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# Activation and function of BH3-only proteins from the Bcl-2 family

Bachelor thesis

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**Declaration:**

I hereby declare that I have written this thesis independently and I have mentioned all the used literature.

Prague, 30.4.2011 .....

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First of all, I wish to thank my supervisor RNDr. Ladislav Anděra, CSc. for his valuable suggestions and comments on the thesis. I would also like to thank my family for encouragement and support.

## Contents

## Abstract

The Bcl-2 (B-cell lymphoma-2) family of proteins comprises crucial regulators of apoptosis in metazoans. In mammals, Bcl-2 family members regulate mitochondrial outer membrane permeabilization (MOMP), which leads to the release of pro-apoptotic molecules from the mitochondrial intermembrane space and subsequent apoptosis. Defects in the Bcl-2 signaling can result in various pathological conditions including cancer. BH3-only proteins form a pro-apoptotic subset of the Bcl-2 family. They mainly act as sensors of various pro-apoptotic stimuli such as cytokine deprivation, genotoxic stress, activated "death receptors" and others. In mammals, over ten BH3-only proteins have been identified, eight of which have been linked to the regulation of MOMP. These "conventional" BH3-only proteins are Bim, Bid, Puma, Noxa, Bad, Hrk, Bik and Bmf. This thesis provides an overview of their origin, structure, activation and involvement in apoptosis.

**Keywords:** apoptosis, MOMP, Bcl-2 family, BH domain, BH3-only proteins, cancer, BH3 mimetics

## Abstrakt

Proteiny z rodiny Bcl-2 (B-cell lymphoma-2) jsou klíčovými regulátory apoptózy u mnohobuněčných živočichů. U savců tyto proteiny regulují permeabilizaci vnější mitochondriální membrány (MOMP), jež vede k uvolnění pro-apoptotických molekul z mezimembránového prostoru mitochondrií a následné apoptóze. Poruchy v signalizaci těmito proteiny mohou u savců vyústit v různé patologické stavy včetně rakoviny. BH3 proteiny tvoří pro-apoptotickou podskupinu Bcl-2 rodiny. Fungují zejména jako senzory různých pro-apoptotických podnětů, jako je například nedostatek cytokinů, genotoxický stres či aktivace "receptorů smrti". U savců bylo identifikováno přes deset BH3 proteinů, z nichž osm se účastní regulace MOMP. Těmito "konvenčními" BH3 proteiny jsou Bim, Bid, Puma, Noxa, Bad, Hrk, Bik and Bmf. Tato práce nabízí přehled o jejich původu, struktuře, aktivaci a funkci v apoptotické signalizaci.

**Klíčová slova:** apoptóza, MOMP, Bcl-2 rodina, BH doména, BH3 proteiny, rakovina, BH3 mimetika

## List of abbreviations

| | |
|---|---|
| AA | amino acid |
| APR | ATL-derived PMA-responsive gene |
| Arg | arginine |
| ATF | cAMP-dependent transcription factor |
| ATL | adult T-cell leukemia |
| Bad | Bcl-2 associated agonist of cell death |
| Bak | Bcl-2-antagonist/killer 1 |
| Bax | Bcl-2-associated X protein |
| Bcl-2 | B-cell lymphoma-2 |
| BH | Bcl-2 homology |
| Bid | BH3 interacting domain death agonist |
| Bik | Bcl-2 interacting killer |
| Bim | Bcl-2 interacting mediator of cell death |
| Bmf | Bcl-2 modifying factor |
| CAD | caspase-activated DNase |
| Cdk5 | cyclin-dependent kinase 5 |
| CK | casein kinase |
| CTL | cytotoxic T-lymphocyte |
| dATP | deoxyadenosine triphosphate |
| DISC | death-inducing signaling complex |
| DKO | double knockout |
| DLC | dynein light chain |
| DNA | deoxyribonucleic acid |
| DRP-1 | dynamin-related protein-1 |
| E | glutamate |
| EGF | epithelilal growth factor |
| ER | endoplasmic reticulum |
| ERAD | ER-associated protein degradation |
| ERK | extracellular-signal-regulated kinase |
| FADD | Fas-associated death domain |
| FasL | Fas ligand |
| FOXO3a | forkhead box O3 |
| G | glycine |
| HIF-1α | hypoxia-inducible factor 1α |
| HPV16 | human papillomavirus-16 |
| CHOP | C/EBP-homologous protein |
| IAP | inhibitor of apoptosis |
| ICAD | inhibitor of CAD |
| IFN-γ | interferon-γ |
| IGF-1 | insulin-like growth factor-1 |
| IL | interleukin |
| IUP | intrinsically unstructured protein |
| JNK | c-Jun N-terminal kinase |
| kDa | kilodalton |

| | |
|---|---|
| KO | knockout |
| L | leucine |
| Lys | lysine |
| MAPK | mitogen-activated protein kinase |
| MEF | mouse embryonic fibroblast |
| MOMP | mitochondrial outer membrane permeabilization |
| MTCH2/MIMP | mitochondrial carrier homologue 2/Met-induced mitochondrial protein |
| NGF | nerve growth factor |
| NMR | nuclear magnetic resonance |
| NS | nervous system |
| OMM | outer mitochondrial membrane |
| p53 | protein 53 |
| p70S6K | 70-kDa ribosomal protein S6 kinase |
| p73 | protein 73 |
| PI3K | phosphatidylinositol 3-kinase |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PMA | phorbol-12-myristate-13-acetate |
| PP1α | protein phosphatase 1α |
| PPA2 | protein phosphatase A2 |
| pRB | retinoblastoma protein |
| PRMT1 | protein arginine methyltransferase 1 |
| Puma | p53-upregulated modulator of apoptosis |
| Ser | serine |
| Smac | second mitochondria-derived activator of caspases |
| TGF-β | transforming growth factor-β |
| Thr | threonine |
| TKO | triple knockout |
| TM | transmembrane |
| TNF | tumor necrosis factor |
| TNFR | tumor necrosis factor receptor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| UV | ultraviolet light |
| VDAC2 | voltage-dependent anion-selective channel protein-2 |
| WT | wild-type |

# 1. Introduction

Apoptosis, a controlled type of cell death, plays essential roles in development, tissue homeostasis and responses to multiple non-physiological conditions in all metazoans. Proteins from the Bcl-2 (B-cell lymphoma-2) family are involved in both major apoptotic pathways, the intrinsic and extrinsic apoptotic pathway. The Bcl-2 family members are highly conserved in metazoans and comprise both pro- and anti-apoptotic members. In vertebrates, the pro-apoptotic subset of the Bcl-2 family encompasses the direct effectors of mitochondrial outer membrane permeabilization (MOMP) and BH3-only proteins, which act as sensors of various apoptotic stimuli. Activated BH3-only proteins inhibit the anti-apoptotic Bcl-2 members and also directly activate effectors of the MOMP. MOMP is the final step in the initiation of apoptosis, leading to the release of cytochrome *c* and other pro-apoptotic proteins from the mitochondrial intermembrane space. The released proteins subsequently trigger events that ultimately lead to proteolytic destruction of the affected cell.

This thesis focuses on the critical evaluation of cellular and physiological roles of eight conventional pro-apoptotic BH3-only proteins, which have been characterized in mammals, namely Bim, Bid, Puma, Noxa, Bad, Hrk, Bik and Bmf. The only homology they share with the rest of the Bcl-2 family is a short BH3 domain, which is essential for interaction with their binding partners from the rest of the family. BH3-only proteins are strictly regulated at the transcriptional level and by multiple posttranslational modifications including proteolytical processing, phosphorylation, ubiquitination and methylation. The activation of BH3-only proteins is triggered by multiple pro-apoptotic stimuli such as cytokine withdrawal, genotoxic stress, activated “death receptors” from the TNFR family, loss of cell-matrix contact and others. Together with other Bcl-2 family members and multiple other cellular pathways, BH3-only proteins form a complex signaling network, defects of which are associated with developmental malfunctions, autoimmune diseases and cancer.

# 2. Apoptosis

Apoptosis was first defined almost four decades ago by John F. Kerr and colleagues as a “mechanism of controlled cell deletion” [1]. It is a strictly controlled process marked by the degradation of cell proteins, membrane blebbing, cell shrinkage, DNA fragmentation and chromatin condensation ultimately leading to the disintegration and removal of the affected cells. The remains of apoptotic cells, so-called apoptotic bodies, are absorbed by phagocytes

or surrounding cells and therefore no traces of such cells are left in the organism. In contrast to necrosis, which is characterized as a disorganized breakdown of cells and tissues and can result in serious conditions such as infection and inflammation, appropriate apoptosis is a vital component of the multicellular organism physiology and is essential for tissue homeostasis, embryonic development and proper functioning of the immune system. Dysregulated apoptosis can turn into a dangerous process involved in numerous pathological conditions including autoimmune diseases, neurodegenerative diseases and cancer. Apoptosis must therefore be perfectly balanced to maintain tissue homeostasis and integrity (reviewed in [2]). The acquired capability of cells to suppress apoptosis is a critical step towards malignant growth, and the ability of tumor cells to evade apoptosis is considered a hallmark of cancer [3].

The importance of balanced apoptosis, both during development and in adults, can be seen in the nervous system (NS). A massive overproduction of neurons as well as other cell types takes place during the embryonic development of NS and the cells that do not establish functional connections or do not receive certain pro-survival signals are eliminated. In contrast, uncontrolled and unscheduled apoptosis of neurons in the adult brain leads to neurodegenerative diseases such as Alzheimer's or Parkinson's disease (reviewed in [4]).

In mammals, apoptosis can be initiated by two major pathways, the intrinsic or the extrinsic pathway. Both of them lead to the activation of caspases, a family of cysteinyl proteases that cleave various cellular substrates after aspartic acid and are one of the final executors of cell destruction. The intrinsic pathway is initiated by a variety of signals including DNA damage, nutrient or cytokine deprivation, loss of contact with the surrounding extracellular matrix, oncogene upregulation and treatment with genotoxic agents. The majority of apoptotic signaling in mammalian cells is carried out by the intrinsic pathway, in which proteins from the Bcl-2 family play a dominant controlling and executing role. The Bcl-2 family comprises both pro-apoptotic and anti-apoptotic members. Pro-apoptotic BH3-only members are activated in response to specific apoptotic stimuli (see in following chapters). One of their major functions is to neutralize the anti-apoptotic members, which act as keepers of mitochondrial outer membrane integrity. Mitochondrial outer membrane permeabilization (MOMP), which is executed by the pro-apoptotic members of the Bcl-2 family Bak and Bax upon the activation of the intrinsic apoptotic pathway, is the final step before the activation of proteolytic pathways and subsequent destruction of the cell components (reviewed in [5]) (Figure 1).

The extrinsic pathway, which is implicated mainly in the immune system, is dependent on the activation of "death receptors" by specific ligands and, depending on cell type, leads to either mitochondria-independent or -dependent apoptosis (reviewed in [6]).

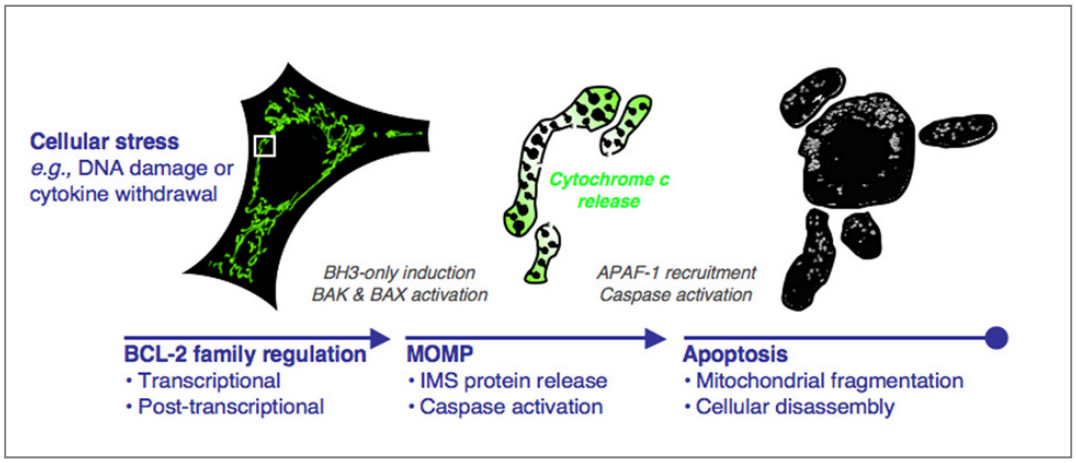


**Figure 1 -** After various cellular stresses, the signaling triggered by activated BH3-only proteins leads to the activation of Bax and Bak. Once activated, Bax and Bak cause mitochondrial outer membrane permeabilization (MOMP) and subsequent apoptosis. *(Adapted from ref. [7])*

### 2.1. Intrinsic apoptotic pathway

The major pathway of apoptosis in vertebrates is termed either the intrinsic or mitochondrial apoptotic pathway. A critical step in this signaling pathway is MOMP, which is under tight control of the Bcl-2 family of proteins. Pro-apoptotic members of the Bcl-2 family termed BH3-only proteins are responsive to various physiological as well as non-physiological signals. For example, BH3-only proteins Puma and Noxa are upregulated after genotoxic stress and are essential for ionizing radiation-induced apoptosis [8]. Another example of a crucial BH3-only protein is Bim, which is activated during multiple steps of the immune system development and maintenance [9]. The major function of the pro-apoptotic BH3-only proteins is to bind and therefore neutralize the anti-apoptotic Bcl-2 proteins, but at least some of them also participate in the direct activation or enhancement of the MOMP-promoting activities of Bax and Bak [10]. The anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-xL are sentinels of outer mitochondrial membrane integrity, because they hold MOMP effectors, pore-forming Bcl-2 members Bak and Bax, in check.

Although the molecular mechanism of MOMP is poorly understood, it leads to the release of soluble proteins, such as cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspases), from the mitochondrial intermembrane space. Once released, cytochrome *c* binds, along with dATP, to cytosolic protein Apaf-1, inducing its

conformational change and thus allowing its assembly into a wheel-like heptameric complex called the apoptosome. Smac/DIABLO neutralizes cytoplasmic proteins from the IAP (inhibitors of apoptosis) family, and blocks their inhibitory effect on activated caspases-3 and -9. The apoptosome binds to pro-caspase-9 and turns it into an active form, caspase-9. After its activation, caspase-9 cleaves downstream effector caspases such as caspase-3. The effector caspases break down a variety of proteins including poly(A) polymerase, retinoblastoma protein (pRB), nuclear lamins and DNA-dependent protein kinase [11]. The effector caspases also activate protein CAD (caspase-activated DNase)/DFF40 by cleaving its inhibitor ICAD/DFF45. When active, CAD cleaves chromosomal DNA into nucleosomal fragments [12].

### 2.2. Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is activated upon the ligation of cell surface proteins called “death receptors”. Death receptors contain a cysteine-rich extracellular domain and an intracellular protein interaction sequence called the death domain. Examples of some well characterized death receptors are FasR/CD95, TNFR1/CD120a or TRAIL receptors DR4 and DR5. Activation of such receptors by ligands from the TNF (tumor necrosis factor) superfamily results in the clustering of receptors’ death domains and the subsequent formation of protein complex DISC (death inducing signaling complex). In a case of Fas/CD95 or DR4/DR5, DISC is composed of several proteins including adapter protein FADD (Fas-associated death domain) and caspase-8, whose recruitment to DISC leads to its autocatalytic cleavage and activation. Activated caspase-8 then cleaves effector caspases, such as caspase-3. As in the intrinsic apoptotic signaling, the activated effector caspases carry out the final stage of the cell demise [6]. Direct caspase-8->caspase-3 axis (so-called type I signaling) is sufficient for triggering cell death only under some conditions (e.g. during activation-induced cell death of T-cells). In the majority of cells, the extrinsic pathway proceeds via type II signaling and needs to be amplified by mitochondrial apoptotic signaling (the intrinsic pathway) via caspase-8 cleavage-mediated activation of the BH3-only protein Bid [13].

An additional mechanism to the extrinsic apoptotic pathway is so-called perforin/granzyme-induced apoptosis, which has been shown to play a role in the immune response. It is an important mechanism (in addition to FasL- or TRAIL-triggered apoptotic signaling), by which cytotoxic T-lymphocytes (CTLs) destroy target cells. After direct contact

with a virus-infected or transformed cell, CTLs release cytoplasmic granules containing granzymes and perforin, causing apoptosis of the target cell in either a caspase-dependent or caspase-independent manner (reviewed in [14]).

## 3. The Bcl-2 family of proteins

The mammalian members of the Bcl-2 family form a group of proteins comprising essential modulators of both the intrinsic and extrinsic apoptotic pathways, which can register multiple input signals, integrate them and subsequently decide whether the cell is supposed to survive or die. The members of the Bcl-2 family are strictly regulated and their expression levels, along with a complex set of posttranslational modifications, modulate the interactions between these functionally opposite proteins.

In order to meet the definition of a Bcl-2 family member, a protein must contain at least one BH (Bcl-2 homology) domain. The multidomain Bcl-2 family members contain up to four BH domains (BH1-BH4) and include anti-apoptotic members (e.g. Bcl-2) and direct effectors (e.g. Bax). BH3-only members contain only one BH domain (BH3) and all are pro-apoptotic. Based on their role in apoptosis, the members of the Bcl-2 family can be divided into two groups: anti-apoptotic members and pro-apoptotic members, subdivided into direct effectors and BH3-only sentinels.

In mammals, the anti-apoptotic members are: **Bcl-2, Bcl-w, Bcl-xL, Mcl-1** and **A1**, the pro-apoptotic are BH3-only proteins **Noxa, Puma, Bim, Bad, Bid, Bmf, Hrk, Bik** and the effector proteins are **Bax** and **Bak** [5]. Here, I focus on these "conventional" Bcl-2 proteins and their role in apoptosis. It should be noted that additional Bcl-2 members such as Bok, Mule, Beclin-1 and BNips exist in mammals, but due to their unsettled role in apoptosis, they are not discussed in this thesis.

When activated, the effector proteins Bax and Bak oligomerize to form pores in the outer mitochondrial membrane, and thus enable soluble protein cytochrome *c* and other pro-apoptotic proteins such as Smac/DIABLO to diffuse from the mitochondrial intermembrane space and to proceed with their pro-apoptotic activities. For the cytochrome *c*, this is the assembly of the Apaf-1 complex and subsequent activation of caspase-9. Pro-apoptotic BH3-only proteins sense various cellular stresses such as DNA damage, nutrient deprivation or endoplasmic reticulum (ER) stress and promote apoptosis by inhibition of the anti-apoptotic proteins and some of them also by direct activation of the Bax/Bak [7].

### 3.1. Discovery of Bcl-2

The first gene coding for a Bcl-2 family member was identified over 25 years ago in human follicular lymphoma. In this type of cancer, a translocation between chromosomes 14 and 18 occurs. Such rearrangement brings the *bcl-2* gene under the transcriptional control of the immunoglobulin enhancer element, causing potent upregulation of the *bcl-2* gene transcription [15]. A few years later using mouse models, it was demonstrated that an overexpressed *bcl-2* gene blocks apoptosis of B-cells and extends the survival of certain hematopoietic cell lines upon growth factor deprivation [16]. Taken together, these studies have established Bcl-2 as an anti-apoptotic protein with proto-oncogenic potential. Since then, the family has significantly grown, and to date, based on the sequence similarity to *bcl-2*, over twenty family members have been identified in humans. Bcl-2 family homologues have since been identified in virtually all metazoans [17].

### 3.2. Evolutionary origin and structure of Bcl-2 family proteins

From the evolutionary perspective, the Bcl-2 family can be subdivided into two groups, each with its own phylogenetic history and structural features (Figure 2-A). Sequence and structural studies revealed that the multidomain anti-apoptotic and pro-apoptotic members (Bcl-2, Bcl-w Bcl-xL, Mcl-1, A1, Bak, Bax) and surprisingly the BH3-only protein Bid probably share a common ancestry. The proteins forming this monophyletic clade are referred to as Bcl-2-like proteins. *Bcl-2-like* genes have been identified or predicted in all metazoans examined so far. On the other hand, no such genes have been found in prokaryotes, fungi and plants [18]. Remarkably, a whole set of Bcl-2 family members was recently characterized in the cnidarian *Hydra magnipapillata* and the Bcl-2 signaling in *Hydra* was found to be much more complex than in higher invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans.* This leads to the conclusion that Bcl-2-like proteins originated in metazoans before the Cambrian explosion and some of them have been lost in certain lines in the course of evolution [19].

Bcl-2-like proteins share similar structural features as all of them contain up to four BH domains (BH1-BH4) and (except for A1 and Bid) a C-terminal trans-membrane (TM) region. A helix-bundle fold of these proteins was first elucidated by resolving the structure of Bcl-xL [20]. The 3D structure of Bcl-xL also provided the first clue as to how it might interact with other Bcl-2 members, as a hydrophobic binding cleft formed by BH1, BH2 and BH3 regions was revealed. The hypothesis that the hydrophobic cleft on Bcl-xL might serve

as the interaction site for its binding partners was later confirmed by the NMR solution structure of Bcl-xL in complex with BH3 region of protein Bak [21] (Figure 2-B).

In contrast, BH3-only proteins (except for Bid) share structural similarity with Bcl-2-like proteins only in the BH3 region and are otherwise structurally unrelated even to each other. It is therefore likely that BH3-only proteins are products of convergent evolution. Interestingly, it is possible that some BH3-only proteins may have arisen by the exonization of transposable elements as the homolog of *noxa* in *Danio rerio* bears *Alu* sequences and therefore may be a product of gene diversity generated by transposones [18]. BH3-only proteins have been identified in vertebrates, nematode *Caenorhabditis elegans* [22], cnidarian *Hydra magnipapillata* [19], *Schistosoma* blood flukes [23], but not in other animals. Finally, BNip1, BNip2 and BNip3, sometimes classified as BH3-only proteins, display several differences in otherwise conserved residues of the BH3 domain and, according to phylogenetic analyses of the Bcl-2 family, they rather form three monophyletic branches within the Bcl-2-like clade [18].

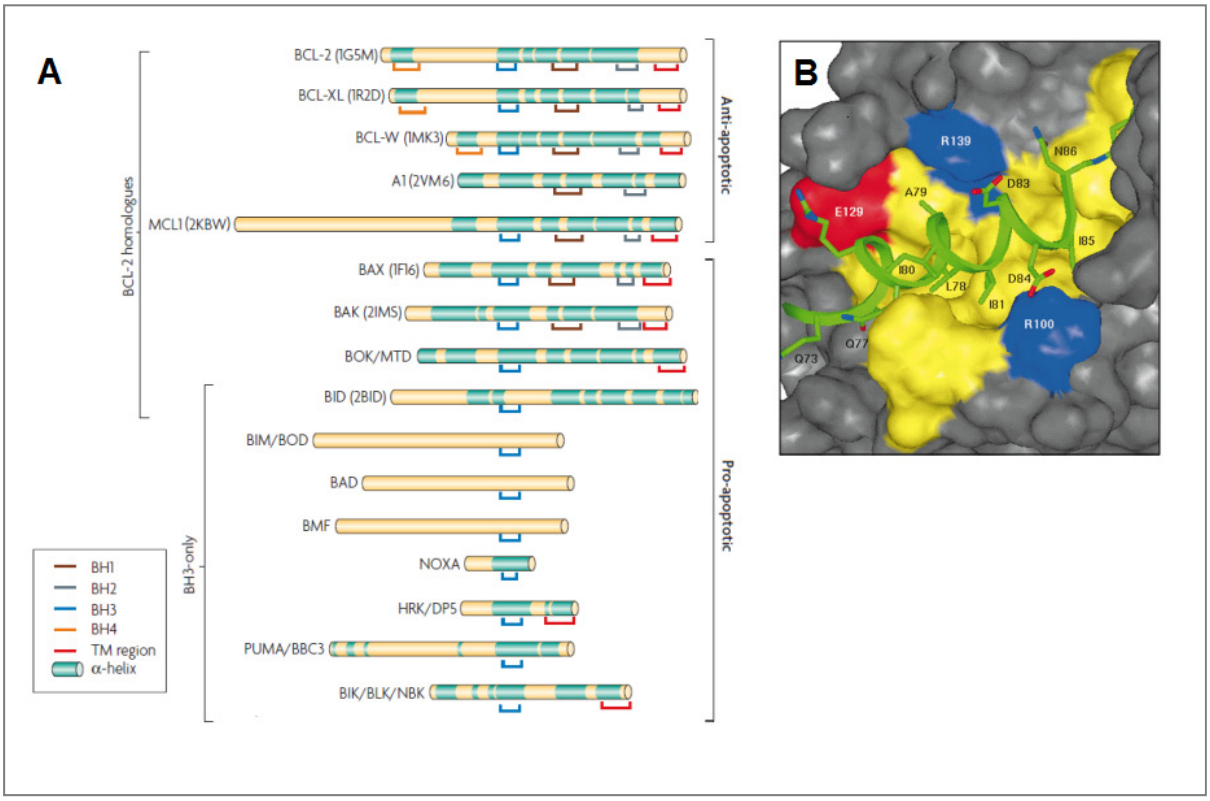


**Figure 2 - (A)** Alignment of the main characterized Bcl-2 family members with highlighted sequence homologies and structural features (Bim, Bad and Bmf are completely unstructured proteins). Currently available PDB IDs (www.pdb.org) are given in parentheses. (*Adapted and modified from ref. [5]).* **(B)** Bcl-xL-Bak peptide complex. BH3 domain of Bak is depicted in green. Hydrophobic binding groove of Bcl-xL is highlighted in yellow. Critical arginines 100 and 139 and glutamic acid 129 are highlighted in blue and red respectively. *(Adapted from ref. [21])*

### 3.3. Interactions between Bcl-2 family members

The anti- or pro-apoptotic nature of particular Bcl-2 family members is well established, but the series of interactions between them, regulating MOMP, is yet to be fully resolved. Investigation of this phenomenon has led to two models describing steps between the activation of pro-apoptotic BH3-only proteins and MOMP.

#### 3.3.1. Indirect activation model

The indirect activation model claims that MOMP is triggered, when BH3-only proteins inhibit the anti-apoptotic Bcl-2 members, and thus uncouple them from binding to the effector proteins Bax and Bak [24]. According to this model, no direct activation of Bax and Bak by BH3-only proteins is necessary. In healthy cells, Bax and Bak must be sequestered and inhibited by the anti-apoptotic Bcl-2 members. In addition to sequestering, Bcl-xL can also actively retrotranslocate Bax from mitochondria into the cytosol [25]. After the apoptotic signal, the binding grooves of the anti-apoptotic proteins become occupied by the BH3 domains of BH3-only proteins. This results in the release of Bax and Bak, their accumulation and clustering in the OMM and subsequent mitochondrial apoptosis (figure 3-A). BH3-only proteins bind the anti-apoptotic proteins selectively, for example Puma, Bid and Bim bind strongly with all anti-apoptotic proteins, whereas Bad binds tightly to Bcl-2, Bcl-xL and Bcl-w but only weakly to A1 and does not bind Mcl-1 at all. Thus, different combinations of BH3-only proteins are needed in different situations [24].

Willis et al. [26] provided support for this model by using *bim*$^{-/-}$*bid*$^{-/-}$ mouse embryonic fibroblasts (MEFs) with RNAi decreased Puma levels to show that Bak/Bax-dependent apoptosis can proceed without the presence of these three proteins, which are considered to be direct activators of Bax/Bak in the direct activation model.

#### 3.3.2. Direct activation model

In the direct activation model, the apoptotic signal transducers from the BH3-only subgroup could be distinguished into two functionally shifted groups: (I) direct activators (and also sensitizers): Bid, Bim and Puma, (II) “pure” sensitizers: Noxa, Bad, Bmf, Hrk and Bik. The model postulates that direct activators, in addition to their competitive inhibition of the anti-apoptotic Bcl-2 proteins, are also able to bind the effectors to enhance their pore-forming activity in the outer mitochondrial membrane (OMM). In healthy cells, the direct activators are sequestered to the anti-apoptotic proteins and therefore no activation of Bax or Bak

occurs. After pro-apoptotic insult, sensitizers are activated and displace the direct activators from the anti-apoptotic proteins. The direct activators then bind Bak and Bax and enhance their oligomerization in the OMM [27] (Figure 3-B).

A recent work argues in favor of this model by showing the direct activation of Bak and Bax by Bid, Bim and Puma in MEFs. Kim et al. [10] showed in this work that upon the interaction with Bim, Bid or Puma, α9 helix of Bax, critical for its insertion into a membrane, is exposed and cytoplasmic Bax can therefore be targeted to mitochondria. After insertion into the OMM, Bax homo-oligomerizes, disrupts the membrane integrity, allowing cytochrome *c* release. The oligomerization is driven by the direct activators as they remain associated with the BH1 domain of Bax. The same mechanism applies to the activation of Bak, with exception of the first step, because Bak is constitutively bound to the OMM, where it is held in a monomeric state by anti-apoptotic Bcl-2 members and also by the OMM protein VDAC2 (Voltage-dependent anion-selective channel protein-2) [10].

Recently generated *bid*$^{-/-}$*bim*$^{-/-}$*puma*$^{-/-}$ triple knockout (TKO) mice displayed the same phenotype observed in *bax*$^{-/-}$*bak*$^{-/-}$ mice (see following chapter) and although other BH3-only proteins were present, multiple cell types were significantly resistant to various apoptotic stimuli [28]. This study underlines a critical role of Bim Bid and Puma in Bax/Bak activation.

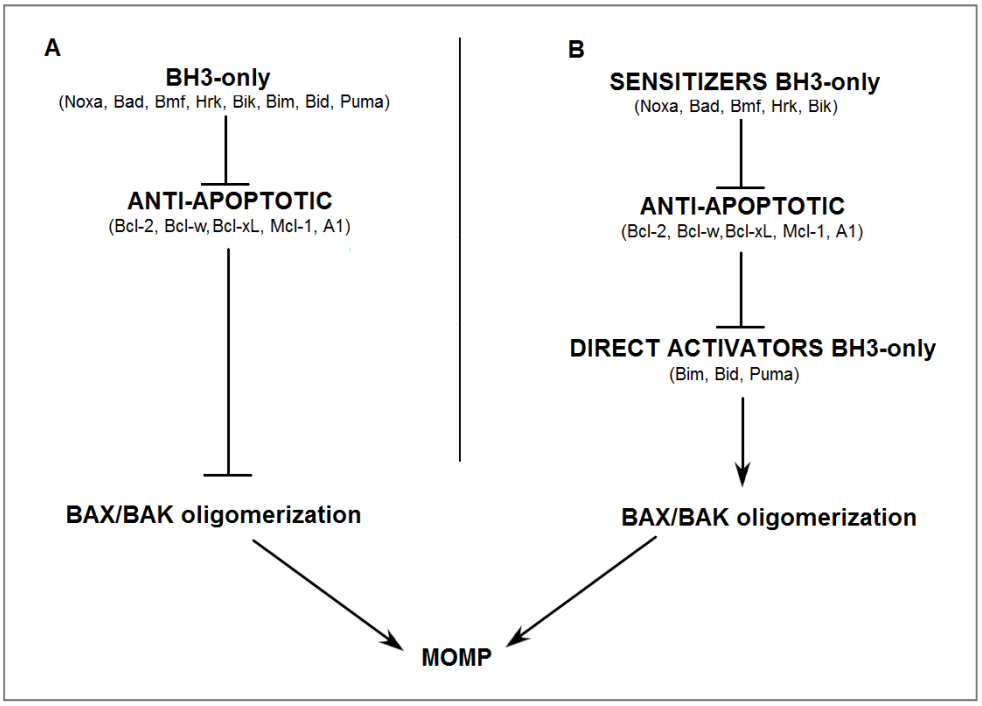


**Figure 3** - Two models of Bax/Bak activation. **(A)** Indirect activation model. Bak and Bax are inhibited by the anti-apoptotic proteins and become active when displaced by BH3-only proteins. **(B)** Direct activation model. The oligomerization of Bax and Bak is driven by the direct activators Bid, Bim and Puma, which are sequestered and inhibited by the anti-apoptotic proteins until displaced by the sensitizers.

### 3.4. Bcl-2 signaling is essential for proper development in mice

Regardless of the exact mechanism of Bax/Bak activation, the importance of the Bcl-2 apoptotic signaling in multicellular organisms is undisputed and knockout experiments in mice revealed involvement of the Bcl-2 family members in multiple physiological processes and responses to various stresses. Because Bak and Bax are direct effectors of MOMP and their activation is the convergent point of all upstream Bcl-2 apoptotic signaling, *bax*$^{-/-}$*bak*$^{-/-}$ double knockout (DKO) mouse represents a model, where no Bcl-2 family member can execute its apoptotic function and the outcome of Bcl-2 signaling is virtually completely abolished. Approximately 90% of *bax*$^{-/-}$*bak*$^{-/-}$ DKO mice died during embryogenesis and those that were viable displayed developmental abnormalities such as persistent interdigital webs, imperforate vaginal openings (Figure 4) and accumulation of neurons, lymphoid and myeloid cells. The animals also displayed defective spermatogenesis, deafness and seizure behavior [29].

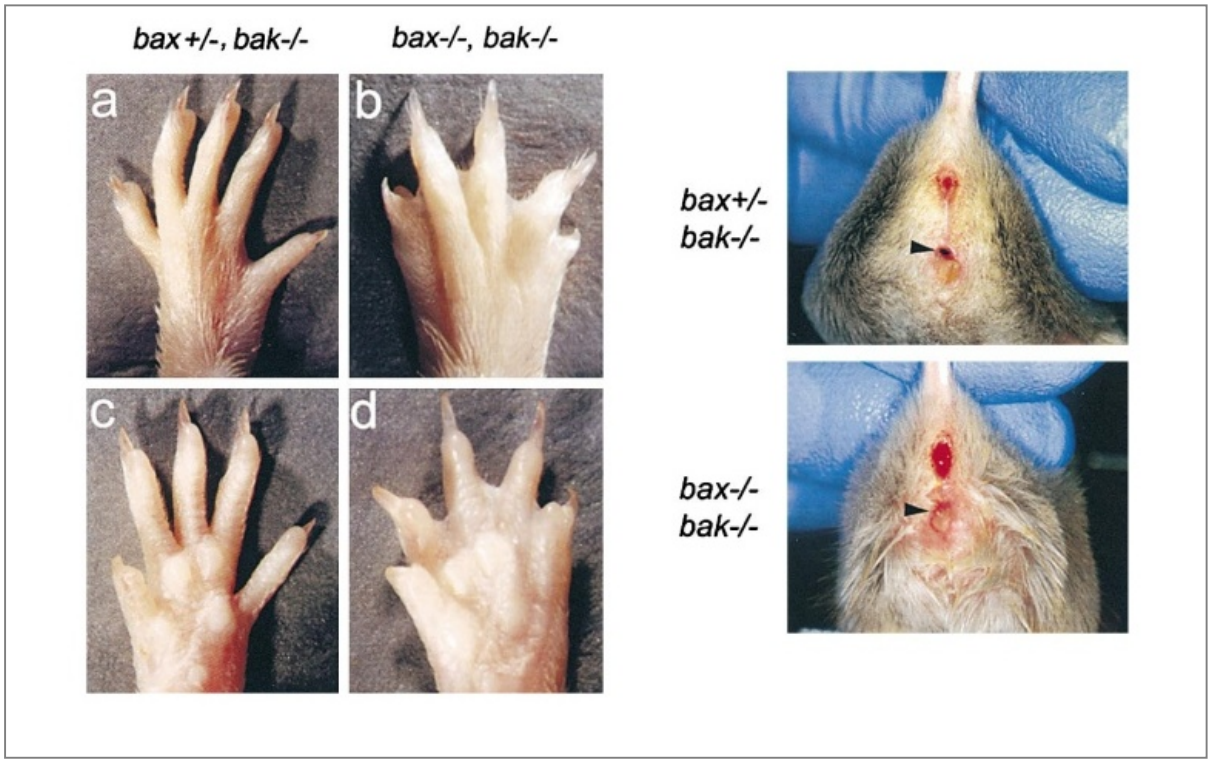


**Figure 4 -** *bax*$^{-/-}$*bak*$^{-/-}$ DKO in mouse results in persistent interdigital webs and imperforated vaginal openings. A single wild-type (WT) allele of *bax* restored WT phenotype. (*Adapted from ref. [29]*)

## 4. BH3-only proteins

BH3-only proteins represent the mediators of various extrinsic and intrinsic apoptotic signaling feeding through their functional interaction with multidomain Bcl-2 proteins mainly into triggering MOMP. Thus similarly as for caspases, their availability and function has to be tightly regulated. As "sentinels" they monitor cellular well-being and can shift affected cells towards apoptosis after developmental cues or environmental stresses. Implication of BH3-only proteins in mammalian physiology has been documented by multiple knockout experiments, which are discussed in following chapters.

As already mentioned, BH3-only proteins carry out their pro-apoptotic function both indirectly and directly; indirectly by neutralizing the anti-apoptotic members of the Bcl-2 family and directly by activating the effector proteins Bak and Bax. As mentioned in chapter 3.3.2, mice with genetically inactivated "BH3-only direct activators" Bim, Bid and Puma, display the same defects that were observed in Bax/Bak DKO mice, suggesting a key role for these three proteins in Bak/Bax oligomerization [28]. BH3-only proteins are characterized by the presence of a sole BH domain (BH3). The BH3 domain is a short amino acid stretch containing motif LxxxGDE, where L and D are conserved in all known mammalian BH3-only proteins (Figure 5-A). The nature of the BH3 domain is responsible for the binding abilities of particular BH3 proteins, and predetermines their selective binding to anti-apoptotic Bcl-2 members. For example, the substitution of two residues in BH3 domain of Noxa resulted in increased affinity to its binding partner Mcl-1 and expanded its binding repertoire to include Bcl-xL, which is normally not bound by the WT Noxa [24].

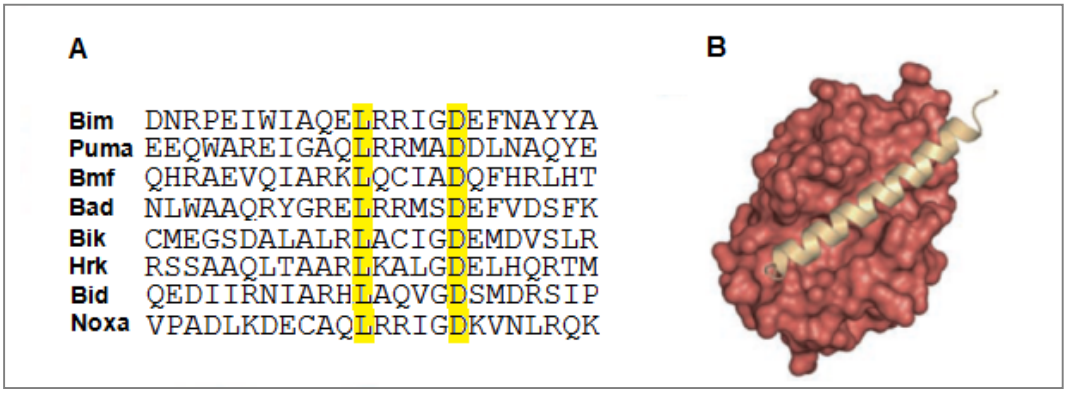


**Figure 5 - (A)** Alignment of the BH3-peptides from eight mammalian BH3-only proteins. (*Adapted and modified from ref. [27]*) **(B)** BH3 peptide of Bim (yellow) in complex with Bcl-xL. (*Adapted from ref. [5]*)

In solution, BH3-only proteins (except for Bid and probably Bik) do not adopt a stable conformation and belong to the class of so-called "intrinsically unstructured proteins" (IUPs).

Upon their association with the anti-apoptotic Bcl-2 members, BH3-only proteins undergo structural transition and their BH3 domain forms an α-helix (Figure 5-B). However, the rest of the protein remains unstructured. Such structural plasticity of BH3-only proteins allows them to bind multiple binding partners [30].

The following chapters provide an overview of the main principles of activation and function of eight mammalian BH3-only proteins. Although BH3-only proteins are involved in multiple processes, including autophagy, maintenance of $Ca^{2+}$ homeostasis, mitochondrial remodeling and glucose metabolism (reviewed in [7]), the aim of this thesis is to summarize their roles in apoptosis.

### 4.1. Bim

At least eighteen variants of Bim (Bcl-2 interacting mediator of cell death) have been described [31], but I shall focus on three major isoforms that are known to play a significant role in cell physiology. These isoforms were described in the original publication reporting Bim discovery and were named, based on their length, as BimS (short, 110 AAs), BimL (long, 140 AAs) and BimEL (extra long, 198 AAs) [32]. All the three proteins promote apoptosis, but with different efficacy, as BimS is the most potent isoform and BimEL the least effective one. BimL and BimEL are expressed in various tissues and cell types including spleen, thymus, testis, skin, neurons, liver and hematopoietic cells, while BimS is expressed only transiently in specific cases or at undetectable levels [33]. Bim has been shown to interact with all major anti-apoptotic Bcl-2 family members [26]. In addition, Bim is able to directly activate effector proteins Bak and Bax [10].

Two principal mechanisms control the availability and activity of Bim. The first one is the transcriptional control of *bim* mRNA expression by several transcription factors. *Bim* expression is regulated by the forkhead transcription factor FOXO3a, which is normally phosphorylated by protein kinase B (PKB/Akt) in the PI3K-Akt pathway. Phosphorylated FOXO3a is unable to translocate to the nucleus and cannot induce the transcription of *bim*. In lymphocytes, upon cytokine withdrawal, PKB is deactivated leading to hypophosphorylation of FOXO3a. FOXO3a then enters the nucleus and drives the transcription of *bim* [34]. Another transcription factor directly inducing *bim* expression is CHOP (C/EBP-homologous protein), which was shown to promote ER stress-mediated apoptosis *in vivo* through *bim* upregulation [35].

The posttranslational control of Bim activity is based on its phosphorylation on four sites (Figure 6). In healthy cells, BimL and BimEL are localized in the cytosol, where they are sequestered to the cytoplasmic dynein light chain DLC1, which is part of the microtubule dynein motor complex, and thus are held in an inactive state [36]. Interaction of BimL and BimEL with DLC1 is mediated via a peptide motif within exon 4, which can be phosphorylated on the Thr-56 site on BimL (Thr-112 in the case of BimEL) by the c-Jun N-terminal kinase (JNK) activated in response to genotoxic stresses, e.g. exposure to UV radiation. Such phosphorylation of BimL/EL disrupts the interaction between BimL/EL and DLC1, allowing Bim to interact with either anti-apoptotic Bcl-2 members or effector proteins Bax and Bak [37]. Moreover, it has been shown in neurons that the dominant-negative c-Jun, a transcription factor downstream of JNK, reduces Bim expression and protects the cells from apoptosis after nerve growth factor (NGF) withdrawal, suggesting that JNK-pathway is also involved in regulation of Bim at the transcriptional level [38].

The longest isoform, BimEL, contains exon 3, which is a target of pro-survival phosphorylation on Ser-55, Ser-65 and Ser-73. In healthy cells, these sites are phosphorylated by ERK in the MAPK pathway and mark BimEL for ubiquitination on Lys-3 and Lys-108, and subsequent proteasomal degradation [39]. The opposing mechanism against pro-survival ERK-mediated phosphorylation of Bim is the activation of protein phosphatase A2 (PPA2), which depho-sphorylates BimEL in response to ER stress, thereby preventing its degradation [35]. An unexpected finding was that ERK can phosphorylate BimEL on Thr-112 in JNK$^{-/-}$ fibroblasts and therefore may have, at least in specific cases, a pro-apoptotic effect, in addition to its predominant pro-survival role in Bim regulation [39].

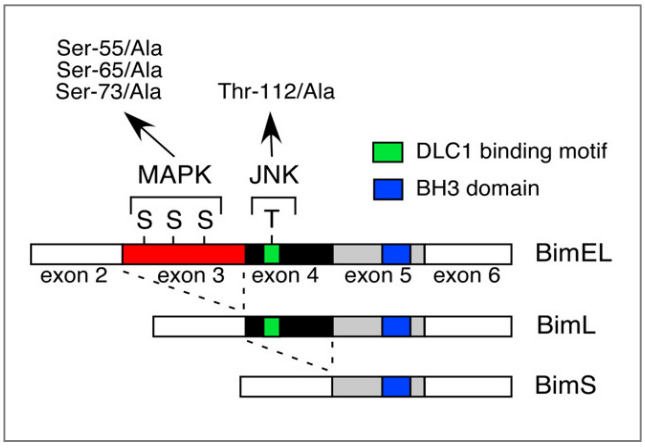


**Figure 6** - Prosurvival phosphorylation of Bim on Ser-55, -65 and -73 by ERK targets Bim for proteasomal degradation. Phosphorylation at Thr-112 by JNK under stress conditions activates Bim by disrupting Bim-DLC1 complex. (*Adapted from ref. [39]*)

Bim plays an important and essential role in the development and regulation of the immune system. *bim*$^{-/-}$ mice display elevated levels of B-cells, both CD4+ and CD8+ T-cells, monocytes and granulocytes and Bim-deficient lymphocytes are resistant to many apoptotic insults such as cytokine withdrawal and taxol treatment. Bim-mediated apoptosis is therefore

involved in both lymphoid and myeloid cell line development and maintenance of cell homeostasis in the immune system [9]. Bim-deficient mice also developed fatal systemic autoimmune diseases and, although detailed molecular mechanisms await elucidation, Bim has been determined as a critical mediator of apoptosis of autoreactive clones in T-cell development [40], apoptosis of T-cells after immune response [41] and apoptosis of developing B-cells with insufficient affinity for antigens [42].

### 4.2. Bid

Bid (BH3 interacting domain death agonist) was described as a BH3-only protein able to heterodimerize with Bcl-2 and Bax. Bid is widely expressed with the highest levels detected in the liver, kidneys, spleen, testis and lungs [43]. In addition to common 195 AAs Bid, three additional isoforms were discovered in humans - BidEL, which is larger (241 AAs) and two shorter variants lacking a BH3 domain (137 AAs BidS and 99 AAs BidES). These variants are probably involved in fine regulation of Bid-induced apoptosis, but their physiological relevance remains unclear [44].

The structure and activation, as well as its unique position in the Bcl-2 signaling, make Bid a rather non-typical BH3-only protein. In solution, Bid has a stable helix-bundle fold that resembles the structure of the multidomain Bcl-2 family members such as Bcl-2 or Bax. As already mentioned in chapter 3.2, Bid shares a common ancestry with these multidomain Bcl-2 members and is not evolutionary related to other BH3-only proteins [18].

Although full-length Bid displays certain pro-apoptotic activity, the fully active form is generated upon its proteolytic processing. The main Bid-processing enzyme is caspase-8 activated in death receptor signaling, which makes Bid an important mediator between the extrinsic apoptotic pathway and MOMP [13]. Given that activated, truncated Bid (tBid) is able to induce apoptosis both indirectly by neutralization of the anti-apoptotic Bcl-2 members and directly by driving Bax/Bak oligomerization in the OMM, it is one the most potent BH3-only death inducers [10, 26].

Although the recruitment of tBid to the OMM has been extensively studied, it still remains unresolved. Initial studies implicated mitochondrial cardiolipin as a main mediator of the insertion of tBid in the OMM [45]. However, some other reports pointed out that OMM proteins, rather than the lipid composition of OMM, play a major role in the mitochondrial targeting of tBid [46]. This is also suggested in a recent study, which identified mitochondrial surface protein MTCH2/MIMP (mitochondrial carrier homologue 2/Met-induced

mitochondrial protein) as a critical mediator of the mitochondrial targeting of tBid *in vivo* [47]. Moreover, truncated Bid becomes N-myristoylated and this modification also apparently enhances its insertion into the OMM [48].

Three sites of phosphorylation were found in mouse Bid (Thr-58, Ser-61 and Ser-64), all in close proximity to the caspase-8 cleavage site (Asp-59). Phosphorylations of these residues are mediated by casein kinase (CK) I and CKII and prevent the cleavage of Bid by caspase-8, and thus inhibit Bid activation [49].

The involvement of Bid in the extrinsic apoptotic pathway *in vivo* was demonstrated in the Bid KO mouse strain. *Bid*$^{-/-}$ animals were born with no developmental defects, but were resistant to apoptosis induced by an antibody against death receptor Fas, most notably in hepatocytes. Whereas WT animals died of hepatocellular apoptosis in 4 hours after the antibody injection, approximately half of *bid*$^{-/-}$ animals remained healthy and the rest displayed only mild liver damage. Also other cell types such as thymocytes and fibroblasts were to some extent protected from Fas antibody-induced apoptosis, but not from other insults such as staurosporine or dexamethasone treatment [50].

### 4.3. Puma

Puma (p53-upregulated modulator of apoptosis) is, similarly to Bim and Bid, one of the most potent pro-apoptotic BH3-only proteins, mainly because of its abilities to strongly bind all the anti-apoptotic Bcl-2 members and directly activate Bax and Bak [10]. *Puma* gene is ubiquitously expressed but its expression is tightly controlled at the transcriptional level. Two main splicing variants of similar properties were characterized, 193 AAs Puma-α, and 131 AAs Puma-β. Two additional isoforms (Puma-γ and Puma-δ) were predicted, but their function remains unknown and, since both of them lacking BH3 domain, their role in apoptosis is most likely insignificant [51].

Four main transcription factors directly contribute to Puma regulation in apoptosis: p53, p73, FOXO3a and E2F1. As apparent from its name, *Puma* was first identified as a gene whose expression was directly induced by tumor suppressor p53 and whose product localized to mitochondria and rapidly induced apoptosis of human fibroblast and multiple cancer cell lines [51]. *Puma* upregulation is the prominent mechanism of p53-mediated apoptosis in response to genotoxic or oncogenic stress. After certain genotoxic stimuli such as γ-irradiation, posttranslational modifications of p53 lead to its stabilization in the nucleus where it directs transcription of multiple genes including *puma* [52].

p73, a protein closely related to p53 was also identified as a direct inducer of *puma* transcription. In response to ionizing radiation, p73 is stabilized by phosphorylation mediated by activated kinase c-Abl and binds the same responsive elements as p53. Moreover, the cancer-specific p73 isoform, ΔNp73, which is able to bind *puma* promoter normally, but lacks the N-terminal transactivation domain and therefore has no transcriptional activity, suppresses both p53- and p73-induced apoptosis [53]. Another transcription factor shown to directly induce *puma* transcription is FOXO3a. This transcription factor, negatively regulated by the PI3K/Akt pathway, can also upregulate *bim* expression (see chapter 4.1), and is activated upon cytokine/growth factor withdrawal [54]. Finally, the transcription factor E2F1, normally held inactive by retinoblastoma tumor suppressor (pRB) is able to positively regulate the expression of several BH3-only proteins, mainly Puma and Noxa, and to lesser extent also Bim and Hrk. This was observed in fibroblasts infected with HPV16 virus expressing protein E7, which disrupts pRB/E2F1 complexes [55].

The posttranslational modification of Puma was reported only recently. Fricker et al. [56] demonstrated that in HeLa cells and MEFs, exogenous Puma is phosphorylated on several serine residues, most importantly on Ser-10, and that these phosphorylations do not affect its interaction with the anti-apoptotic Bcl-2 members, but rather decrease its stability. The authors argue that the phosphorylation might mark Puma for degradation in the proteasome pathway as it is the case in other BH3-only member Bim. However, it is not known which pathways are responsible for Puma phosphorylation and what is the relevance of such modification in Puma-mediated apoptosis.

The significance of Puma in both p53-dependent and cytokine withdrawal -dependent apoptosis was demonstrated in *puma*$^{-/-}$ mice [52]. Although *puma*$^{-/-}$ mice were fully viable, their thymocytes as well as postnatally developing nervous tissue were almost completely resistant to γ-irradiation-mediated apoptosis, and as the same effects were observed in *p53*$^{-/-}$ mice, it is likely that, at least in some tissues, Puma is the main mediator of p53-dependent apoptosis in response to γ-irradiation. Moreover, apoptosis in response to *c-Myc* oncogene upregulation was also highly suppressed in *puma*$^{-/-}$ mice. Damage- or p53-independent role of Puma in regulating cell homeostasis was demonstrated in cytokine-deprived primary myeloid cells. While 90% of the myeloid cells from WT animals died upon cytokine withdrawal in 3 days, only 10% of the myeloid cells from *puma*$^{-/-}$ mice died under the same conditions [52]. Overall, although Puma is non-essential for development, it is a protein of exceptional importance for apoptosis triggered under non-physiological conditions such as cytokine withdrawal and genotoxic stress.

### 4.4. Noxa

*Noxa* was originally found as a gene of unknown function upregulated in adult T-cell leukemia (ATL) cells after exposure to phorbol-12-myristate-13-acetate (PMA) and termed *APR* (ATL-derived PMA-responsive gene) [57]. After ten years, a homolog of *APR* was found in MEFs and its product was described as a BH3-only protein inducible by tumor suppressor p53. The protein was termed Noxa, which means *damage* in Latin, and its expression was detected in the mouse brain, thymus, spleen, lung, kidney and testis [58]. Activated Noxa binds most potently Mcl-1 and A1 [24]. Two additional splicing variants of Noxa were characterized, both lacking BH3 domain [59].

Similarly to Puma, Noxa has been determined as a mediator of p53-dependent apoptosis and also some other transcription factors, such as E2F1 and FOXO3a contribute to the upregulation of Noxa under similar circumstances as in the case of Puma [55, 60]. In response to DNA damage, elevated levels of both Noxa and Puma drive cells to apoptosis. However, the contribution of Noxa was shown to be limited because its deletion did not protect mouse thymocytes and fibroblasts from apoptosis induced by γ-irradiation, whereas *puma*$^{-/-}$ cells were highly resistant [8]. Interestingly, another experiment revealed that Puma is a predominant mediator of γ-irradiation-induced apoptosis, while Noxa plays a major role in apoptosis triggered by UV-irradiation in mouse fibroblasts and skin keratinocytes [61].

Another process, which implicates Noxa, is hypoxia-induced apoptosis. Noxa is upregulated in early stages of the response to brain ischemic injury and its suppression protects the brain from ischemic-induced cell death. In hypoxia-induced apoptosis, Noxa is directly upregulated by transcription factor HIF-1α (hypoxia inducible factor-1α), which normally protects cells from hypoxia, but in severe cases of oxygen deprivation rather induces apoptosis [62].

Bortezomib and Eeyarestatin are drugs which cause ER stress through inhibition of proteasome and ERAD (ER-associated protein degradation) and display cytotoxic activity in multiple cancers. Recently described mechanism of cytotoxicity of these drugs seems to be dependent on Noxa induction. In this case, Noxa upregulation is mediated by transcription factors ATF3 and ATF4 and requires deubiquitination of histone H2A [63]. Another mechanism of bortezomib-induced death of cancer cells in response to proteasome inhibition is direct induction of Noxa by c-Myc [64].

Noxa is phosphorylated by Cdk5 on Ser-13 in hematopoietic cells upon sufficient levels of glucose. After this phosphorylation, Noxa is sequestered to cytosolic multiprotein

complexes and loses its pro-apoptotic activity. In these so far uncharacterized complexes, Noxa stimulates glucose metabolism and therefore actively contributes to cell survival [65].

*Noxa* KO in mice did not result in any apparent developmental abnormalities. The animals were normally viable and displayed moderately increased resistance in multiple cell types against γ-irradiation-induced apoptosis and significant resistance of keratinocytes to UV-irradiation. A comparison of *noxa*$^{-/-}$, *puma*$^{-/-}$and *noxa*$^{-/-}$*puma*$^{-/-}$animals proved that in the case of p53-dependent apoptosis, Noxa has rather complementary function to Puma, which plays the predominant role in such responses. This could be due to the limited potential of Noxa in terms of binding the anti-apoptotic Bcl-2 proteins and its inability to directly activate Bax and/or Bak [8].

### 4.5. Bad

Bad (Bcl-2 associated agonist of cell death) was discovered in 1995 and although its BH3 domain was not initially identified, its pro-apoptotic activity as well as the ability to bind anti-apoptotic Bcl-2 proteins were immediately recognized [66]. The expression of Bad was detected in all examined rat tissues and two additional splicing variants were reported [67].

The activation of Bad in apoptosis is related to its posttranslational modifications. Bad inactivation is mediated mainly by its sequestering by the cytosolic 14-3-3 proteins and phosphorylations on sites Ser-112 and Ser-136 in mouse Bad were found to be critical for such interaction [68]. Another way to inactivate Bad is phosphorylation of Ser-155 within its BH3 domain. This modification has no effect on 14-3-3- Bad interaction, but directly prevents Bad from binding to the anti-apoptotic Bcl-2 proteins [69].

Bad can be phosphorylated in response to sufficient nutrient or cytokine/growth factor levels by several kinases. In mice, phosphorylation on Ser-112 is mediated by the Ras-MAPK pathway [70], while Ser-136 is phosphorylated by the PI3K-Akt pathway [71] and PKA kinase activated by IL-3 [71, 72]. Ser-155 is also a target of PKA [73]. Another kinase responsible for Ser-136 phosphorylation is p70S6K (70-kDa ribosomal protein S6 kinase) whose activation is dependent on IGF-1 (insulin-like growth factor-1) [74]. Bad is therefore an important convergent point for signaling pathways monitoring cellular proliferation and availability of nutrients.

Several phosphatases are known to sensitize cells to apoptosis by disrupting Bad-14-3-3 complex via Bad dephosphorylation. Calcineurin dephospohorylates Bad in response to non-physiological $Ca^{2+}$ levels [75]. PP1α (protein phosphatase 1α) and PP2A

dephosphorylate Bad upon insufficient levels of IL-2 and IL-3, respectively [76, 77]. Upon its activation, Bad translocates to mitochondria where it preferentially interacts with Bcl-2, Bcl-xL and Bcl-w [24]. Bad was recently identified as the first member of the Bcl-2 family, which is regulated by arginine methylation. Methylation of human Bad at Arg-94 and Arg-96 by PRMT1 (protein arginine methyltransferase 1) negatively regulates phosphorylation on Ser-99 (Ser-136 in mice) and thus prevents Bad from being inactivated by 14-3-3 [78].

Consistent with the pro-survival role of Bad phosphorylation, knock-in mice with Bad in Ser-112, Ser-136 and Ser-155 changed to alanines displayed increased responsiveness to multiple apoptotic stimuli, decreased levels of lymphocytes and cultured neurons died earlier than WT cells when treated with IGF-1 [79].

Although thymocytes and fibroblasts derived from *bad*$^{-/-}$ mice were not resistant to cytokine withdrawal-induced apoptosis when compared to WT cells, epithelial cells were unresponsive to their deprivation of EGF (epithelial growth factor). *Bad*$^{-/-}$ mice displayed no significant developmental abnormalities. However, Bad turned out to be a potential tumor suppressor in the immune system, since aging Bad-deficient mice developed B-cell lymphomas more frequently than WT animals and their T- and B-cell lines were more sensitive to tumorigenesis induced by γ-irradiation [80].

### 4.6. Hrk

Hrk/harakiri/DP5 was independently discovered by two groups. The first group discovered Hrk as a protein induced during apoptosis of sympathetic neurons cultured in the absence of NGF (nerve growth factor) [81]. The second group described Hrk as a binding partner of Bcl-2 and Bcl-xL in HeLa cells [82]. The expression of Hrk is restricted mainly to the nervous system; low levels were detected in the liver, lungs, kidneys and pancreas. Hrk is a weak inducer of apoptosis since it preferentially binds only Bcl-2, Bcl-xL and possibly A1 [24].

Due to its weak pro-apoptotic potential and highly specific expression, Hrk plays only a limited role in cell physiology. It was reported that Hrk is able to induce apoptosis of hematopoietic cell lines upon cytokine deprivation [83]. However, this was later denied by another group, which failed to detect Hrk expression under the same conditions. Using *hrk*$^{-/-}$ mice they also found no role for Hrk in cytokine-deprived hematopoietic cells, as the cells derived from these animals were normally responsive to cytokine deprivation [84]. A potential role for Hrk was found in type-1 diabetes, which is caused by the destruction of

pancreatic β-cells mediated by pro-inflammatory cytokines such as IL-1β and IFN-γ. *In vitro* experiment showed that in β-cells treated with these cytokines, Hrk is directly upregulated by c-Jun and the cells undergo Hrk-dependent apoptosis [85].

c-Jun-dependent regulation of Hrk was observed also in the nervous system, where Hrk plays a more prominent role [86]. Hrk expression is upregulated during apoptosis of neurons after several different stimuli such as potassium deprivation [86], NGF deprivation, axotomy [87] and exposure to amyloid β-peptide [88]. However, its sole expression is unable to induce apoptosis in potassium-deprived neurons derived from *bim*$^{-/-}$*bid*$^{-/-}$*puma*$^{-/-}$ mice [28], suggesting only a supportive role of Hrk in neuronal apoptosis. *Hrk*$^{-/-}$ mice developed normally and displayed only moderately delayed apoptotic responses in several neuronal populations [87].

### 4.7. Bik

Bik (Bcl-2 interacting killer) was the first protein recognized as a BH3-only member of the Bcl-2 family. It was discovered as a death-inducing protein interacting with Bcl-2 and its homologues [89]. Unlike most of the other BH3-only proteins, Bik appears similarly as Bid to have a stable conformation in solution and contains a C-terminal TM region [30].

Expression of Bik in mouse is tissue specific with the highest levels detected in the liver, lungs, heart, kidneys, B- and T-cell lines and endothelial cells. Lower levels were detected in the spleen, skeletal muscle and salivary glands [90].

Activated Bik operates as a pro-apoptotic protein by two distinct mechanisms. As a classical BH3-only protein, it neutralizes the anti-apoptotic Bcl-2 family members, most potently Bcl-2 and Bcl-xL. The second mechanism by which Bik sensitizes cells to apoptosis is based on $Ca^{2+}$ release from ER, which is mediated by the fraction of Bik anchored in the ER membrane. Released $Ca^{2+}$ serves as a signal for the recruitment of DRP-1 (dynamin related protein-1) to the OMM and subsequent remodeling of the mitochondrial cristae, which enables cytochrome *c* to be released more efficiently from the mitochondrial intermembrane space during apoptosis [91]. Besides Bik, another BH3-only protein, Puma, was shown to promote $Ca^{2+}$ release from ER during apoptosis [92].

Several transcription factors contribute to Bik expression in response to different apoptotic stimuli. P53 upregulates Bik in response to adenoviral E1A oncogene [93] and Smad transcription factors activated by TGF-β (transforming growth factor-β) induce *bik*

transcription in B-cells [94]. In several cancer cell lines treated with chemotherapeutics, Bik was upregulated in E2F-dependent manner [95].

Human Bik is phosphorylated on two residues (Thr-33 and Ser-35). The kinase responsible for Bik phosphorylation is probably a casein kinase II (CKII) -related enzyme. Substitution of Thr-33 and Ser-35 by alanines resulted in decreased pro-apoptotic activity of Bik, but the underlying mechanism of this lower apoptotic activity is unknown [96].

In *bik*$^{-/-}$ mice, no developmental malfunctions were observed and no examined cell type displayed changed sensitivity to multiple apoptotic stimuli, suggesting that the role of Bik in apoptosis is only additional or overlapping with that of other BH3-only proteins [90]. Despite the non-essential role of Bik in development and apoptotic responses, its deletion has been observed in human renal cell carcinoma, indicating Bik as a potential tumor suppressor [97]. Moreover, Bik seems to play a prominent role in apoptotic responses to protein synthesis inhibition caused by adenoviral infection or bacterial pathogens [98].

### 4.8. Bmf

Bmf (Bcl-2 modifying factor), the last and also "youngest" BH3-only member discussed in this thesis, was discovered ten years ago. Bmf expression has been detected in many tissues and cell lines including liver, kidneys, lungs, pancreas, testes, lymphoid cell lines, myeloid cell lines and fibroblasts. Bcl-2, Bcl-xL, Bcl-w and Mcl-1 were identified as likely binding partners for Bmf [99]. Two additional splicing variants were reported, both without BH3-domain [100].

The activation of Bmf proceeds in a similar manner as in the case of Bim. In healthy cells, Bmf is associated with the cytoskeleton and therefore unable to bind the anti-apoptotic Bcl-2 proteins. While Bim is associated with microtubules via DLC1, Bmf binds to DLC2, which is part of the myosin V motor complex associated with the actin cytoskeleton [99]. After apoptotic stimulus, such as UV-irradiation or anoikis, Bmf is phosphorylated by JNK kinase at Ser-74, which lies in the DLC2 binding domain, and rapidly translocates to mitochondria, where it binds the anti-apoptotic Bcl-2 proteins [37, 99]. Anoikis is a term for apoptosis triggered by the detachment of cells from the surrounding matrix. The loss of cell-matrix interaction affects many aspects of cell physiology, from which JNK activation and actin filaments perturbation probably participate in Bmf activation. (reviewed in [101]).

Bmf-deficient mice showed significantly (over 50 %) increased counts of B-cells at various stages of development, suggesting a role for Bmf in B-cell homeostasis. The cells

were also resistant to glucocorticoid treatment and histone deacetylase inhibitor. Interestingly, anoikis, a process involving Bmf activation in breast cancer cells, was not delayed in *bmf*$^{-/-}$ colon epithelial cells and nor was changed the sensitivity of *bmf*$^{-/-}$ MEFs to UV-irradiation. Another interesting finding in *bmf*$^{-/-}$ mice was the high susceptibility of the aging animals to γ-irradiation-induced thymic lymphoma, which was roughly the same as in *p53*$^{+/-}$ mice [102].

### 4.9. BH3 mimetics

Considering that insensitivity to apoptotic stimuli has been observed in virtually all types of cancer, Hanahan and Weinberg [3] postulated that evasion of apoptosis is one of the capabilities a cell needs to acquire in order to turn cancerous. The fact that Bcl-2 was originally discovered in human follicular lymphoma immediately suggested its possible involvement in tumorigenesis [15]. Since then, the structural studies and a great progress in understanding the role of BH3-only proteins in the Bcl-2 signaling provided a theoretical basis for designing molecules that would sensitize cancer cells to apoptosis by targeting anti-apoptotic Bcl-2 members.

One of these so-called "BH3 mimetics" is ABT-737 (Figure 7-A), a small organic molecule that mimics the BH3 domain of Bad and binds the hydrophobic grooves of Bcl-2, Bcl-xL and Bcl-w. In combination with other chemotherapeutics such as paclitaxel, ABT-737 significantly sensitizes multiple cancer cell lines to apoptosis. More importantly, as a single agent, ABT-737 was shown to potently kill human lymphoid cancer cells and induce rapid and complete regression of small cell lung carcinoma xenografts in mice (Figure 7-B) [103]. Currently ongoing phase I clinical trials of Navitoclax (ABT-263), which is an orally bioavailable derivative of ABT-737, have so far shown promising results and acceptable side effects in patients with solid tumors [104].

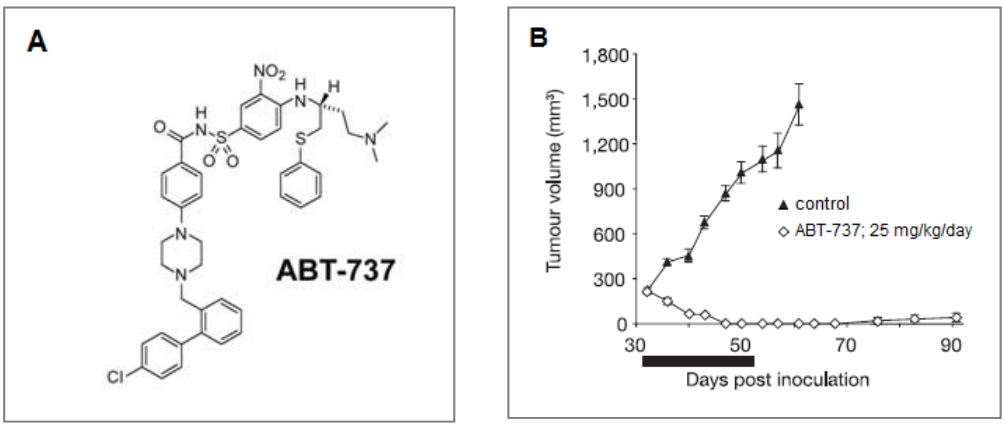


**Figure 7** - **(A)** Chemical structure of ABT-737 **(B)** Complete regression of small cell lung carcinoma xenografts in mice treated with ABT-737 (*Adapted from ref. [103]*)

Another example of a molecule that potently antagonizes Bcl-2 anti-apoptotic members is gossypol (Figure 8), a naturally occurring compound contained in cotton plant seeds (*Gossypium*). Especially its (-)-enantiomer (also known as AT-101) has been shown to induce apoptosis in cancers including leukemia and prostate cancer among others. Therapeutic use of gossypol is currently being evaluated in phase I/II of the clinical trials [105, 106].

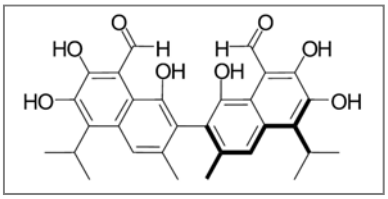


**Figure 8** – Chemical structure of (-)-gossypol (*adapted from ref. [105]*)

## 5. Conclusions

Despite their presence in several invertebrates such as nematodes and cnidarians, BH3-only proteins are unique by means that they have been directly linked to mitochondrial apoptosis only in vertebrates; apart from mammals, BH3-only homologues have also been characterized in birds and fishes [18]. A need for robust and multilateral apoptotic responses throughout the life of vertebrates could explain the partial redundancy and overlapping functions of some BH3-only proteins in these complex organisms.

A huge advance in understanding the Bcl-2 family signaling in mammals has been made since the discovery of founding member Bcl-2 in 1985. Especially *in vivo* studies in mice identified the Bcl-2 family members as crucial regulators of apoptosis.

BH3-only proteins, which trigger signaling events leading to MOMP upon their activation by various pro-apoptotic stimuli, have been extensively studied during the last fifteen years. Although a complex regulation of BH3-only proteins on both transcriptional and posttranslational level has been already uncovered, relatively little is known about the detailed mechanism by which these proteins induce apoptosis.

Interesting is the debate about whether they just passively block anti-apoptotic Bcl-2 proteins or in addition they also participate in the formation of Bax/Bak megachannels in the OMM by their direct functional interaction with Bax or Bak. Two recent publications argue in favor of the latter hypothesis. The first one illustrates a mechanistic basis of direct activation of Bax/Bak by putative direct activators Bim, Bid and Puma [10]. In the second work, published by the same group, these claims were supported using $bim^{-/-}bid^{-/-}puma^{-/-}$ mice, which showed almost completely suppressed apoptosis in multiple cell types despite the presence of other BH3-only proteins [28]. Intriguingly, a recent report suggests that even BH3-only proteins other than Bid, Bim and Puma, mainly Noxa and Bmf, may to some extent contribute to direct activation of Bax/Bak [107]. However, since this was demonstrated only

in a simplified *in vitro* assay with BH3 peptides but not full length proteins, further investigation is required. On the other hand, it has also been shown, both *in vitro* and *in vivo*, that in certain situations, Bak/Bax oligomerization is independent of the direct activators. This comes, for example, from recent observation in thrombocytes, where MOMP induced by Bcl-xL knockout proceeded independently of Bim, Bid and Puma [108]. Hence, it appears that both models are legitimate, depending on the cellular context and the nature of apoptosis-inducing stimuli.

It is problematic to generalize particular findings on BH3-only proteins, because their activation and function is also dependent on cell type and apoptotic stimulus. For example, Bmf mediates anoikis in breast cancer cells [99], but not in colon epithelial cells [102]. Similarly, Noxa induces apoptosis in response to UV-irradiation in keratinocytes, but not in other cell types, where it plays only a complementary role to Puma [61].

BH3-only proteins and in fact the entire Bcl-2 family represent a textbook example of "breeding" the top-quality basic research into its practical applicability. Although our understanding of the structure-functional relations within the Bcl-2 family is still far from complete, current knowledge has allowed the development of a new generation of anti-tumor drugs – BH3 mimetics. Some of these promising compounds such as gossypol or Navitoclax are currently undergoing phase I/II of clinical trials.

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