

Chapter 2

Exploiting BH3 Mimetics for Cancer Therapy

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Abstract In apoptotic cells, the transcriptional induction or posttranslational activation of Bcl-2-homology domain-3 (BH3)-only proteins triggers the activation of the pro-apoptotic pore-forming proteins Bax and Bak. All members of this subgroup of the Bcl-2 family share a nine amino acid BH3-domain which binds to a hydrophobic groove of anti-apoptotic Bcl-2 family members that comprises residues of their BH1, BH2 and BH3 domains. These observations led to the development of BH3 mimetics, a class of small-molecule inhibitors targeting the BH3-binding domain of the pro-survival Bcl-2 family members, thereby facilitating/activating Bax/Bak-dependent apoptosis. In addition, BH3 mimetics can displace the pro-autophagic BH3only protein Beclin-1 from a complex with pro-survival Bcl-2 family members to induce autophagy. BH3 mimetics hold great promise for

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the treatment of cancer and currently, a large variety of natural and synthetic BH3 mimetics are characterized in preclinical studies and developed in clinical studies in an aim to exploit their therapeutic potential for the treatment of cancer.

Keywords Bcl-2 family • BH3-only proteins • Programmed cell death • Apoptosis • Autophagy • Cell death resistance • Cancer • Mitochondrial membrane permeabilization • Intrinsic apoptosis pathway • Caspases • Stress signaling • Target specificity

1 Introduction

Apoptosis is an evolutionary conserved process enabling multicellular organisms to eliminate damaged and unwanted cells by executing a cellular suicide program. Defects in apoptotic signalling pathways and overexpression of anti-apoptotic genes play fundamental roles in the development of cancer (Hanahan and Weinberg 2000; Lowe and Lin 2000; Evan and Vousden 2001; Igney and Krammer 2002; Fulda and Debatin 2004). The caspase family of aspartate proteases are central executioners of apoptotic cell death (Alnemri et al. 1996; Nicholson 1999; Degterev et al. 2003; Fischer et al. 2003) and two major caspase-activating pathways predominate. Activation of caspases can occur either after ligation of death ligands to their cell surface receptors (extrinsic pathway) or after the release of pro-apoptotic factors from mitochondria (intrinsic pathway) (Hengartner 2000; Fulda and Debatin 2006). In the intrinsic pathway, activation of the initiator caspase-9 occurs via binding of adaptor protein apoptotic protease activating factor-1 (Apaf-1) to the caspase recruitment domain (CARD) (Garrido et al. 2006). The association of caspase-9 and Apaf-1 and subsequent apoptosome formation is triggered by the pro-apoptotic factor cytochrome c. Release of cytochrome c from mitochondria is therefore the key regulatory step in the mitochondrial apoptosis pathway (Martinou and Green 2001) (Fig. 1).

1.1 *Bcl-2 Family Members: Arbiters of Cell Survival*

Pro- and anti-apoptotic members of the Bcl-2 family are key regulators of apoptotic and non-apoptotic cell death. The Bcl-2 family proteins can be classified into three subfamilies: (i) the BH3-only proteins which have only one domain in common, the alpha helical BH3 domain; (ii) the Bax-like proteins which contain three such domains (BH1,2,3) and (iii) the Bcl-2-like proteins which contain 4 domains (BH1-4) (Kroemer et al. 2007). Bax and Bax-like protein Bak trigger mitochondrial permeabilisation which is required for the release of pro-apoptotic factors from the mitochondria into the cytosol (Kroemer et al. 2007) (Fig. 2).

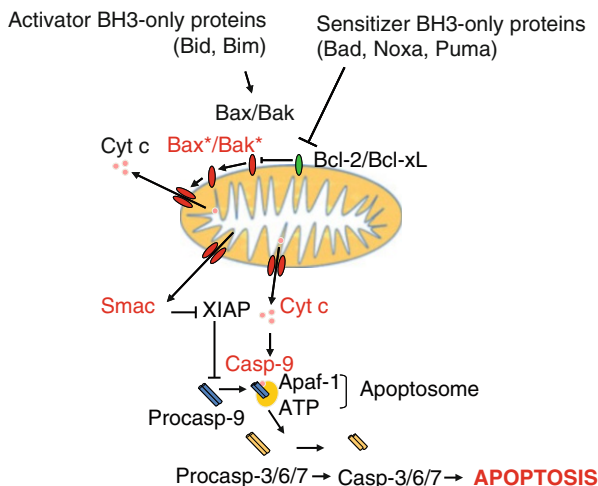


Fig. 1 Bcl-2 family members positively and negatively regulate the activation of the intrinsic apoptosis signalling pathway. In non-apoptotic cells, activation of Bax or Bak is inhibited by direct binding of anti-apoptotic Bcl-2 family members (Bcl-2-like proteins) such as Bcl-2 and Bcl-x_L. Upon apoptosis induction, the transcriptional or posttranslational activation of Bcl-2-homology domain-3 (BH3)-only proteins subsequently triggers the activation of Bax and Bak. Activator BH3-only proteins (Bid, Bim) directly bind to and oligomerize Bax and Bak (activated Bax and Bak are denoted as Bax* and Bak*). Sensitizer BH3-only proteins (Bad, Noxa, PUMA) bind to and neutralize the pro-survival Bcl-2 family members, thereby displacing them from Bax and Bak to facilitate MOMP, cytochrome c release and subsequent activation of initiator caspase-9 and downstream effector caspase induction. Abbreviations: *Apaf-1* apoptotic peptidase activating factor 1, *Bax* Bcl-2-associated X protein, *Bak* Bcl-2-antagonist/killer 1, *Bcl-2* B-cell lymphoma 2, *Bcl-w* Bcl2-like-2, *Bcl-x_L* Bcl-x long, *Bid* BH3 interacting domain death agonist, *Cyt c* cytochrome c, *Mcl-1* myeloid cell leukaemia sequence 1, *Procasp* procaspase, *Casp* caspase, *Smac* second mitochondrial activator of caspases, *tBid* truncated Bid, *XIAP* X-linked inhibitor of apoptosis

In non-apoptotic cells, activation of Bax or Bak is inhibited by direct binding of anti-apoptotic Bcl-2 family members (Bcl-2-like proteins) such as Bcl-2 and Bcl-x_L (Ranger et al. 2001; Cory and Adams 2002; Adams and Cory 2007; Danial 2007). Both Bcl-2 and Bcl-x_L normally reside in the outer mitochondrial membrane, but to a less extent are also localized to the ER membrane and nuclear envelope, facing the cytosol. Bcl-2 family members can contain a carboxy-terminal hydrophobic transmembrane (TM) domain in addition to up to four Bcl-2 homology domains (BH1-4) corresponding to α -helical regions in the proteins. While homodimerization of Bcl-2 and Bcl-x_L involves a head- to- tail interaction, heterodimerization of Bcl-2/Bcl-x_L with Bax/Bak is performed in tail- to- tail fashion and requires a pocket formed by the BH1, BH2, and BH3 region of Bcl-2/Bcl-x_L as well as a central region in Bax/Bak where the BH3 domain is located. Since the molecular cloning of Bcl-2 by Korsmeyer and colleagues, there has been an ever increasing interest in the role of Bcl-2 in drug resistance and its exploitation as a

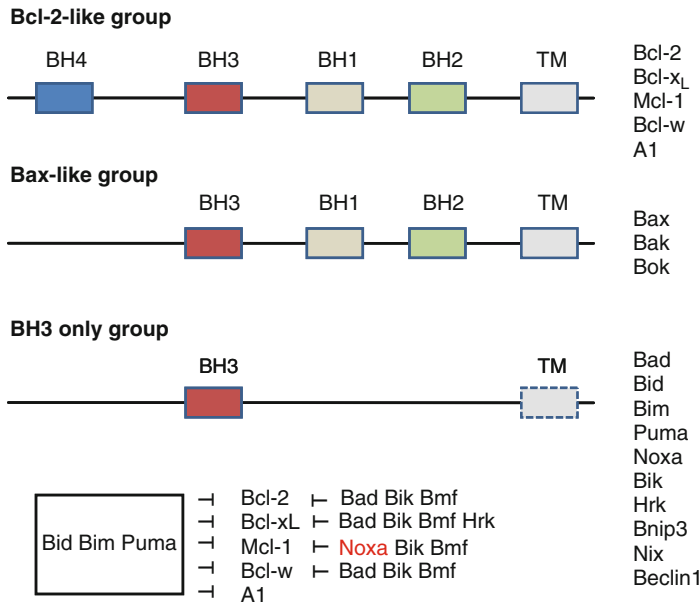


Fig. 2 Pro- and anti-apoptotic members of the Bcl-2 family. The Bcl-2 family proteins can be classified into three subfamilies: (i) the anti-apoptotic Bcl-2-like proteins which contain 4 Bcl-2 homology domains (BH1-4), (ii) the Bax-like proteins which contain three such domains (BH1,2,3) and (iii) the BH3-only proteins which have only the BH3 domain and in some cases a transmembrane (TM) domain, but otherwise share very little structural homology. The BH3-only proteins serve to couple diverse death and stress stimuli to the mitochondrial death program. Activated BH3-only proteins differ in their binding affinities to the pro-survival Bcl-2 proteins, e.g. Noxa which has an especially high affinity to Mcl-1

drug target. In mammalian cells, there are five pro-survival Bcl-2 family members (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w and A1 [Bfl-1]) (Fig. 2) and there is abundant evidence that overexpression of anti-apoptotic Bcl-2 family members constitutes a general hallmark of haematological malignancies and solid tumours. While the role of the four major pro-survival members Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w in tumorigenesis and therapy resistance is well established, the potential role of A1 is only beginning to be unravelled (Vogler 2012).

Upon apoptosis induction, the transcriptional or posttranslational activation of Bcl-2-homology domain-3 (BH3)-only proteins subsequently triggers the activation of Bax and Bak. In addition to Bax and Bak, The BH3 domains of BH3-only proteins can also bind to the hydrophobic groove of the anti-apoptotic Bcl-2 family members. All members of the BH3-only subgroup share a nine amino acid BH3-domain, but otherwise possess very little structural homology. Members of this subgroup include Bim, Bid, Bad, PUMA, Noxa, Hrk, and Bmf (Huang and Strasser 2000; Bouillet and Strasser 2002; Puthalakath and Strasser 2002). The role of the individual BH3-only family members is to couple specific upstream stress signals (e.g. DNA damage, ER stress, proteasomal stress) to the intrinsic pathway of apoptosis.

There are two competing models for activation of Bak and Bax by BH3-only proteins (Green 2006). In the direct activation model proposed by Letai et al., BH3-only proteins termed as activators (Bid, Bim) directly bind to and oligomerize Bax and Bak. In the indirect activation model, BH3-only proteins denoted as sensitizers (Noxa, PUMA, Hrk, Bmf) bind to and neutralize the pro-survival Bcl-2 family members, thereby displacing them from Bax and Bak to facilitate MOMP (Fig. 1). Sensitizer BH3 proteins therefore decrease the apoptotic threshold in cells already primed to death.

Induction of apoptosis is a major mechanism by which most chemotherapeutic drugs and radiation kill tumour cells. However, conventional cancer therapies fail to mediate their effects in a target-specific fashion. This chapter focuses on BH3 mimetics, a new class of small molecule inhibitors targeting the BH3-binding domain of the pro-survival Bcl-2 family members (Lessene et al. 2008; Kang and Reynolds 2009). Since pro-survival Bcl-2 family members are known to be overexpressed in a wide variety of human malignancies and since the intrinsic pathway of apoptosis is implicated in the cell death-inducing effects of most chemotherapeutic drugs as well as gamma irradiation, BH3 mimetics are perceived to be highly promising anti-cancer drugs and apoptosis sensitizers. As outlined in detail below, there are several synthetic and natural BH3 mimetics with different binding profiles to the pro-survival Bcl-2 family members (Bcl-2, Bcl-x_L, myeloid cell leukaemia-1 [Mcl-1], Bcl-w [Bcl2-like-2], A1). Most of these BH3 mimetics target Bcl-2 and Bcl-x_L. The highly selective inhibitor ABT-737 and its orally applicable derivative ABT-263 target Bcl-2, Bcl-x_L and Bcl-w with high affinity, but not Mcl-1 (Oltersdorf et al. 2005; van Delft et al. 2006; Chonghaile and Letai 2008). Gossypol and Obatoclax (GX15-070, Geminx) are so-called pan-Bcl-2 inhibitors targeting the four major pro-survival Bcl-2 family members Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w. In contrast to the experimental and clinical progress regarding these various inhibitors targeting the pro-survival Bcl-2 family members and therefore mimicking the function of sensitizing BH3 only proteins, BH3 mimetics that directly activate Bax and Bak are currently in the early stages of preclinical development. One exception to this classification is Obatoclax which may act both as a sensitizer and an activator BH3 mimetic.

Of note, the pro-survival Bcl-2 family members interact with a large number of additional molecules not directly involved in regulation of the intrinsic pathway of apoptosis and these interactions may also be affected by BH3 mimetics. For example, it has been demonstrated that Bcl-2 activates the anti-apoptotic nuclear factor- κ B (NF- κ B) pathway by a signalling mechanism that involves Raf-1/MEKK-1-mediated activation of IKK β . In addition, the BH3 binding groove of Bcl-2 was shown to interact with a mitochondrial pool of glutathione (GSH), the major cellular ROS scavenger and this interaction is thought to contribute to the antioxidant function of Bcl-2 (Zimmermann et al. 2007). In line with this hypothesis, BH3 mimetics were shown to disrupt the Bcl-2/GSH interaction in neurons and to suppress the transport of GSH into isolated brain mitochondria (Zimmermann et al. 2007).

In addition to their role in apoptosis, pro-survival Bcl-2 family members are also modulators of autophagy. Autophagy is a form of cellular self-digestion in which cellular constituents are engulfed in double-membrane containing vesicles called autophagosomes (Codogno and Meijer 2005; He and Klionsky 2009). Their

vesicular content is subsequently digested by lysosomal proteases after fusion of autophagosomes with lysosomes (Codogno and Meijer 2005; Kimura et al. 2007). Autophagy is a complex, multistep process which is genetically regulated by the ~30 Atg genes discovered hitherto in mammals. Bcl-2-like proteins can form a complex with the core autophagy regulator Beclin-1 (Atg6) (Pattingre et al. 2005; Pattingre and Levine 2006; Maiuri et al. 2007), and formation/dissociation of this complex plays an important role in modulating autophagy in healthy cells and tumour cells. Interestingly, Beclin-1 is a BH3 protein incapable of inducing apoptosis. The Bcl-like proteins sequester Beclin-1 via binding to its BH3 domain and prevent it from forming a multiprotein complex essential for vesicle nucleation during the early steps of the autophagic process, thereby inhibiting autophagy. Consequently, BH3 mimetics are capable to activate both apoptosis and autophagy (Hetschko et al. 2008). Recently it was also demonstrated that the mitochondrial pool of Bcl-2 can inhibit autophagy by sequestering the pro-autophagic factor AMBRA1 (Pattingre et al. 2005; Strappazzon et al. 2011).

Autophagy is normally involved in regulated turnover of long-lived proteins and damaged organelles, but the net effects of autophagy on cell death are highly contextual and may depend on the extent of autophagy. Autophagy may comprise a primordial pro-survival stress response, e.g. under conditions of nutrient deprivation where it serves to ensure energy balance, but there is also evidence that enforced, prolonged over-activation of autophagy can lead to autophagic cell death (type II cell death), i.e. massive cellular self-digestion via the autophagosomal-lysosomal pathway beyond the point allowing cell survival (Edinger and Thompson 2004; Gozuacik and Kimchi 2004; Codogno and Meijer 2005; Degenhardt et al. 2006; Gozuacik and Kimchi 2007) and it is tempting to speculate that autophagy may possibly act as a backup for apoptosis in apoptosis-deficient cells.

2 BH3 Mimetics Employed in Preclinical Studies

HA14-1 was initially identified in a computer screen based on the structure of Bcl-2 and binds Bcl-2 with an IC_{50} value of ~9 μ M as determined in competitive fluorescence polarization assays with a conjugated peptide derived from the BH3 domain of Bak (Wang et al. 2000). HA14-1 does not target the other major anti-apoptotic Bcl-2 family members Bcl-x_L, Mcl-1 and Bcl-w (Table 1). HA14-1 is capable to induce an apoptotic type of cell death in cellular models of various types of cancer, including haematopoietic malignancies, colon cancer, glioblastoma and neuroblastoma and can enhance the cell killing effects of several chemotherapeutic agents including doxorubicin, bortezomib and dexamethasone (Sinicrope et al. 2004; Manero et al. 2006). In line with its proposed action as a Bcl-2 inhibitor, HA14-1 was shown to activate Bax, caspase-9 and downstream effector caspase caspase-3 and its killing effects were reduced, but not completely abrogated in Bax/Bak-deficient cells. One major drawback of HA14-1 is its limited stability, as HA14-1 has been shown to decompose very rapidly in solution (~15 min). Therefore, several HA14-1

Table 1 BH3 mimetics employed in preclinical and clinical studies

	Drug	Target	Stage of development	References
Selective inhibitors	HA14-1	Bcl-2	Preclinical	Kang and Reynolds (2009)
	BH3-Is	Bcl-x _L	Preclinical	Kang and Reynolds (2009)
	YC137	Bcl-2	Preclinical	Bodur and Basaga (2012)
	S1	Bcl-2, Mcl-1		Bodur and Basaga (2012)
	Antimycin A	Bcl-2, Bcl-x _L	Preclinical	Kang and Reynolds (2009)
	ABT-737	Bcl-2, Bcl-x _L , Bcl-w	Preclinical	Lessene et al. (2008)
	ABT-263	Bcl-2, Bcl-x _L , Bcl-w	Phase I/II	Kögel et al. (2010)
Pan-Bcl-2 inhibitors	Gossypol/AT-101	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Phase I/II	Kögel et al. (2010)
	TM-1206	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Preclinical	Lessene et al. (2008)
	TW-37	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Preclinical	Bodur and Basaga (2012)
	Apogossypolone	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Preclinical	Bodur and Basaga (2012)
	BI-97C1	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Preclinical	Bodur and Basaga (2012)
	Obatoclax	Bcl-2, Bcl-x _L , Bcl-w, Mcl-1	Phase I/II	Kögel et al. (2010)

derivatives have been developed. Tian and colleagues have synthesized sHA14-1, a chemically modified derivative with improved stability (Tian et al. 2008). Another more stable HA14-1 derivative is SV30 (Weyland et al. 2011). SV30-induced cell death was reported to (at least partially) require the mitochondrial pathway of apoptosis (Weyland et al. 2011). A third HA14-1 analogue is EM20-25 (Milanesi et al. 2006). Similar to HA14-1 and sHA14-1, EM20-25 can synergize with other apoptotic stimuli to enhance tumour cell death. However, the killing mechanisms of the three novel derivatives are ill-defined and may also be related to off target effects of the drugs. In line with this notion, sHA14-1 was shown to induce calcium release from the ER prior to activation of the intrinsic apoptosis pathway (Hermanson et al. 2009).

BH3-Inhibitors (BH3-Is) are a series of preclinical small molecule inhibitors that have been identified in a high-throughput screen based on disruption of the interaction of Bak with the BH3 domain of a recombinant glutathione-S-transferase (GST)–Bcl-xL fusion protein and they target both Bcl-2 and Bcl-xL (Degterev et al. 2001). They bind to Bcl-2, Bcl-x_L and Mcl-1 in the micromolar range (Table 2), and are capable to activate the intrinsic pathway of apoptosis and to synergize with cancer drugs and radiation. Similar to HA14-1 and its derivatives, BH3-Is however seem to also exert off target effects.

YC137 is a cell-permeable naphthoquinone compound with low micromolar affinity for Bcl-2 (Real et al. 2004). YC137 was reported to selectively induce apoptosis in Bcl-2-overexpressing cells and to sensitize them to DNA damaging agents. Another promising novel BH3 mimetic in preclinical development is S1 that binds both Bcl-2 and Mcl-1 at low nanomolar concentrations to induce the intrinsic apoptotic pathway and exerts significantly lower toxicity in Bax/Bak-deficient cells than in wild type (wt) control cells (Zhang et al. 2011).

Antimycin A is a streptomyces-derived BH3 mimetic and natural inhibitor of the ubiquinone–cytochrome c oxidoreductase complex at the mitochondrial respiration chain. Molecular docking simulations suggest that Antimycin A also binds to the BH3 binding groove of anti-apoptotic Bcl-2 proteins (Tzung et al. 2001). The 2-methoxy-Antimycin A3 derivative may act as a bona fide BH3 mimetic because it lacks the inhibitory function of Antimycin A on mitochondrial respiration. Antimycin A3 also was shown to exert significant *in vivo* anti-tumour activity (Cao et al. 2007).

3 BH3 Mimetics in Clinical Development

3.1 ABT-737 and ABT-263

The most advanced and best-characterized synthetic Bcl-2 inhibitor is the Bad-like BH3 mimetic ABT-737 (Abbott Laboratories) (Tables 1 and 2) which was rationally designed with the aid of structure-activity relationship (sAR) analysis of ligand binding to the hydrophobic groove of BCL-xL by a nuclear magnetic resonance

Table 2 Binding affinities of BH3 mimetics to the major four pro-survival Bcl-2 family members

Compound	K_i (nM)				Fluorescence polarization assay	References
	Bcl-2	Bcl-x _L	Mcl-1	Bcl-w		
ABT-737	<1	<0.5	ND	ND	Bad- and Bax-FITC peptides	Vogler et al. (2009a)
ABT-737	120	64	>20,000	24	Bid-FITC peptide	Vogler et al. (2009a)
ABT-263	<1	<0.5	550	<1		Vogler et al. (2009a)
Gossypol	ND	500	ND	ND	Bad-FITC peptide	Vogler et al. (2009a)
Gossypol	320	480	180	ND	Bid-FAM peptide	Vogler et al. (2009a)
Gossypol	280	3,030	1,750	1,400	Bid-FITC peptide	Vogler et al. (2009a)
Apogossypolone	35	660	25	ND		Vogler et al. (2009a)
BL-97C1	320	310	200	ND	Bak-FITC peptide	Wei et al. (2010)
Obatoclox	1,110	4,690	2,000	7,010	Bid-FITC peptide	Vogler et al. (2009a)
HA14-1	9,000	ND	ND	ND	Bak peptide	Vogler et al. (2009a)
BH3I-1	ND	2,400	ND	ND	Bak-Oregon Green peptide	Vogler et al. (2009a)
BH3I-1	1,140	5,860	2,170	2,330	Bid-FITC peptide	Vogler et al. (2009a)
Antimycin A	2,950	2,700	2,510	4,570	Bid-FITC peptide	Vogler et al. (2009a)
TW-37	290	1,110	260	ND	Bid-FAM peptide	Vogler et al. (2009a)
TW-37	120	1,100	260	ND	Bid-FAM peptide	Vogler et al. (2009a)

Adapted from Vogler et al. (2009a)

(NMR)-based screening approach followed by structure-based drug design (Oltersdorf et al. 2005). A-385358 (compound 73R), a predecessor of ABT-737 derived from this approach, has K_i values of 0.8 nM against Bcl-xL and 67 nM against Bcl-2 (Shoemaker et al. 2006; Wendt et al. 2006). It also shows cell killing activity in tumour cells overexpressing BCL-XL. A-385358 synergizes with multiple pro-apoptotic cancer drugs *in vitro* but its pro-apoptotic effects were shown to be blunted in the presence of serum. Subsequent studies led to the discovery of ABT-737, a molecule with high affinity for BCL-2 and BCL-xL (IC₅₀ values of <1 nM and <0.5 nM, respectively)(Oltersdorf et al. 2005). These sub-nanomolar affinities of ABT-737 for Bcl-2 and Bcl-xL are significantly lower than for most other BH3 mimetics. Similar to the BH3 domain of Bad, ABT-737 binds to Bcl-2, Bcl-x_L and Bcl-w, but not to Mcl-1 or A1 (Zhai et al. 2006). The pro-apoptotic activity of ABT-737 has been shown to be tightly correlated to the respective expression levels of the pro-survival Bcl-2 family members. In line with the high affinity of ABT-737 for Bcl-2, cancer cells with overexpression of endogenous Bcl-2 are particularly vulnerable to ABT-737-induced apoptosis whereas cells with high expression of Mcl-1 are resistant to the drug.

A major limitation of ABT-737 is that it is not orally bioavailable, but further modifications of ABT-737 have resulted in the development of the orally available derivative ABT-263 (Navtioclax), which retains its high affinity binding to Bcl-2, Bcl-x_L and Bcl-w (Tse et al. 2008) (Table 2). In xenograft models of SCLC, ABT-263 was able to completely eradicate the tumours while it significantly enhanced the efficacy of standard therapies in models of B-cell lymphoma and multiple myeloma (Shoemaker et al. 2008). ABT-263 is currently evaluated in phase I/II trials for SCLC, leukaemia and lymphoma, both as a monotherapy and in combination with other anti-cancer drugs or with monoclonal antibodies (rituximab and erlotinib). Despite the high binding selectivity of ABT-263, its on-target effects are crucial for its safety profile and the maximum tolerated dose of the drug. Because Bcl-x_L plays a pivotal role for cell survival of thrombocytes, the major adverse side effect of ABT-263 observed in the clinic is thrombocytopenia. In a phase I study, monotherapy with ABT-263 was shown to reduce pathologic lymphocytosis, lymphadenopathy, and splenomegaly in patients suffering from relapsed or refractory CLL (Roberts et al. 2012). The therapeutic efficacy of ABT-263 +/- rituximab on relapsed or refractory leukaemia/lymphoma patients is currently investigated in phase II trials. Another phase II multi-centre trial is currently recruiting chemonaive CLL patients to assess the clinical impact of ABT-263 in combination with rituximab in previously untreated patients (www.clinicaltrials.gov).

Structurally the pro-survival Bcl-2 family members can be divided into two categories. Bcl-2, Bcl-x_L and Bcl-w are structurally closely related, whereas the accessibility of the BH3 binding pocket of Mcl-1 and A1 is structurally different from the other three family members. Mcl-1 plays a pivotal role in resistance to the Bcl-2/Bcl-x_L/Bcl-w-specific inhibitors ABT-737 and ABT-263. The pro-apoptotic activity of ABT-737 was shown to negatively correlate with Mcl-1 expression in various cancer models and suppression of Mcl-1 expression was shown to abrogate

ABT-737 resistance, e.g. in acute myeloid leukaemia (AML) cells with high endogenous expression of Mcl-1 (Konopleva et al. 2006). Therefore, the combination of ABT-263 with drugs targeting the expression/stability of Mcl-1 (e.g. sorafenib, maritoclast) may be a feasible approach for future clinical studies. Indeed, one ongoing phase I clinical study in patients with solid tumours addresses the combined effects of ABT-737 and the EGFR receptor erlotinib known to suppress expression of Mcl-1 (Chen et al. 2012). In addition, expression profiling of Bcl-2 family members (Bcl-2, Bcl-x_L, Mcl-1, Bim, Noxa etc.) may help to rationally design future clinical trials with ABT-263 and combinatorial treatments by selecting patients most likely to benefit from the therapy.

3.2 Gossypol and Its Derivatives

The pro-survival Bcl-2 proteins serve partially redundant functions and considerable evidence suggests that – depending on the Bcl-2 expression profile of tumours – inactivation of all Bcl-2-like proteins may significantly enhance the efficiency of Bcl-2-targeted therapy. This may either be achieved by combining a more selective inhibitor such as the Bcl-2/Bcl-x_L/Bcl-w inhibitor ABT-737/ABT-263 with another drug interfering with the expression of Mcl-1 or targeting it for degradation, or alternatively with pan-Bcl-2 inhibitors, targeting all four major Bcl-2-like proteins. Gossypol is a natural polyphenolic compound and BH3 mimetic derived from cottonseeds which was initially identified as an antifertility agent in China during the 1950s, and possesses pro-apoptotic effects in various *in vivo* and *in vitro* models (Wolter et al. 2006; Ko et al. 2007; Meng et al. 2008; Paoluzzi et al. 2008). Gossypol acts as a pan-Bcl-2 inhibitor and can inactivate Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w (Lessene et al. 2008; Kang and Reynolds 2009) (Tables 1 and 2). There are two enantiomers of Gossypol, (+)-Gossypol and (–)-Gossypol, the latter being more potent as an inhibitor of tumour growth (Lessene et al. 2008). (–)-Gossypol (AT-101, Ascenta) has shown single-agent activity in various types of cancer (Lessene et al. 2008; Kang and Reynolds 2009). In cancer cells with an intact apoptotic machinery, (–)-Gossypol has been reported to induce the intrinsic pathway of apoptosis and apoptotic cell death (Wolter et al. 2006; Balakrishnan et al. 2008; Meng et al. 2008; Paoluzzi et al. 2008). In contrast, cell death triggered by Gossypol largely seems to depend on induction of autophagic cell death in apoptosis-deficient malignant glioma cells and prostate cancer cells (Lian et al. 2010; Voss et al. 2010). (–)-Gossypol has nanomolar affinities to Bcl-2 ($K_i = 320$ nM), Bcl-x_L ($K_i = 480$ nM) and Mcl-1 ($K_i = 180$ nM) (Wang et al. 2006).

(–)-Gossypol has good pharmacokinetic properties and appears to exhibit manageable (mainly gastrointestinal) toxicity, and demonstrated single-agent activity in a phase I/II trial in castrate-resistant prostate cancer (Liu et al. 2009). In another study, AT-101 was given in combination with topotecan and a partial response of this combined therapy was observed in patients with relapsed SCLC, although this

study unfortunately did not include a group of patients treated only with topotecan (Heist et al. 2010). Another phase II clinical trial data obtained from advanced and metastatic NSCLC patients revealed no clinical benefit of AT-101 in comparison to standard therapy in regard to overall survival (Ready et al. 2011). The potential clinical impact of AT-101, either as a monotherapy or in combination with other anti-cancer drugs, is currently further investigated in phase I/phase II clinical trials of leukaemia, lymphoma, NSCLC and prostate cancer (www.clinicaltrials.gov). Similar to ABT-737, subjecting patients to expression profiling of Bcl-2 family members may aid the rational design of future clinical trials.

In addition to (–)-Gossypol, a series of Gossypol derivatives are developed in an aim to further improve the therapeutic efficacy and dampen toxic side effects. Structure-based design strategies led to the development of the Gossypol derivative TM-1206 (Tang et al. 2008), which binds to Bcl-2, Bcl-x_L and Mcl-1 proteins with K_i values of 0.11, 0.639 and 0.15 μM, respectively. Employing molecular models of the (–)-Gossypol/Bcl-2 complex allowed the structure-based design of the novel Gossypol analogue and benzenesulfonyl derivative TW-37 (Wang et al. 2006) which binds with submicromolar/low micromolar affinity to Bcl-2 (0.29 μM), Bcl-x_L (1.11 μM), and to Mcl-1 (0.26 μM) (Wang et al. 2006). *In vitro* studies confirmed the on-target effects of TW-37 which disrupts the interaction between Bax and truncated Bid (tBid) with the pro-survival Bcl-2 family members Bcl-2, Bcl-x_L and Mcl-1 (Mohammad et al. 2007). In human endothelial and pancreatic cancer cells, TW-37 interestingly was also demonstrated to inhibit the pro-angiogenic and pro-metastatic activities of Bcl-2 which may be mediated by activation of the NF-κB pathway (Zeitlin et al. 2006; Wang et al. 2008).

Removal of two reactive aldehyde groups that are held responsible for the toxic side effects of (–)-Gossypol observed in clinical studies gave rise to Apogossypolone which retains activity against anti-apoptotic Bcl-2 family proteins *in vitro* (Arnold et al. 2008). Apogossypolone binds Bcl-2, Bcl-x_L and Mcl-1 with nanomolar affinities (Table 2) and was shown to induce apoptosis either alone or in combination with DNA damaging cancer drugs *in vitro* and *in vivo* and showed *in vivo* activity in xenograft models (Arnold et al. 2008). In addition to direct inhibition of pro-survival Bcl-2 proteins and similar to (–)-Gossypol, Apogossypolone was suggested to down-regulate Bcl-2, Bcl-x_L and Mcl-1 expression at the protein level. Further attempts to improve the target specificity by molecular docking computer screens led to synthesis of BI-97C1 (Sabutoclax) (Wei et al. 2010), a very promising, optically pure Apogossypol derivative with submicromolar target affinity. BI-97C1 inhibits the binding of BH3 peptides to Bcl-x_L, Bcl-2 and Mcl-1 with IC₅₀ values of 0.31, 0.32 and 0.20 μM, respectively (Table 2). The compound also potently inhibits cell growth of human prostate cancer, lung cancer, and lymphoma cell lines with EC₅₀ values of 0.13, 0.56, and 0.049 μM, respectively, shows little cytotoxicity in Bax/Bak DKO cells (Wei et al. 2010). Recently, BI-97C1 was also reported to enhance mda-7/IL-24-induced apoptosis of prostate cancer cells and to reduce tumour growth in a xenograft model *in vivo* (Dash et al. 2011). The potential clinical impact of BI-97C1 has not been investigated so far.

3.3 Obatoclax

Obatoclax (GX15-070, discovered by Gemin X, now Cephalon), a polypyrrrole Pan-Bcl-2 inhibitor was identified in a high throughput screening of natural compound libraries followed by lead optimization (Shore and Viallet 2005). Obatoclax binds to Bcl-2, Bcl-xL and Mcl-1 with low micromolar affinities (Table 2) and was reported to exert *in vitro* and *in vivo* single agent activity in various types of cancer. Activation and mitochondrial translocation of Bax, mitochondrial depolarization, cytochrome c release, subsequent activation of caspase-9 and caspase-3 were reported to precede apoptosis induced by Obatoclax. Interestingly, Obatoclax was suggested to induce apoptosis by direct activation of Bax in a cell-free system, suggesting that it may act both as a sensitizer BH3 mimetic neutralizing the anti-apoptotic Bcl-2 proteins as well as an activating BH3 mimetic (Smoot et al. 2010). Obatoclax demonstrated single-agent activity in a phase I clinical study in CLL and is currently investigated in phase I/II studies of relapsed or refractory leukaemia, myeloma, lymphoma and SCLC, both as a monotherapy and in combination with other cancer drugs (www.clinicaltrials.gov). Preliminary reports on the outcomes of phase I/II trials appear to be promising. Antineoplastic activity was observed in 2 out of 5 pre-treated advanced non-Hodgkin's lymphoma (NHL) patients. Improved platelet and haemoglobin counts of chronic lymphocytic leukaemia (CLL) and myelofibrosis patients suffering from thrombocytopenia and anaemia were also reported. Although Obatoclax is generally well tolerated, the major observed side effect of the drug appears to be central nervous system (CNS) toxicity including ataxia, euphoria, and confusion.

4 Killing Mechanisms of BH3 Mimetics: The Clinical Perspective

The target specificity of many currently available BH3 mimetics has been put into question recently (van Delft et al. 2006; Vogler et al. 2009b). The combined findings of two comparative studies demonstrated that HA14-1, BH3I-1, antimycin A, chelerythrine, (–)-Gossypol, Apogossypol, Obatoclax, and EM20-25 induced significant cell death in Bax/Bak-deficient MEFs, whereas ABT-737 was shown to be highly dependent on the expression of Bax, Bak and caspase-9 to induce apoptosis (van Delft et al. 2006; Vogler et al. 2009b). These Bax/Bak-independent toxicities indicate that the other inhibitors might not behave solely as BH3 mimetics but might have additional cellular targets implicated in activating cell death. Conversely, it was proposed that ABT-737 may be the only bona fide BH3 mimetic inducing apoptotic cell death in a purely target-specific manner. In the aforementioned studies, Bax/Bak-deficient or Caspase-9-deficient cells were used as an experimental model to analyse the target-specificity of BH3 mimetics. However, the established role of pro-survival Bcl-2 family members in regulation of non-apoptotic forms of cell

death and the interaction of Bcl-2-like proteins with proteins not involved in apoptosis (as outlined below), should be taken into account for the interpretation of these results. In addition, a Bax/Bak-independent, alternative mode of apoptosis activation by the BH3 mimetic Gossypol has been proposed, namely a Gossypol-induced conformational change in Bcl-2 which converges it into a pro-apoptotic molecule activating the mitochondrial pathway of apoptosis (Lei et al. 2006). It is currently unknown whether other Bcl-2 inhibitors are potentially capable to induce a similar change in Bcl-2 conformation.

In addition to their role in apoptosis, all pro-survival Bcl-2 family members are negative endogenous regulators of autophagy. They serve to sequester and inactivate the pro-autophagic BH3 only protein Beclin-1 and consequently, all BH3 mimetics are potential activators of autophagy. Despite its proposed target selectivity, pleiotropic pro-autophagic effects on multiple autophagic signalling pathways have been shown to be induced by ABT-737 recently (Malik et al. 2011). The extent of autophagy induction by BH3 mimetics appears to be highly diverse, however. In this regard, the pan-Bcl-2 inhibitor (–)-Gossypol was shown to be a more potent activator of autophagy than the BH3 mimetics HA14-1 and ABT-737 (Voss et al. 2010). As outlined in the Introduction, autophagy may exert both protective and pro-death effects depending on the respective cellular context. Therefore, autophagy induced by BH3 mimetics may contribute to the cell death observed in apoptosis refractory cells such as glioblastoma cells and Bax/Bak-deficient MEFs. Indeed, in Bax/Bak-deficient cells and glioblastoma cells, autophagy was shown to be required for triggering necroptotic cell death induced by Obatoclax and autophagic cell death triggered by (–)-Gossypol, respectively (Bonapace et al. 2010; Voss et al. 2010). In the case of Obatoclax, the drug was reported to reactivate the sensitivity of multidrug-resistant childhood ALL cells to glucocorticoid treatment and other anti-cancer drugs by inducing an autophagy-dependent necroptotic type of cell death. This type of cell death required the expression of receptor-interacting protein (RIP-1) kinase and cylindromatosis (turban tumour syndrome) (CYLD), both of which are critically involved in necroptosis and was inhibited by knock-down of Beclin-1 (Bonapace et al. 2010). In most tumours and haematological malignancies however, autophagy appears to be a pro-survival stress response and therefore rather limits the therapeutic effects of chemotherapy and radiation. The clinical use of autophagy inhibitors (e.g. chloroquine) together with BH3 mimetics may therefore be a useful strategy to potentiate apoptosis in these apoptosis-proficient malignancies.

The issue of target selectivity may also have important implications for therapy. While highly selective inhibitors offer obvious advantages for mechanistic, experimental studies, at the moment it is still open to debate whether an ultra high target selectivity of Bcl-2 inhibitors actually is an advantage from a purely clinical perspective. On the one hand, an apparent disadvantage of inhibitors with lower selectivity/higher off target effects lies in the increased possibility for non-mechanism based toxicity issues in normal tissue. Despite this notion, the adverse effects of BH3 mimetics with proposed off target mechanisms such as (–)-Gossypol and Obatoclax have been shown to be manageable so far in the clinic. It is even

conceivable that the off target effects (e.g. Bcl-2 independent ROS generation) may actually contribute to the therapeutic efficacy of the drugs and therefore enhance their clinical efficacy. Due to the mechanism-based selection pressure, drug resistance in tumours treated with highly selective inhibitors also may acquire more rapidly. Therefore, highly selective inhibitors may be most successful when applied in combination with other drugs/compounds targeting drug resistance mechanisms such as enhanced Mcl-1 expression in ABT-737-treated malignancies.

5 Outlook

Despite the fact that activation of apoptosis is a major mechanism by which most chemotherapeutic drugs and radiation kill tumour cells, conventional cancer therapy does not allow target-specific intervention in death and survival signalling pathways which appears to be the most reasonable strategy to overcome the intrinsic apoptosis resistance of cancer cells. The ultimate goal for molecular, apoptosis-based therapies is to develop specific drugs selectively targeting various signalling components of pro- and anti-apoptotic pathways in an aim to limit unwanted toxicity in normal tissues and to trigger tumour-selective cell death. BH3 mimetics are an exciting new class of cancer drugs that hold great promise to fulfil these criteria because of their relatively limited toxic side effects in comparison to conventional chemotherapy/radiotherapy. In addition to their anti-tumour activities when used as monotherapy, a vast number of preclinical data suggest BH3 mimetics may be highly useful tools to reduce patient drug loads when applied in synergistic therapies with conventional cancer drugs and radiotherapy. The results of ongoing clinical trials will shed further light on the clinical impact of these novel anti-cancer agents in patients suffering from relapsed, refractory, metastatic or advanced stage cancers. Future trials with chemo-naïve patients will also allow to scrutinize the clinical potential of BH3 mimetics in early stage/untreated cancer. The rational design of trials with BH3 mimetics may also increase the attention on molecular profiling, such as expression profiling of Bcl-2 family members in an aim to select patients most likely to benefit from the therapy.

Future strategies will aim at designing even more specific Bcl-2 inhibitors to further reduce their off target effects in an aim to limit the toxicity of these compounds in normal cells. Very recently, the structure-based design of a novel, highly potent and specific small-molecule inhibitor of Bcl-2/ Bcl-x_L (compound 21) was published. The design of this inhibitor was developed from a novel chemical scaffold and the crystal structures of Bcl-x_L complexed with the Bad BH3 peptide. The novel compound binds to both Bcl-x_L and Bcl-2 with $K_i < 1$ nM and inhibited cell growth in two small-cell lung cancer cell lines with IC₅₀ values of 60–90 nM.

Pro-survival Bcl-2 proteins share a functional redundancy in antagonizing cell death, and there is considerable evidence that parallel inhibition of all pro-survival Bcl-2 proteins holds great promise to increase therapeutic efficacy. The pan-Bcl-2 inhibitors (–)-Gossypol and Obatoclax are already under clinical investigation.

Several new compounds are in preclinical development and time will tell if these novel Gossypol derivatives such as the highly promising BI-97C1 will advance to clinical trials. In addition to the currently available published material, ongoing efforts in drug development may yield even better, high affinity pan-Bcl-2 inhibitors in the future. Future clinical studies may also focus on the intelligent design of combined therapies employing BH3 mimetics with more restricted binding profiles and other, more selective drugs, e.g. ABT-263 with agents targeting the expression/stability of Mcl-1.

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