcl-2, a subset of these oligonucleotides were bispecific and reduced the expression of both proteins by more than 80% in prostate cancer PC3 cells [136]. This effect was correlated with a strong increase in chemosensitivity to paclitaxel and a decrease in IC<sub>50</sub> values by >90%.

An improvement over existing antisense technology has recently been disclosed (WO2005061710) [87]. To enhance stability and cellular uptake of an oblimersen-based sequence, the inventors tested a large series of LNA/DNA gapmers. Gapmers having the formula 5′-[(DNA/RNA)<sub>0-1</sub>-(LNA-LNA\*)<sub>2-7</sub>-(DNA/RNA/LNA\*)<sub>4-14</sub>-(LNA/LNA\*)<sub>2-7</sub>-(DNA/RNA)<sub>0-1</sub>]-3′ (wherein LNA\* designates an LNA analogue nucleotide) and containing at least two LNA or LNA\* linked by a phosphorothioate group represented the best combination and were granted patent protection. In a xenograft model of 518A2 human melanoma cells transplanted into SCID mice, the gapmer corresponding to “SEQ ID 15” showed an effectiveness comparable to that of Genasense® using a 4 times lower dose, the improved efficiency being related to higher stability. Identification of novel target sites on the “druggable” genes of the Bcl-2 family (as published in patent WO2004046327) [83] combined with improvements in oligonucleotide stability (by modifying the oligonucleotide structure or conjugating it to confer more advantageous pharmacokinetic properties) should open new perspectives in cancer treatment. Moreover, although emphasis was put on *bcl-2*, the antisense technology may be used to assess other genes of the Bcl-2 family, different from *bcl-2/x<sub>L</sub>* (e.g. *bax*, *bid*, *mcl-1* and *bfl-*

1) and their roles in diseases. In that respect, antisense oligonucleotides targeted to the genes encoding Bfl-1/A1 and Mcl-1 were claimed in patent (WO0040595A1) [88] and may be useful in treatment of some malignancies [137,138].

#### **IV-2-2-RNA Interference Strategy to Down-Regulate Bcl-2 Protein Levels**

A second, more recent technology based on RNA interference has emerged in the medical and cancer treatment field. RNA interference (RNAi) is a phenomenon in which a double stranded RNA (dsRNA) introduced or naturally expressed in a cell triggers posttranscriptional gene silencing, through sequence-specific degradation of homologous mRNA [139]. Briefly, the presence of long dsRNA (>30 bp) in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short interfering RNA (siRNAs) which are typically 21-27 nucleotides long [140]. An endonuclease complex called RISC (RNA-induced silencing complex) then incorporates an siRNA, and cleaves (through an RNase III-like activity) a target RNA that has a region complementary to the siRNA sequence.

The successful use of siRNA to downregulate gene expression in several model systems has led to many attempts to extend this methodology to potential therapeutic purposes [141], including manipulation of *bcl-2* expression. A patent issued in 2002 first described the use of 3 short dsRNA against the human *bcl-2* gene to initiate cell death in YAP C pancreatic carcinoma cells; however, overall levels of induced cell death were low (WO2055692A2) [89]. Further studies demonstrated efficient downregulation of Bcl-2 protein level and apoptosis induction by other siRNAs targeted at *bcl-2*. For example, siRNAs for *bcl-2* sensitized MCF-7 breast cancer cells to chemotherapeutic agents such as etoposide or doxorubicine [142] and a 19-nt oligonucleotide was shown to effectively kill HeLaB2 and BGC-823 tumor cells and induce significant tumor growth inhibition in mice bearing liver tumors [143]. Based on research done on antisense oligonucleotides, Sirna Therapeutics described a range of chemical modifications that may be useful to fine-tune critical characteristics of siRNA (short interfering nucleic acids), such as increased resistance to nuclease degradation *in vivo* and/or improved cellular uptake (WO03070969A3) [90]. In the same patent application, inventors demonstrated that application of 25 nM siRNA oligonucleotides on A549 human lung carcinoma cells reduced *bcl-2* expression by more than 50 %. Hence, the amount of siRNA oligonucleotides required for specific gene silencing is extremely small compared with antisense DNA (see, for example, [144] for comparison). A siRNA was claimed by Nippon Shinyaku Co. that similarly limited tumor growth on A549 xenografted nude mice after subcutaneous administration to the tumor zone (WO2004106511) [91]. A series of rationally designed siRNA knocking down *bcl-2* expression with remarkable efficacy (and thus termed "hyperfunctional" siRNAs) were disclosed elsewhere (WO2004045543) [92]. Although functional consequences are not addressed in the patent, it is noteworthy that a number of these siRNAs almost completely abolished *bcl-2* expression at 300 pM concentration. Future developments may include the combination of different siRNAs with such very low effective dose, as it

might be more efficient to use a pool of siRNAs directed to a particular target rather than using a single siRNA. Moreover, it would be interesting to target other types of endogenous antagonists of apoptosis (e.g. IAPs) in addition to *bcl-2*-related genes in order to optimize the response of cancer cells towards chemotherapy induced cell death [142]. Last, in addition to siRNA, RNAi can also involve small RNA-mediated gene silencing *via* microRNA (miRNAs). In contrast to siRNAs, miRNAs are coded by endogenous genes. Recent studies have shown that *bcl-2* was the target of *miR-15* and *miR-16* and that downregulation of *bcl-2* by these microRNAs triggered apoptosis. Interestingly, *miR-15* and *miR-16* are frequently deleted or downregulated in chronic lymphocytic leukemia (CLL) [145,146]. Accordingly, use of *miR-15* and *miR-16* in diagnosis of CLL was claimed in patent (WO2004043387) [93]. Blocking the function of miRNAs directed at other genes directly or indirectly involved in control of mito-chondrial apoptosis holds important potential for the understanding and treatment of human disease.

#### **IV-3-Peptide-Based Bcl-2 Family Inhibitors**

Downregulation of gene expression by antisense oligonucleotides or RNAi approaches represent novel, promising strategies to overcome apoptosis resistance in cancer cells. In addition to nucleic acid-based applications, current research projects focus on small peptides to prevent abnormal cell death activation or abnormal cell accumulation.

##### **IV-3-1-BH3 Peptides as Inhibitors of Anti-Apoptotic Bcl-2 Proteins**

An attractive approach for achieving functional inhibition of prosurvival Bcl-2 proteins is to mimic the activity of pro-apoptotic members, and in particular BH3-only proteins. Based on the finding that peptides comprising the BH3 domain of pro-apoptotic Bcl-2 family members act as specific death inducers, Stanley Korsmeyer was first to patent agonistic BH3 peptides from Bad and Bid in 1999 (WO9916787) [94]. Proof-of-concept experiments subsequently conducted at Boston's Dana Farber Cancer Institute provided strong evidence that peptides derived from the BH3 domains of Bid, Bad or Bim can promote Bax/Bak-dependent cytochrome c release from mitochondria of cancer cells. Cell-permeable peptides were disclosed that fuse a D-isomer arginine homopolymer (termed r8) to the BH3 domains of Bid or Bad (WO2004022580) [95]. The r8-BH3 peptides induced apoptosis of neuroblastoma cells *in vitro* and *in vivo* in a mouse xenograft model [147]. Second-generation BH3 peptides with improved pharmacological properties such as protease-resistance, enhanced -helicity, cell-permeability and increased binding potency to Bcl-2 proteins were developed (WO2005044839) [96]. Therein, the inventors envisioned utilizing olefin metathesis strategy for BH3 peptide synthesis and generated Bid BH3 -helices stabilized by an all-hydrocarbon staple (referred to as 'Stabilized Alpha-Helices of Bcl-2 domains' or 'SaHBs'). The Bid-derived SaHB activated apoptosis in leukaemia cell lines and inhibited the growth of human leukaemia xenografts in mice [148].

As with SAHBs, several other drug discovery programs focused their efforts on converting biologically active BH3

peptides into clinically and commercially viable agents. Patent (WO2004058804) [97] provides a set of conformationally-constrained and proteolysis-resistant BH3 peptidomimetics based on the Bim protein. As an example, a conformationally-restrained peptide with a hexane linker tethering two glutamic acid residues is described. This semi-rigid peptide has four times more helicity and displays a 40-fold higher affinity for recombinant Bcl-w than an unconstrained wild-type Bim 12-mer. In patent (WO 2006000034) [98] issued two years later, the inventors envision to conjugate the peptides to moieties that target specific cell surface molecules on cancer cells, in order to minimize unwanted side effects in healthy tissues. There are examples of this approach being used successfully: for instance, tumor cells overexpressing the luteinizing hormone-releasing hormone (LHRH) receptor were killed by a Bak-derived BH3 peptide conjugated to a synthetic LHRH peptide [149]. Furthermore, Xigen SA recently tried to apply for a patent on D-retro-inverso analogues of BH3 peptides (comprising a Tat transduction domain) that caused cytotoxic effects on a variety of tumor cells *in vitro* including cervical cancer and murine insulinoma cell lines (WO2006056370) [99]. Cyclic lactam bridged BH3 peptide analogues with increased helical structure were also reported [150]; however apparently no patent has been filed to cover the invention. Although these compounds represent a significant achievement in BH3 domain engineering, their efficacy remains to be clearly assessed in animal models.

As previously mentioned, one major advantage of the BH3 domain is the apparent flexibility of its consensus signature, which allows rationale design or discovery of various BH3-like peptides with minimum sequence and structure requirements, Fig. (3). A BH3 peptide from human Bax, or closely related sequences designed upon the [AILMV]-[ARK]-[ILMVSTKR]-[ILMV]-G-[DE]-[DE]

pattern were described in patent application (WO0100670) [100]. Similarly, synthetic peptides from human Bad-BH3 or consisting of sequence [Y-(X)<sub>3</sub>-L-(X)<sub>4</sub>-D-X-F-V ] (plus additional flanking residues at each terminus) were developed by Abbott Laboratories (WO0220568) [101]. Such Bad BH3 peptides were shown to fit into the hydrophobic pocket of Bcl-x<sub>L</sub> [29] and this knowledge contributed to the development of a high-throughput fluorescence polarization assay for identifying small molecule Bcl-x<sub>L</sub> inhibitors [151]. (WO2005014638) [102] and (WO 2006082304 [103] published in French) disclose a similar fluorescence polarization assay [152] for screening inhibitors of peptide-protein interactions involving BH3-like peptides and Bcl-2, Bcl-x<sub>L</sub> and/or Bcl-w. In these patents, the interacting peptides were identified using the yeast two-hybrid system and found to have the consensus sequence [(X)<sub>8</sub>-L-(X)<sub>4</sub>-D-(X)<sub>10</sub>]. The Leu and Asp residues lying within this consensus sequence are equivalent to Leu<sup>151</sup>/Asp<sup>156</sup> on Bad BH3 domain [29], Leu<sup>78</sup>/Asp<sup>83</sup> on the Bak protein [30] and similar amino acid pairs on Bim (Leu<sup>94</sup>/Asp<sup>99</sup>) [153] and Bid (Leu<sup>14</sup>/Asp<sup>19</sup>) [154], i.e. BH3 amino acids which closely contact the hydrophobic groove of Bcl-x<sub>L</sub>, Fig. (4). Interestingly, a peptide sequence from Beclin (GTMENLSRRLKVTGDLFDIMSGQTDV), a protein required for autophagy, was identified through a similar yeast two-hybrid screen as a Bcl-2-interacting partner and was found to exhibit identical residues (WO 2006082303) [104]. There was also a similar case in patent (WO2006062173) [105] where a peptide having the aminoacid sequence YQVARM<sup>LRRVADQMAS</sup> is reported to compete *in vitro* with Bad in binding to Bcl-x<sub>L</sub>, providing further evidence that this minimal BH3 signature can serve as an efficient template for drug discovery. As stated in patent (WO2006034454) [106], a next step could be the design of agents with improved specificity for anti-apoptotic Bcl-2 proteins, i.e. that can discriminate against the BH3-

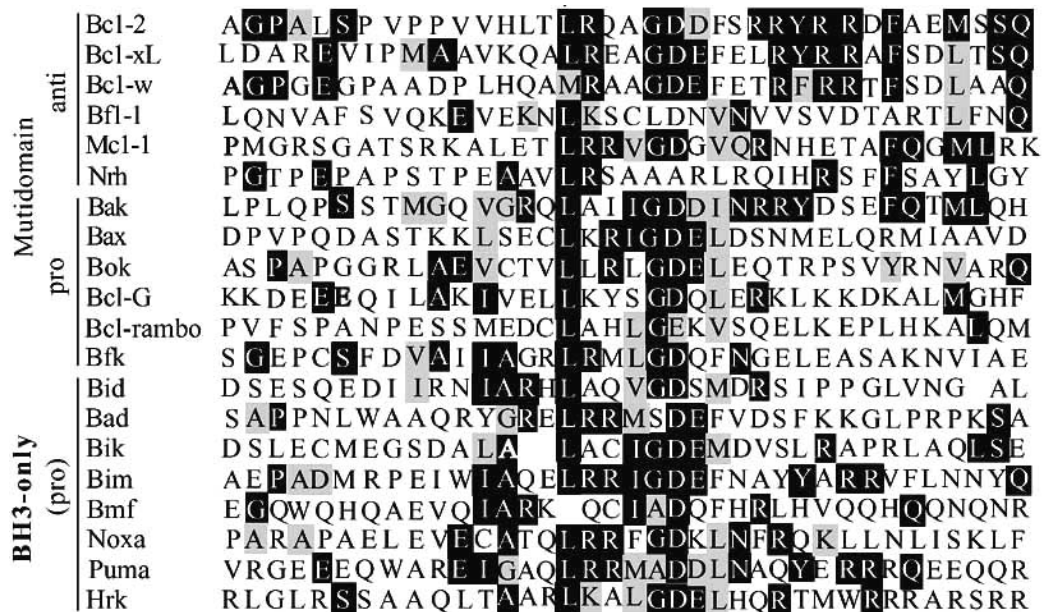
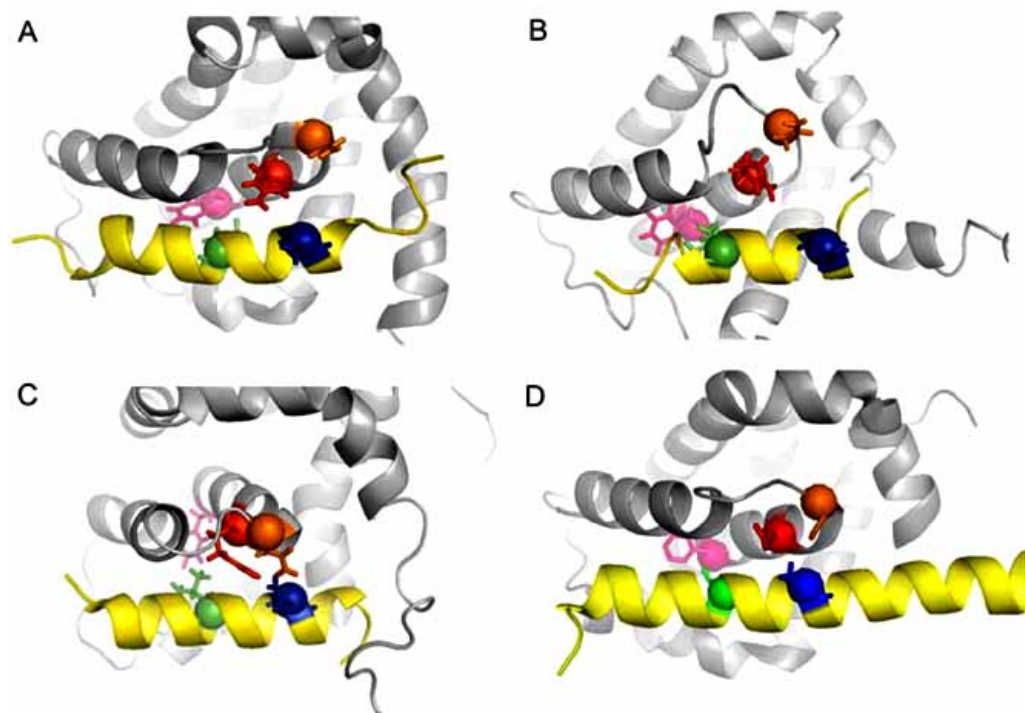


Fig. (3). Alignment of the BH3 domains of multidomain and BH3-only Bcl-2 family members.

Asterisks indicate the Leu and Asp conserved positions discussed in the text. These residues are important for contacting a hydrophobic groove on antiapoptotic Bcl-2 family members (Figure 4).



**Fig. (4). BH3-centric view of the binding between BH3 peptides and full-length multidomain prosurvival Bcl-2 family proteins.**

Isolated BH3 peptides bind to the hydrophobic groove formed by the BH1, BH2 and BH3 domains of Bcl-xL and Bcl-w. The ribbon representations show the Bcl-xL/Bad (A), Bcl-xL-Bak (B), Bcl-w/Bid (C) and Bcl-xL/Bim (D) complexes. The Leu and Asp conserved positions discussed in the text (see also Figure 3) are colored in green and blue, respectively. The Asn, Arg and Phe residues conserved in the BH1 domains of Bcl-xL (NWGRIVAFFSL) and Bcl-w (NWGRLVAFFVL) and that contribute to binding to BH3 peptides are depicted for information. Orange: Asn; Red: Arg; Pink: Phe. Structural data indicate that other residues on Bcl-xL/Bcl-w are important as well for interacting with BH3 peptides [29,30,90,91]. Note that the different BH3 helices are rotated slightly within the binding pockets of Bcl-xL/w when the distances between labelled residues are compared.

binding pocket of pro-apoptotic members such as Bak or Bax, and that may be used for treating diseases involving apoptosis inhibition while reducing potential side effects.

#### ***IV-3-2-Strategies Directed at Post-Translational Events Involving Bcl-2 Family Members***

##### **Modulation of Bax Translocation**

The apoptotic protein Bax normally resides in the cytosol of healthy cells. In response to death stimuli, Bax undergoes a conformational change and translocates to mitochondrial membranes. Several proteins have been shown to interact directly with Bax and promote Bax inactivation in the cytosol. The DNA repair factor Ku70 is one such protein which prevents Bax from relocating to mitochondria and inducing apoptosis [155,156]. Under normal growth conditions, Ku70 in its unacetylated form sequesters Bax in the cytosol, and cell damage causes Ku70 acetylation (by CBP or PCAF) and Bax release. Ku70 suppression of the Bax-mediated cell death pathway offers new perspectives for the control of inappropriate apoptosis, as exemplified by patent (WO03074667) [107] and [41]. In this invention, cell-permeable peptides based on the Ku70-Bax inhibiting domain (VPMLKE) are disclosed that inhibit the interaction of Bax and Ku70 in a dose-dependent manner and prevent Bax-mediated apoptosis. Furthermore, in an invention by Sinclair and Cohen (WO2006007411) [108], methods and compositions for modulating Ku70 acetylation are provided.

Combining these agents with CBP or PCAF inhibitors is an attractive strategy for reducing apoptosis, although specific and potent inhibitors have yet to be identified and characterized for both enzymes.

Humanin (a 24-aminoacid secreted peptide) is another factor that blocks Bax translocation to mitochondria and subsequent cell death [40]. Humanin also suppresses apoptosis by binding to Bid or BimEL (an isoform of Bim) and preventing Bax activation induced by these BH3-only proteins [157, 158]. Methods were reported by the Burnham Institute for identifying compounds that may modulate the Humanin/Bax (or Bid) interaction (patent WO03046205) [109]. In addition, the invention provides a mitochondrial-derived Humanin polypeptide and antibodies that bind to Humanin for therapeutic and diagnostic purposes. Curagen Corporation is also applying for a patent covering novel humanin-like polypeptides (WO2006019365) [110]. As Humanin was reported to prevent neuronal cell death in Alzheimer disease [159], -amyloid toxicity in human cerebrovascular smooth muscle cells [160] and prion-peptide toxicity in rat cortical neurons, Humanin derivatives may be of great interest for the treatment of neurodegenerative diseases. However, the risk of developing cancer should be adequately assessed because up-regulation of the mitochondrial 16S mRNA (which encodes Humanin) was suggested in some malignancies [161].

Last, Bax itself carries regulatory sequences controlling the exposition of its membrane-targeting domains, some of which represent potential targets for selective cytoprotection or cytotoxicity. In particular, Bax possess an amino-terminal sequence termed ART (for apoptosis-regulating targeting sequence) that prevents Bax insertion into the outer mitochondrial membrane in cells that are not committed to die [162]. (WO0020446) [111] published in 1999 relates to a Bax protein lacking an ART domain and its use to induce apoptosis of cancer cells. Conversely, the invention features a method for identifying potentially cell-protective compounds that specifically bind to the ART domain and inhibit Bax insertion into the mitochondrial membrane. In the same line of idea, N-terminal cleavage of Bax by calpain has been shown to produce a truncated molecule of 18 kDa with enhanced pro-apoptotic activity [163]. Use of the resulting Bax p18 fragment, which contains the ART sequence, was claimed for killing tumor cells (WO0203921) [112]. Similarly, researchers at Keio University filed a patent application claiming His-Tat-ART peptides (containing the minimal ART sequence PTSSEQIM) and their use to suppress the translocation of Bax to mitochondria (WO2004113367) [113].

#### Changes in the Phosphorylation State of Bcl-2 Family Proteins

Several phosphatases such as protein phosphatase 1 alpha (PP1), PP2A and calcineurin have been shown to interact with Bcl-2 [37,164,165]. Bcl-x<sub>L</sub> and Bcl-w, the two closest homologues of Bcl-2, also bind to PP1 [166,167], as does the BH3-only protein Bad [166,168]. PP1 has been reported to be a Bad phosphatase in a variety of cytokine-dependent cell lines and, consistent with a role for Bad as a sensitizing BH3-only protein, dephosphorylation of Bad by PP1 may allow it to interact with the prosurvival Bcl-2 family members and initiate the mitochondrial cell death pathway [169,170]. Researchers at the Institut Pasteur identified PP1-binding motifs within the BH3 and BH1 domains of the Bcl-2/x<sub>L</sub>/w proteins and within the Bad BH3 domain. A set of peptides were disclosed which mimic these motifs and inhibit the interactions between the catalytic subunit of PP1 (PP1c) and Bcl-x<sub>L</sub>/Bcl-w, or interfere with the formation of the Bcl-2/Bad/PP1c trimolecular complex (patent WO03096022) [114]. In a follow-up study, a cell-penetrating peptide derived from the Bad PP1c-docking sequence was shown to promote apoptosis in tumor cell lines [171].

PP2A is another phosphatase that has been involved in the control of the Bcl-2/Bad phosphorylation status. PP2A induces dephosphorylation and inactivation of Bcl-2 [164]. In addition, PP2A dephosphorylates Bad upon growth factor withdrawal, leading to inhibition of Bcl-2 and apoptosis [172, 173]. Therefore, PP2A activates Bad and inhibits Bcl-2, and constitutes an attractive target for induction of apoptosis in cancer cells that rely on Bcl-2 or specific growth factors for their survival. A distinct patent application (WO04035611) [115] filed by the Institut Pasteur describes Bcl-2-derived sequences able to associate with PP2A. These peptides were obtained by using the SPOT synthesis method as originally described by Frank and Overwing in 1996 (reviewed in [174]). Interesting for both PP1 and PP2A

inhibition, an assay for measuring the amounts of Bcl-2/Bad complexes was described in patent application (WO04087887) [116] and might prove useful for patient monitoring purposes.

Lastly, proapoptotic activity of BAD is decreased by phosphorylation (at different sites including Ser-112 and Ser-116) and subsequent sequestration by 14-3-3 proteins [175,176]. Taken together with the previous data on Bcl-2 and Bad dephosphorylation, these findings suggest that the combined use of inhibitors of the ERK or PI3K/Akt cascades (that lead to BAD phosphorylation) and activators of protein phosphatase 1/2 may be a potential anticancer strategy.

#### **IV-4- NON-BH3 ACTIVE PEPTIDES DERIVED FROM BCL-2 FAMILY PROTEINS**

##### *IV-4-1-1-Pro-Apoptotic Peptides*

The BH3-only proteins Noxa and Puma have been identified as transcriptional targets of the tumor suppressor protein p53 [177,178]. Recent studies in mice suggested that Noxa is a major effector of p53-mediated cell death [179-181]. In "Cell-killing peptide" (patent WO06001582), the inventor describes a polyarginine transduction domain fused to a KLLNLISKLF peptide (r8-MTD). This sequence present at the carboxy-terminus of the Noxa protein has been reported to function as a mitochondria targeting domain [182]. The r8-MTD peptide was shown to display clear cytotoxic effects in a range of cancer cell lines at 10 μM concentration and was more efficient at killing HeLa cells than TRAIL or cisplatin. It is worth noting that the KLLNLISKLF helix is clearly amphipathic, with both charged and hydrophobic residues; therefore, although the mode of action of CKP is unclear, it is likely to involve Bcl-2 family independent mechanisms, such as non-selective destabilization of the mitochondrial outer membrane, and subsequent release of apoptogenic proteins.

##### *IV-4-1-2-Anti-Apoptotic Peptides*

The BH4 domain is well conserved among mammalian anti-apoptotic members of the Bcl-2 family, and interacts principally with proteins outside of the Bcl-2 family such as Raf-1, calcineurin and paxillin [183-186]. Bcl-2 lacking the BH4 motif is no longer anti-apoptotic [120], and proteolytic cleavage of Bcl-2 by caspase-3 or calpain produces a fragment deleted of the BH4 region which shows proapoptotic activity [187,188]. Tsujimoto's group showed that the BH4 domain of Bcl-x<sub>L</sub> was able to close VDAC, an outer mitochondrial channel, and that HIV Tat-conjugated BH4 peptides protected HeLa cells from etoposide-induced apoptosis [189]. Accordingly, the group applied for a patent on BH4-fused synthetic peptides and their use in treating degenerative diseases (WO0148014) [118]. Tat-BH4 peptides were shown to be readily internalized in a variety of cells, to prevent apoptosis *in vitro* and *in vivo* in several animal models including ischemia/reperfusion, DNA damage and sepsis-induced T-cell apoptosis [190-193]. Thus, although less extensively studied than the BH3 suicide domain, the BH4 domain has demonstrated important functions in the regulation of prosurvival Bcl-2 proteins, and may prove useful in treating diseases characterized by excessive apoptosis. However, the BH4 domain has been less clearly defined than the other BH domains, which

**Table 2. Selection of Relevant Patents Covering Genes, Proteins and Methods Related to Bcl-2 Family Proteins. NF = Not Found**

	Patent	Year	Title	Applicant/Inventors	Cells/tissues	Reference No.
<b>Nucleic acid based strategy</b>						
<i>Antisense against Bcl-2-related genes</i>						
	WO9427426A1	1994	Methods of Using Bcl-2 for the Therapeutic Treatment And Prevention of Diseases	La Jolla Cancer Research Foundation; <i>Reed, JC.</i>	PC12, 32D, IMR-5, B50	[79]
	WO9508350A1	1995	Regulation of Bcl-2 Gene Expression	Reed, JC; <i>Reed, JC.</i>	697, SH-DHL-4, 32D, RS11846, DOHH2, MCF-7	[80]
	WO0217852A2 WO0217852A3	2002	Methods of Treatment of A Bcl-2 Disorder Using Bcl-2 Antisense Oligomers	Genta Incorporated <i>Warrel, RP; Klem, RE., Fingert, H.</i>	Melanoma metastases, skin, lung, liver	[81]
	WO04056971	2004	Methods of Treatment of a Bcl-2 Disorder Using Bcl-2 Antisense Oligomers	Genta Incorporated <i>Warrel, RP.</i>	Melanoma metastases, skin, lung, liver	[82]
	WO04046327	2004	Inhibitory Oligonucleotides Targeted to Bcl-2	Genta Salus Llc ; <i>Chen, Z., Ruffner, DE., Prakash, R., Koehn, R.</i>	A-2780R, PC-3	[83]
	WO9814172A1	1998	Inhibition of Bcl-2 Protein Expression by Liposomal Antisense Oligodeoxynucleotides	Board of Regents, The University of Texas System; <i>Tormo, M., Tara, AM., Lopez-Berenstein G., McDonnel, TJ.</i>	Johnson, Jurkat, Raji, Daudi, LNCaP, NIH3T3, RKO	[84]
	WO05012357	2005	Bcl-2 Splicing Variants	The University of York; <i>Jiang, M.</i>	LoVo, SW48, HCT116	[85]
	WO0020432A1	2000	Antisense Modulation of Bcl-X Expression	ISIS Pharmaceuticals, Inc.; <i>Bennett, CF; Dean, NM., Monia, BP., Nickoloff, BJ., Zhang, Q.</i>	SEM-K2 (leukemia xenograft), HUVEC, A549, HeLa, T-24, HEK, NHDF	[86]
	WO05061710	2005	Oligomeric Compounds for the Modulation of Bcl-2	Santaris Pharma A/S; <i>Frieden, M., Hansen, JB., Ørum, H., Westergaard, M., Thruue, CA.</i>	15PC3, HeLa, 518A2 (melanoma xenograft), PC3 (prostate xenograft)	[87]
	WO0040595A1	2000	Antisense Modulation of Novel Anti-Apoptotic Bcl-2-Related Proteins	ISIS Pharmaceuticals, INC.; <i>Ackermann, EJ., Bennett, CF., Dean, NM., Marcusson, EG.</i>	SEM-K2 (leukaemia xenograft), C8161	[88]
<i>RNA interference</i>						
	WO02055692A2	2002	Method for Inhibiting the Expression of a Target Gene and Medicament for Treating a Tumor Disease	Ribopharma AG; <i>Kreutzer, R., Limmer, S., Vornlocher, HP., Hadwiger, P., Geick, A., Ocker, M., Herold, C., Schuppan, D.</i>	YAP-C	[89]

(Table 2) Contd....

	Patent	Year	Title	Applicant/Inventors	Cells/tissues	Reference No.
	WO03070969A3	2003	RNA Interference Mediated Inhibition of Bcl2 Gene Expression Using Short Interfering Nucleic Acid (SINA)	Sirna Therapeutics, Inc. <i>Mcswiggen, J., Beigelman, L.</i>	A549	[90]
	WO04106511	2004	Oligo Double-Stranded RNA Inhibiting the Expression of Bcl-2 and Medicinal Composition Containing the same	Nippon Shinyaku CO., Ltd. <i>Yano, J., Hirabayashi, K., Nakagawa, S.</i>	NF	[91]
	WO04045543	2004	Functional and Hyperfunctional SIRNA	Dharmacon, Inc. ; <i>Anastasia, K., Angela, R., Devin, L., William, M., Stephen, S.</i>	HEK293, DU145, HeLa	[92]
	WO04043387	2004	Compositions And Methods For Cancer Diagnosis And Therapy	Thomas Jefferson University; <i>Croce, CM., Calin, GA.</i>	CLL, expression in kidney, prostate, liver, skeletal muscle, bone marrow, leukaemia cells	[93]
<b>Peptide-based strategy</b>						
<i>BH3-like peptides</i>						
	WO9916787	1999	Cell Death Agonists	Washington University <i>Korsmeyer, SJ.</i>	MEF, FL5.12, 2B4	[94]
	WO04022580	2004	Bh3 Peptides And Method of Use Thereof	Dana-Farber Cancer Institute, Inc.; <i>Korsmeyer, SJ., Letai, A.</i>	FL5.12, Jurkat	[95]
	WO05044839	2005	Stabilized Alpha Helical Peptides and uses thereof	Dana-Farber Cancer Institute, Inc.; <i>Walensky, LD., Korsmeyer, SJ., Verdine, G.</i>	Jurkat, REH, MV4;11 leukemia xenograft, SEM-K2 leukemia xenograft	[96]
	WO04058804	2004	Peptides and therapeutic uses thereof	Walter and Eliza Hall Institute of Medical Research; <i>Baell, J., Huang, D., Smith, B.J., Street, IP.</i>	Lymphocytes, D3, DoHH2	[97]
	WO06000034	2006	Conjugates and therapeutic uses thereof	The Walter and Eliza Hall Institute of Medical Research; <i>Baell, J., Wei, AH., Scanlon, DB.</i>	Lymphocytes, D3, DoHH2	[98]
	WO06056370	2006	Fusion Protein Comprising a Bh3-Domain of a BH <sub>3</sub> -Only Protein	Xigen S.A.; <i>Bonny, C., Coquoz, D.</i>	HeLa, TC-3, C33A	[99]
	WO0100670	2001	BH3 Modified Peptides	Societe de Conseils de Recherches et D'Applications Scientifiques, S.A.S.; <i>Morgan, BA., Prevost, G., Cotter, TG., Finnegan, N.</i>	PC3, DU145	[100]

(Table 2) Contd....

	Patent	Year	Title	Applicant/Inventors	Cells/tissues	Reference No.
	WO0220568	2002	Mutant peptides derived from Bad and their use to identify Substances which bind to a Member of the Bcl-2 Family of Proteins	Abbott Laboratories; <i>Fesik, SW., Meadows, RP., Joseph, MK., Olejniczak, ET., Petros, AM., Nettesheim, DG., Swift, KM., Matayoshi, E., Zhang, H.</i>	NF	[101]
	WO05014638	2005	Novel Peptide interacting with Anti-Apoptotic Proteins of a Bcl-2 Family	LES Laboratoires Servier Hybrigenics ; <i>Geneste, O., Hickman, J., Bennett, R., Rain, JC.</i>	NF	[102]
	WO06082304	2006	Novel Peptides which interact with Anti-Apoptotic Members of The Family of Bcl-2 Proteins and use thereof	Les Laboratoires Servier Hybrigenics; <i>Geneste, O., Hickman, J., Bennett, R., Rain, JC.</i>	NF	[103]
	WO06082303	2006	Motif of Beclin Protein which Interacts with Anti-Apoptotic Members of the Family of Bcl-2 Proteins and use thereof	LES Laboratoires Servier Hybrigenics; <i>Geneste, O., Hickman, J., Bennett, R., Rain, JC.</i>	NF	[104]
	WO06062173	2006	Bcl-XI Heterodimer Inhibitory Peptide Enhancing The Effect Of Anticancer Agent And Method Of Screening The Same	Keio University; <i>Yanagawa, H., Tsuji, T.</i>	NF	[105]
	WO06034454	2006	Bcl-2 Family Member And Bh-3 Only Proteins For Use In Development Of Peptidomimetics	The Trustees Of The University OF Princeton <i>Shi, Y.</i>	NF	[106]
<i>Bax mitochondrial translocation</i>						
	WO03074667	2003	Ku-70-Derived Bax-Suppressing Peptides And Use Thereof For The Protection Of Damaged Cells	Blood Center Research Foundation; <i>Matsuyama, S.</i>	HEK293T, MCF-7, U87-MG, LNCaP, MEFs	[107]
	WO06007411	2006	Methods And Compositions For Modulating Bax-Mediated Apoptosis	President And Fellows Of Harvard College; <i>Sinclair, DA., Cohen, HY.</i>	HEK293T, MEFs	[108]
	WO03046205	2003	Methods For Identifying Modulators Of Apoptosis	The Burnham Institute; <i>Reed, JC., Guo, B.</i>	CSM14.1, HEK293T, HCT116, COS7, SF268	[109]
	WO06019365	2006	Novel Humanin-Like Polypeptides And Polynucleotides And Their Methods Of Use	Curagen Corporation; <i>Ramesh, K., Xiaozhong, Q.</i>	PC12	[110]
	WO0020446	2000	Bax-Mediated Apoptosis Modulating Reagents And Methods	Mcgill University; <i>Shore, GC., Goping, IS.</i>	NF	[111]
	WO0203921	2002	Bax Fragment Induced Tumor Cell Death	University Of South Florida; <i>Dou, P., Gao, G.</i>	Jurkat, MCF-7	[112]
	WO04113367	2004	Peptide having Apoptosis-Inhibiting Activity	Keio University; <i>Imoto, M., Tabe, H., Tashiro, E., Ohmori, Y.</i>	HEK293T, HeLa	[113]

(Table 2) Contd....

Patent	Year	Title	Applicant/Inventors	Cells/tissues	Reference No.
<i>Bcl-2/Bad phosphorylation</i>					
WO03096022	2003	Screening for peptides inhibiting Pp1c Binding to Bcl-2, Bcl-Xl and Bcl-W Proteins	Institut Pasteur; Garcia, A., Cayla, X., Rebollo, A.	Ts1, COS	[114]
WO04035611	2004	Peptides binding the Phosphatase 2a Protein and Polynucleotides Encoding Same	Institut Pasteur; Garcia, A., Dessauge, F., Cayla, X., Rebollo, A.	NF	[115]
WO04087887	2004	Intracellular complexes as biomarkers	Monogram Biosciences, Inc. Singh, S., Badal, MY., JIN, X., Salimi-Moosavi, H.	MCF-7	[116]
<i>Other peptides</i>					
WO06001582A1	2006	Cell-killing peptide	Chosun University Kim, TH.	HeLa, HCT116, MCF-7 breast cancer xenograft	[117]
WO0148014	2001	BH4-fused polypeptides	Shionogi & Co., Ltd. Shimizu, S., Tsujimoto, Y.	NF	[118]

renders rationale design of BH4-like peptides more difficult. In addition, lack of knowledge about the structural and sequence determinants for interaction with regulatory proteins outside of the Bcl-2 family may be an obstacle for the development and application of BH4 mimetics.

#### CURRENT & FUTURE DEVELOPMENTS

This report provided an assessment of patent activity surrounding the Bcl-2 family of apoptotic regulators, including nucleic acid- and peptide-based strategies (see Table 2 for a summary). Although the antisense molecule Genasense<sup>®</sup>, the most advanced candidate in the clinical pipeline, has moved into phase 3, there are only few nucleic acid- and peptide-based biotherapeutics related to the Bcl-2 family currently in preclinical studies (compared to chemical compounds). Though it is too early to compare the two approaches and to estimate which will offer the best solution for a given application, both have to deal with some fundamental problems such as cellular uptake, tissue delivery, stability, etc. More years of investigation will be needed before the place of these strategies in patient-tailored therapies can be fully defined.

A number of points regarding the Bcl-2 family suggest avenues for future research. First, future work should also aim at developing drugs targeting selected members of the Bcl-2 family, as recently initiated for the multidomain antiapoptotic subfamily [194]. In that respect, BH3-derived peptides are strong candidates for selective inhibition since specific BH3-only proteins were shown to have differing specificities for prosurvival members [28, 195]. Moreover, because membrane association and/or integration are critical for the function of Bcl-2 family multidomain members, the development of innovative drugs may be hampered by the lack of structural information about their membrane-bound or membrane-inserted conformation. The design of peptides

able to interfere with the activity of Bcl-2 proteins at the membrane level, by inhibiting membrane translocation (i.e. conformational changes), integration or oligomerization, is worthy of further attention, because it could give fine control over permeabilization of the outer mitochondrial membrane. Last, it will be useful to elucidate precisely the role of the different isoforms of the Bcl-2 family genes as isoform-specific inhibitors may be of interest for treating some particular diseases.

#### ACKNOWLEDGEMENTS

AA is recipient of a fellowship from the Centre National de la Recherche Scientifique (CNRS). This work was supported by the Association pour la Recherche sur le Cancer (ARC), the Ligue Nationale Contre le Cancer and the Région Rhône-Alpes. Patents were searched through the WIPO website (<http://www.wipo.int/pctdb/en/>).

#### REFERENCES

- [1] Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002, 2: 647-56.
- [2] Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000, 100: 57-70.
- [3] Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004, 116: 205-19.
- [4] Liu X, Kim CN, Yang J, Jemerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996, 86: 147-57.
- [5] Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome C-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000, 102: 33-42.
- [6] Verhagen AM, Ekert PG, Pakusch M, *et al.* Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000, 102: 43-53.
- [7] Jiang X, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 2004, 73: 87-106.
- [8] Suzuki Y, Imai Y, Nakayama H, *et al.* A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001, 8: 613-21.

- [9] Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003, 22: 8590-8607.
- [10] Aouacheria A, Brunet F, Gouy M. Phylogenomics of life-or-death switches in multicellular animals: Bcl-2, BH3-Only, and BNip families of apoptotic regulators. *Mol Biol Evol* 2005, 22: 2395-2416.
- [11] Woo JS, Jung JS, Ha NC, *et al.* Unique structural features of a BCL-2 family protein CED-9 and biophysical characterization of CED-9/EGL-1 interactions. *Cell Death Differ* 2003, 10: 1310-19.
- [12] Chou JJ, Li H, Salvesen GS, Yuan J, Wagner G. Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell* 1999, 96: 615-24.
- [13] Suzuki M, Youle RJ, Tjandra N. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 2000, 103: 645-54.
- [14] Huang Q, Petros AM, Virgin HW, Fesik SW, Olejniczak ET. Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. *Proc Natl Acad Sci USA* 2002, 99: 3428-33.
- [15] Huang Q, Petros AM, Virgin HW, Fesik SW, Olejniczak ET. Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. *J Mol Biol* 2003, 332: 1123-30.
- [16] Denisov AY, Madiraju MS, Chen G, *et al.* Solution structure of human BCL-w: modulation of ligand binding by the C-terminal helix. *J Biol Chem* 2003, 278: 21124-28.
- [17] Antonsson B, Conti F, Ciavatta A, *et al.* Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997, 277: 370-72.
- [18] Minn AJ, Velez P, Schendel SL, *et al.* Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 1997, 385: 353-57.
- [19] Schendel SL, Azimov R, Pawlowski K, *et al.* Ion channel activity of the BH3 only Bcl-2 family member, BID. *J Biol Chem* 1999, 274: 21932-36.
- [20] Schlesinger PH, Gross A, Yin XM, *et al.* Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc Natl Acad Sci USA* 1997, 94: 11357-62.
- [21] Hinds MG, Smits C, Fredericks-Short R, *et al.* Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death Differ* 2006 [Epub ahead of print].
- [22] McDonnell JM, Fushman D, Milliman CL, Korsmeyer SJ, Cowburn D. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell* 1999, 96: 625-34.
- [23] Zinkel SS, Hurov KE, Ong C, *et al.* A role for proapoptotic BID in the DNA-damage response. *Cell* 2005, 122: 579-91.
- [24] Kamer I, Sarig R, Zaltsman Y, *et al.* Proapoptotic BID is an ATM effector in the DNA-damage response. *Cell* 2005, 122: 593-603.
- [25] Lum JJ, Bauer DE, Kong M, *et al.* Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 2005, 120: 237-48.
- [26] Wei MC, Zong WX, Cheng EH, *et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001, 292: 727-30.
- [27] Letai A, Bassik MC, Walensky LD, *et al.* Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2002, 2: 183-92.
- [28] Kuwana T, Bouchier-Hayes L, Chipuk JE, *et al.* BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005, 17: 525-35.
- [29] Petros AM, Nettesheim DG, Wang Y, *et al.* Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Sci* 2000, 9: 2528-34.
- [30] Sattler M, Liang H, Nettesheim D, *et al.* Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 1997, 275: 983-86.
- [31] Day CL, Chen L, Richardson SJ, *et al.* Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. *J Biol Chem* 2005, 280: 4738-44.
- [32] Hinds MG, Lackmann M, Skea GL, *et al.* The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity. *EMBO J* 2003, 22: 1497-1507.
- [33] Dlugosz PJ, Billen LP, Annis MG, *et al.* Bcl-2 changes conformation to inhibit Bax oligomerization. *EMBO J* 2006, 25: 2287-96.
- [34] Jeong SY, Gaume B, Lee YJ, *et al.* Bcl-x(L) sequesters its C-terminal membrane anchor in soluble, cytosolic homodimers. *EMBO J* 2004, 23: 2146-55.
- [35] Zhang Z, Lapolla SM, Annis MG, *et al.* Bcl-2 homodimerization involves two distinct binding surfaces, a topographic arrangement that provides an effective mechanism for Bcl-2 to capture activated Bax. *J Biol Chem* 2004, 279: 43920-28.
- [36] Chau BN, Cheng EH, Kerr DA, Hardwick JM. Aven, a novel inhibitor of caspase activation, binds Bcl-xL and Apaf-1. *Mol Cell* 2000, 6: 31-40.
- [37] Shibasaki F, Kondo E, Akagi T, McKeon F. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. *Nature* 1997, 386: 728-31.
- [38] Wang HG, Rapp UR, Reed JC. Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* 1996, 87: 629-38.
- [39] Ohtsuka T, Ryu H, Minamishima YA, *et al.* ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol* 2004, 6: 121-28.
- [40] Guo B, Zhai D, Cabezas E, *et al.* Humanin peptide suppresses apoptosis by interfering with Bax activation. *Nature* 2003, 423: 456-61.
- [41] Sawada M, Hayes P, Matsuyama S. Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. *Nat Cell Biol* 2003, 5: 352-57.
- [42] Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ. VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* 2003, 301: 513-17.
- [43] Bouillet P, Strasser A. BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci* 2002, 115: 1567-74.
- [44] Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999, 3: 287-96.
- [45] Puthalakath H, Villunger A, O'Reilly LA, *et al.* Bmf, a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001, 293: 1829-32.
- [46] Clem RJ, Cheng EH, Karp CL, *et al.* Modulation of cell death by Bcl-XL through caspase interaction. *Proc Natl Acad Sci USA* 1998, 95: 554-59.
- [47] Fadeel B, Hassan Z, Hellstrom-Lindberg E, *et al.* Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. *Leukemia* 1999, 13: 719-28.
- [48] Blagosklonny MV. Unwinding the loop of Bcl-2 phosphorylation. *Leukemia* 2001, 15: 869-74.
- [49] del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997, 278: 687-89.
- [50] Akgul C, Moulding DA, Edwards SW. Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications. *Cell Mol Life Sci* 2004, 61: 2189-99.
- [51] Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988, 335: 440-42.
- [52] Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 1985, 228: 1440-43.
- [53] Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Can.* 2005, 5: 876-85.
- [54] Letai A. Pharmacological manipulation of Bcl-2 family members to control cell death. *J Clin Invest* 2005, 115: 2648-55.
- [55] Walensky LD. BCL-2 in the crosshairs: tipping the balance of life and death. *Cell Death Differ* 2006, 13: 1339-50.
- [56] Tsujimoto, Y, Croce, C.A.: US5015568 (1991) and US5202429 (1993).
- [57] Thompson, C.B., Boise, L.H., Nunez, G.: WO9500642 (1995).
- [58] Cory, S., Adams, J.M., Gibson, L.M., Holmgreen, S.P.: WO9735971 (1997).
- [59] Shin, H.S., Sung, Y.C., Hong, S. II. Choi, S.S., Yun, J.W., Choi, E.K., Park, I.C.: WO9630513 (1996).
- [60] Craig, R.W.: WO9429330 (1994).
- [61] Ruben, S.M., Duan, D.R., Ni, J.: WO0157060 (2001).
- [62] Reed, J.C., Ke, N., Godzik, A.: WO02072601 (2002).
- [63] Chittenden, T.D., Lutz, R.J.: US5656725 (1997).
- [64] Korsmeyer, S.J.: US5691179 (1997).

- [65] Hsueh, A.J.W., Hsu, S.Y., WO9924453 (1999) and Ruben S.M., Duan D.R., Ni J.: WO9924453 (1999).
- [66] Donoho, G., Hilbun, E., Turner, C.A. Jr., Friedrich, G., Abuin, A., Zambrowicz, B., Sands, A.T.: WO0157213 (2001).
- [67] Tschopp, J., Hofmann, K.: WO0248353 (2002).
- [68] Coultas, L., Strasser, A.: WO04037857 (2004).
- [69] Stegh, A., Kim, H., Depinho, R.A., Chin, L.: WO04096991 (2004).
- [70] Korsmeyer, S.J.: WO9809980 (1998).
- [71] Korsmeyer, S.J.: WO9613614 (1996).
- [72] Horne, W.A., Oltersdorf, T.: WO9812328 (1998).
- [73] Alnemri, E.S.: WO9950414 (1999).
- [74] Cory, S., Adams J., Huang, D.C.S. O'connor, L., Strasser, A., Puthalakath, H., O'reilly, L.: WO9914321 (1999).
- [75] Strasser, A., Puthalakath, H., Villunger, A., Coultas, L., Beaumont, J., O'reilly, L., Huang, D.C.S.: WO02097094 (2002).
- [76] Clarke, M.F., Wicha, M., Nunez, G., Han, J., Inohara, N.: WO9830101 (1998).
- [77] Levine, B.C.: WO1998011256 (1998).
- [78] Lee, R-M., Dai, Q., Chen, J., Liu, J.: WO04081200 (2004).
- [79] Reed, J.C.: WO9427426A1 (1994).
- [80] Reed, J.C.: WO9508350A1 (1995).
- [81] Warrel, R.P. Jr., Klem, R.E., Fingert, H.: WO0217852A2 (2002) and WO0217852A3 (2002).
- [82] Warrel, R.P.: WO04056971 (2004).
- \*[83] Chen, Z., Ruffner, D.E., Prakash, R., Koehn, R.: WO04046327 (2004).
- [84] Tormo, M., Tara, A.M., Lopez-Berestein, G., McDonnell, T.J.: WO9814172A1: (1998).
- [85] Milmer, J., Jiang, M.: WO05012357 (2005).
- [86] Bennett, C.F., Dean, N.M., Monia, B.P., Nickloff, B.J., Zhang, Q.: WO0020432A1 (2000).
- [87] Frieden, M., Hansen, J.B., Oerum, H., Westergaard, M., Thru, C.A.: WO05061710 (2005).
- [88] Ackermann, E.J., Bennett, C.F., Dean, N.M., Marcusson, E.G.: WO0040595A1: (2000).
- [89] Kreutzer, R., Limmer, S., Vornlocher, H.-P., Hadwiger, P., Geick, A., Ocker, M., Herold, C., Schuppan, D.: WO02055692A2 (2002).
- \*[90] Mcswiggen, J., Beigelman, L.: WO03070969A3 (2003).
- [91] Yano, J., Hirabayashi, K., Nakagawa, S.: WO04106511 (2004).
- \*[92] Anastasia, K., Angela, R., Devin, L., William, M., Stephen, S.: WO04045543 (2004).
- [93] Croce, C.M., Calin, G.A.: WO04043387 (2004).
- [94] Korsmeyer, S.J.: WO9916787 (1999).
- [95] Korsmeyer, S., Letai, A.: WO04022580 (2004).
- \*[96] Walensky, L.D., Korsmeyer, S.J., Verdine, G.: WO05044839 (2005).
- [97] Baell, J., Huang, D., Smith, B.J., Street, I.P.: WO04058804 (2004).
- [98] Baell, J., Wei, A.H., Scanlon, D.B.: WO06000034 (2006).
- [99] Bonny, C., Coquoz, D.: WO06056370 (2006).
- [100] Morgan, B.A., Prevost, G., Cotter, T.G., Finnegan, N.: WO0100670 (2001).
- [101] Fesik, S.W., Meadows, R.P., Joseph, M.K., Olejniczak, E.T., Petros, A.M., Nettesheim, D.G., Swift, K.M., Matayoshi, E., Zhang, H.: WO0220568 (2002).
- [102] Geneste, O., Hickman, J., Bennett, R., Rain, J.-C.: WO05014638 (2005).
- \*[103] Geneste, O., Hickman, J., Rain, J.-C.: WO06082304 (2006).
- [104] Geneste, O., Hickman, J., Rain, J.-C.: WO06082303 (2006).
- [105] Yanagawa, H., Tsuji, T.: WO06062173 (2006).
- [106] Shi, Y.: WO06034454 (2006).
- [107] Matsuyama, S.: WO03074667 (2003).
- [108] Sinclair, D.A., Cohen Haim, Y.: WO06007411 (2006).
- [109] Reed, J.C., Guo, B.: WO03046205 (2003).
- \*[110] Ramesh, K., Xiaozhong, Q.: WO06019365 (2006).
- [111] Shore, G.C., Goping, I.S.: WO0020446 (2000).
- [112] Dou, P., Gao, G.: WO0203921 (2002).
- [113] Imoto, M., Tabe, H., Tashiro, E., Ohmori, Y.: WO04113367 (2004).
- [114] Garcia, A., Cayla, X., Rebollo, A.: WO03096022 (2003).
- [115] Garcia, A., Dessauge, F., Cayla, X., Rebollo, A.: WO04035611 (2004).
- [116] Singh, S., Badal, M Y., Jin, X., Salimi-M.H.: WO04087887 (2004).
- [117] Kim, T.H.: WO06001582A1 (2006).
- [118] Shimizu, S., Tsujimoto, Y.: WO0148014 (2001).
- [119] Gewirtz AM, Sokol DL, Ratajczak MZ. Nucleic acid therapeutics: State of the art and future prospects. *Blood* 1998, 92: 712-36.
- [120] Stein CA, Subasinghe C, Shinozuka K, Cohen JS. Physico-chemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Research* 1988, 16: 3209-21.
- [121] Blake KR, Murakami A, Miller PS. Inhibition of rabbit globin mRNA translation by sequence-specific oligodeoxyribonucleotides. *Biochemistry* 1985, 24: 6132-38.
- [122] Monia BP, Lesnik EA, Gonzalez C, *et al.* Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* 1993, 268: 14514-22.
- [123] Gryaznov S, Skorski T, Cucco C, *et al.* Oligonucleotide N3'->P5' phosphoramidates as antisense agents. *Nucleic Acids Res* 1996, 24: 1508-14.
- [124] Elayadi AN, Corey DR. Application of PNA and LNA oligomers to chemotherapy. *Curr Opin Invest Drugs* 2001, 2: 558-61.
- [125] Kurreck J, Wyszko E, Gillen C, Erdmann VA. Design of antisense oligonucleotides stabilized by locked nucleic acids. *Nucleic Acids Res* 2002, 30: 1911-18.
- [126] Reed JC, Stein C, Subasinghe C, *et al.* Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. *Cancer Res* 1990, 50: 6565-70.
- [127] Miyashita T, Reed JC. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res* 1992, 52: 5407-11.
- [128] Grover R, Wilson GD. Bcl-2 expression in malignant melanoma and its prognostic significance. *Eur J Surgical Oncol* 1996, 22: 347-49.
- [129] Jansen B, Schlagbauer-Wadl H, Brown BD, *et al.* bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med* 1998, 4: 232-34.
- [130] Cotter FE, Johnson P, Hall P, *et al.* Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. *Oncogene* 1994, 9: 3049-55.
- [131] Chanan-Khan A. Bcl-2 antisense therapy in hematologic malignancies. *Curr Opin Oncol* 2004, 16: 581-85.
- [132] Jansen B, Wacheck V, Heere-Ress E, *et al.* Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet* 2000, 356: 1728-33.
- [133] Pepper C, Hooper K, Thomas A, Hoy T, Bentley P. Bcl-2 antisense oligonucleotides enhance the cytotoxicity of chlorambucil in B-cell chronic lymphocytic leukaemia cells. *Leuk Lymphoma* 2001, 42: 491-98.
- [134] Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Nat Acad Sci USA* 1986, 83: 5214-18.
- [135] Hoffmann R, Seidl T, Neeb M, Rolink A, Melchers F. Changes in gene expression profiles in developing B cells of murine bone marrow. *Genome Res* 2002, 12: 98-111.
- [136] Yamanaka K, Rocchi P, Miyake H, *et al.* A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells. *Mol Cancer Ther* 2005, 4: 1689-98.
- [137] Choi SS, Park IC, Yun JW, *et al.* A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. *Oncogene* 1995, 11: 1693-98.
- [138] Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005, 37: 267-71.
- [139] Fire A, Xu S, Montgomery MK, *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998, 391: 806-11.
- [140] Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001, 409: 363-66.
- [141] Sullenger BA, Gilboa E. Emerging clinical applications of RNA. *Nature* 2002, 418: 252-58.
- [142] Lima RT, Martins LM, Guimaraes JE, Sambade C, Vasconcelos MH. Specific downregulation of bcl-2 and xIAP by RNAi

- enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. *Cancer Gene Ther* 2004, 11: 309-16.
- [143] Fu GF, Lin XH, Han QW, *et al.* RNA interference remarkably suppresses bcl-2 gene expression in cancer cells *in vitro* and *in vivo*. *Cancer Biol Ther* 2005, 4: 822-29.
- [144] Zangemeister-Witke U, Leech SH, Olie RA, *et al.* A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. *Clin Cancer Res* 2000, 6: 2547-55.
- [145] Calin GA, Dumitru CD, Shimizu M, *et al.* Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 2002, 99: 15524-29.
- [146] Cimmino A, Calin GA, Fabbri M, *et al.* miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA* 2005, 102: 13944-49.
- [147] Goldsmith KC, Liu X, Dam V, *et al.* BH3 peptidomimetics potently activate apoptosis and demonstrate single agent efficacy in neuroblastoma. *Oncogene* 2006, 25: 4525-33.
- [148] Walensky LD, Kung AL, Escher I, *et al.* Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. *Science* 2004, 305: 1466-70.
- [149] Dharap SS, Minko T. Targeted proapoptotic LHRH-BH3 peptide. *Pharm Res* 2003, 20: 889-96.
- [150] Yang B, Liu D, Huang Z. Synthesis and helical structure of lactam bridged BH3 peptides derived from pro-apoptotic Bcl-2 family proteins. *Bioorg Med Chem Lett* 2004, 14: 1403-06.
- [151] Zhang H, Nimmer P, Rosenberg SH, Ng SC, Joseph M. Development of a high-throughput fluorescence polarization assay for Bcl-x(L). *Anal Biochem* 2002, 307: 70-75.
- [152] Owicki JC. Fluorescence polarization and anisotropy in high throughput screening: perspectives and primer. *J Biomol Screen* 2000, 5: 297-306.
- [153] Liu X, Dai S, Zhu Y, Marrack P, Kappler JW. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. *Immunity* 2003, 19: 341-52.
- [154] Denisov AY, Chen G, Sprules T, *et al.* Structural model of the BCL-w-BID peptide complex and its interactions with phospholipid micelles. *Biochemistry* 2006, 45: 2250-56.
- [155] Cohen HY, Lavu S, Bitterman KJ, *et al.* Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* 2004, 13: 627-38.
- [156] Sawada M, Sun W, Hayes P, *et al.* Ku70 suppresses the apoptotic translocation of Bax to mitochondria. *Nat Cell Biol* 2003, 5: 320-29.
- [157] Luciano F, Zhai D, Zhu X, *et al.* Cytoprotective peptide humanin binds and inhibits proapoptotic Bcl-2/Bax family protein BimEL. *J Biol Chem* 2005, 280: 15825-35.
- [158] Zhai D, Luciano F, Zhu X, *et al.* Humanin binds and nullifies Bid activity by blocking its activation of Bax and Bak. *J Biol Chem* 2005, 280: 15815-24.
- [159] Hashimoto Y, Niihura T, Tajima H, *et al.* A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta. *Proc Natl Acad Sci USA* 2001, 98: 6336-41.
- [160] Jung SS, Van Nostrand WE. Humanin rescues human cerebrovascular smooth muscle cells from Abeta-induced toxicity. *J Neurochem* 2003, 84: 266-72.
- [161] Maximov V, Martynenko A, Hunsmann G, Tarantul V. Mitochondrial 16S rRNA gene encodes a functional peptide, a potential drug for Alzheimer's disease and target for cancer therapy. *Med Hypotheses* 2002, 59: 670-73.
- [162] Goping IS, Gross A, Lavoie JN, *et al.* Regulated targeting of BAX to mitochondria. *J Cell Biol* 1998, 143: 207-215.
- [163] Gao G, Dou QP. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem* 2000, 80: 53-72.
- [164] Deng X, Ito T, Carr B, Mumby M, May WS, Jr. Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatins 1 is mediated by direct interaction with protein phosphatase 2A. *J Biol Chem* 1998, 273: 34157-63.
- [165] Ayllon V, Cayla X, Garcia A, *et al.* Bcl-2 targets protein phosphatase 1 alpha to Bad. *J Immunol* 2001, 166: 7345-52.
- [166] Ayllon V, Martinez AC, Garcia A, Cayla X, Rebollo A. Protein phosphatase 1alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *EMBO J* 2000, 19: 2237-46.
- [167] Ayllon V, Cayla X, Garcia A, Fleischer A, Rebollo A. The anti-apoptotic molecules Bcl-xL and Bcl-w target protein phosphatase 1alpha to Bad. *Eur J Immunol* 2002, 32: 1847-55.
- [168] Salomoni P, Condorelli F, Sweeney SM, Calabretta B. Versatility of BCR/ABL-expressing leukemic cells in circumventing proapoptotic BAD effects. *Blood* 2000, 96: 676-84.
- [169] Malissein E, Verdier M, Ratinaud MH, Troutaud D. Activation of Bad trafficking is involved in the BCR-mediated apoptosis of immature B cells. *Apoptosis* 2006, 11: 1003-12.
- [170] Gonzalez-Polo RA, Soler G, Alvarez A, Fabregat I, Fuentes JM. Vitamin E blocks early events induced by 1-methyl-4-phenylpyridinium (MPP+) in cerebellar granule cells. *J Neurochem* 2003, 84: 305-15.
- [171] Guergnon J, Dessauge F, Dominguez V, *et al.* Use of penetrating peptides interacting with PP1/PP2A proteins as a general approach for a drug phosphatase technology. *Mol Pharmacol* 2006, 69: 1115-24.
- [172] Tzivion G, Avruch J. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J Biol Chem* 2002, 277: 3061-64.
- [173] Datta SR, Dudek H, Tao X, *et al.* Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997, 91: 231-41.
- [174] Frank R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *J Immunol Methods* 2002, 267: 13-26.
- [175] Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 1996, 87: 619-28.
- [176] Datta SR, Katsov A, Hu L, *et al.* 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell* 2000, 6: 41-51.
- [177] Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001, 7: 683-94.
- [178] Oda E, Ohki R, Murasawa H, *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000, 288: 1053-58.
- [179] Kiryu-Seo S, Hirayama T, Kato R, Kiyama H. Noxa is a critical mediator of p53-dependent motor neuron death after nerve injury in adult mouse. *J Neurosci* 2005, 25: 1442-47.
- [180] Villunger A, Michalak EM, Coultas L, *et al.* p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003, 302: 1036-38.
- [181] Shibue T, Takeda K, Oda E, *et al.* Integral role of Noxa in p53-mediated apoptotic response. *Genes Dev* 2003, 17: 2233-38.
- [182] Seo YW, Shin JN, Ko KH, *et al.* The molecular mechanism of Noxa-induced mitochondrial dysfunction in p53-mediated cell death. *J Biol Chem* 2003, 278: 48292-99.
- [183] Huang DC, Adams JM, Cory S. The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* 1998, 17: 1029-39.
- [184] Bonnefoy-Berard N, Aouacheria A, Vershelde C, *et al.* Control of proliferation by Bcl-2 family members. *Biochim Biophys Acta* 2004, 1644: 159-68.
- [185] Sorenson CM. Interaction of bcl-2 with Paxillin through its BH4 domain is important during ureteric bud branching. *J Biol Chem* 2004, 279: 11368-74.
- [186] Reed JC, Zha H, Aime-Sempe C, Takayama S, Wang HG. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. *Adv Exp Med Biol* 1996, 406: 99-112.
- [187] Vance BA, Zacharchuk CM, Segal DM. Recombinant mouse Bcl-2(1-203). Two domains connected by a long protease-sensitive linker. *J Biol Chem* 1996, 271: 30811-15.
- [188] Grandgirard D, Studer E, Monney L, *et al.* Alphaviruses induce apoptosis in Bcl-2-overexpressing cells: evidence for a caspase-mediated, proteolytic inactivation of Bcl-2. *EMBO J* 1998, 17: 1268-78.
- [189] Shimizu S, Konishi A, Kodama T, Tsujimoto Y. BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. *Proc Natl Acad Sci USA* 2000, 97: 3100-3105.

- [190] Hotchkiss RS, McConnell KW, Bullok K, *et al.* TAT-BH4 and TAT-Bcl-xL peptides protect against sepsis-induced lymphocyte apoptosis *in vivo*. *J Immunol* 2006, 176: 5471-77.
- [191] Dietz GP, Bahr M. Delivery of bioactive molecules into the cell: the Trojan horse approach. *Mol Cell Neurosci* 2004, 27: 85-131.
- [192] Ono M, Sawa Y, Ryugo M, *et al.* BH4 peptide derivative from Bcl-xL attenuates ischemia/reperfusion injury thorough anti-apoptotic mechanism in rat hearts. *Eur J Cardiothorac Surg* 2005, 27: 117-21.
- [193] Sugioka R, Shimizu S, Funatsu T, *et al.* BH4-domain peptide from Bcl-xL exerts anti-apoptotic activity *in vivo*. *Oncogene* 2003, 22: 8432-40.
- [194] Zhai D, Jin C, Satterthwait AC, Reed JC. Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. *Cell Death Differ* 2006, 13: 1419-21.
- [195] Chen L, Willis SN, Wei A, *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005, 17: 393-403.