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Bcl-2 Inhibitors: Emerging Drugs in Cancer Therapy

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Abstract: Dose-limiting toxicity to healthy tissues is among the major hurdles in anticancer treatment along with intrinsic or acquired multi-drug resistance. Development of small molecule inhibitors (SMI) specific for antiapoptotic Bcl-2 proteins is a novel approach in a way that these antagonists are aimed to interfere with specific protein-protein interactions unlike conventional chemo-/radiotherapies. SMIs of antiapoptotic Bcl-2 proteins are assumed to compete with proapoptotic Bcl-2s to occupy BH3 docking grooves on the surfaces of antiapoptotic family members. Instead of directly initiating cell death, these inhibitors are intended to decrease apoptotic threshold in tumor cells that were already primed to death. In this regard, antiapoptotic Bcl-2 protein SMIs have the advantage of lower normal tissue toxicity relative to conventional anticancer therapies that interfere with general mechanisms including DNA synthesis, mitosis and tyrosine kinase activity. Besides, Bcl-2 antagonists were shown to potentiate efficacies of established drugs in several hematological malignancies and solid tumors which render them promising candidates for combination anticancer therapy. Utilizing these SMIs in such a way may prove to decrease the patient drug load by diminishing the required chemo-/radiotherapy dose. This review summarizes and compares BH3 mimetics on the basis of specificity, mode of action and efficacy, as well as providing remarks on their therapeutical potential and routes of development in near future.

Keywords: ABT-737, anticancer, apoptosis, Bcl-2, BH3 mimetic, cancer, gossypol, small molecule inhibitor, obatoclax.

INTRODUCTION

Novel therapeutic approaches that target specific signaling pathways with small cell-permeant molecules have emerged as one of the novel foci in cancer treatment. Fueled by the concept that the more specifically we target the tumor specific pathways, the less the patients suffer from toxicity, a number of novel monoclonal antibodies and tyrosine kinase inhibitors found their ways into the clinicals recently. A major concern of currently applied anticancer therapies is to cope with intrinsic/acquired resistance against the established regimens. In this regard, to explore low-toxicity profile small molecules that synergize with established therapies may help overcome such limitations.

Following the targeted therapy concept coming into cancer research scene, it did not take much time for prosurvival Bcl-2 proteins to draw the attention of the researchers as they evidently help cancer cells evade apoptosis. Overexpression of antiapoptotic Bcl-2 family proteins has been implicated in both pathology and drug resistance in various hematopoietic malignancies and solid tumors [1-4]. Computer based screening of small compound libraries and rational drug design based on unveiled structures of Bcl-2/xL provide opportunities to test the efficacies of several small molecule inhibitors (SMIs) of prosurvival Bcl-2 proteins in different cancer models. Thanks to X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, we are now capable of modeling the engagement of the hydrophobic surface cleft of prosurvival Bcl-2 proteins by their proapoptotic Bcl-2 counterparts.

SMIs of antiapoptotic Bcl-2 proteins are basically aimed to activate the intrinsic (mitochondrial) apoptotic pathway which is triggered by intracellular signals [5]. On the contrary, extrinsic apoptotic pathway is activated through extracellular death ligand binding to the receptors on cell surface. Whilst sharing common downstream executionary protease activity mediated by caspases 3, 6 and 7, the effector caspases of intrinsic and extrinsic apoptotic pathways are known to be caspases 9 and 8, respectively. As the central regulators of intrinsic apoptotic activity together with their proapoptotic partners, prosurvival Bcl-2 family proteins contain shared Bcl-2 homology domains 1-4 (BH1-4) each spanning around 20 amino acids [6] (Fig. **1**). Highly conserved BH1, BH2 and BH3 domains are involved in the formation of a hydrophobic BH3 docking groove harboring binding sites for BH3 containing proapoptotic Bcl-2 proteins [7, 8]. Sequestration of BH3-only proapoptotic proteins such as Bim, Puma, Noxa, and Bmf or multi-domain proapoptotic Bax/Bak by prosurvival Bcl-2s works in favor of cellular survival. BH3 mimetics are aimed to compete with proapoptotic Bcl-2 proteins to engage prosurvival ones which would supposedly cause release of either Bax/Bak or activator BH3-only proteins from prosurvival Bcl-2s. Once freed from prosurvival Bcl-2s proapoptotic Bcl-2 proteins mediate Bax/Bak oligomerization, mitochondrial outer membrane (MOM) permeabilization and subsequent apoptogenic molecule release that activates downstream caspase machinery (Fig. **2**). So called activator BH3-only proteins Bim, Bid and Puma are considered to be capable of directly activating Bax and Bak to initiate apoptotic events while the other BH3-only proteins work solely by neutralizing antiapoptotic Bcl-2 proteins [9].

The basic logic behind the design of anti-sense oligonucleotides or SMIs against Bcl-2 and its antiapoptotic fellow proteins is to diminish the apoptotic threshold that is mainly determined by saturation of antiapoptotic Bcl-2 molecules by their proapoptotic counterparts. SMIs of antiapoptotic Bcl-2 proteins are supposed to specifically induce apoptosis in cancerous human cells. Such tumor specificity is based on the assumption that survival of cancer cells depends on expression of antiapoptotic guardians such as Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1 to a greater extent than that of healthy cells. The internal milieu of cancerous cell deviates from normal in that death signals exist in excess compared to survival signals as a result of severe DNA damage, defective checkpoint regulation or disrupted energy metabolism. Accordingly, constitutively activated apoptotic pathways that remain dormant in transformed cells reset the apoptotic threshold rendering those primed to death. By making cancer cells more sensitive to both intrinsic apoptotic signals and insults triggered by established anticancer regimens, Bcl-2 antagonists may also help reduce the required radio-/chemotherapeutic dose and related toxicity to healthy cells.

Mammalian cells have a delicately regulated survival/cell death network composed of interconnected signaling pathways. Because of the hardship to isolate multiple death signaling pathways in these cells it may not be easy to establish the mechanistic basis for actions of different BH3 mimetics. Cell death/growth inhibition pathways including caspase-dependent intrinsic or extrinsic pathways, caspase-independent apoptosis, autophagy, necrosis or senescence cross-talk with each other. An appropriate way to interrogate the causality of an apoptotic stress induced by a Bcl-2 antagonist is to use a model which lacks Bax/Bak expression. Absolute requirement for Bax/Bak for cytotoxic effect together

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with demonstration of mitochondrial transmembrane potential ($\Delta\psi_m$) loss and caspase-9 activation could be evaluated as indicative of BH3 mimetic activity which requires that apoptosis is triggered solely by mitochondrial apoptotic pathway. The term "mechanism-based toxicity" refers to the activation of intrinsic apoptotic pathway as the primary cell death mechanism throughout this review. On the other hand, "nonmechanism-based toxicity" depicts the existence of a cell death mechanism other than intrinsic apoptotic pathway as the primary trigger.

It is of note that any interaction of prosurvival Bcl-2s with molecules other than proapoptotic Bcl-2 counterparts may also be affected by Bcl-2 antagonists. Other than their well-established classical apoptotic roles on MOM, antiapoptotic Bcl-2 proteins have also been implicated in several other survival pathways in mammalian cells. These prosurvival guardians were suggested to regulate mitochondrial inner membrane (MIM) permeability, MOM integrity and shown to possess antiautophagic and antisenescencent features [10-13]. A relevant connection exists between Bcl-2 and autophagy which is mediated by another Bcl-2 binding protein, Beclin-1. This autophagic initiator was shown to be restrained by antiapoptotic Bcl-2 proteins Bcl-2, Bcl-xL, Bcl-w and Mcl-1 through its BH3 domain [14, 15]. Upon released from prosurvival Bcl-2s Beclin-1 binds to phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) to facilitate autophagosome formation [16]. Autophagy is a major intracellular signaling for degradation and recycling of proteins and organelles. However, role of autophagy in cancer is controversial because of its dual circumstantial nature. Autophagy could either work as a cell suicidal mechanism against radiation or stress or may turn cell protective in response to starvation, hormones or other stresses [17-19].

Unrelated to its survival function, Bcl-2 was also associated with proangiogenic and prometastatic features. Bcl-2 proangiogenic pathway was shown to be mediated by NF-κB-dependent upregulation of proangiogenic chemokines CXCL1 and CXCL8 in human endothelial cells exposed to proangiogenic vascular endothelial growth factor (VEGF) [20]. In addition, ability of Bcl-2 to activate NF-κB and upregulate its metastasis-associated target genes matrix metalloproteinase-9 (MMP-9) and VEGF was shown in pancreatic cancer cells [21]. Hence, inhibition of protein-protein interactions of Bcl-2 might also result in loss of invasion and angiogenesis capacity in tumor cells.

Fluorescent or NMR-based competitive inhibition assays show that newly discovered or tailored SMIs directed against prosurvival Bcl-2 targets represent an affinity range that spans from subnanomolar to low micromolar Ki/Kd values. Preclinical and clinical investigations confirm that Bcl-2 antagonists are relatively less toxic than common chemotherapies as expected from their mode of action. This review comprises a collection of wide-scale comparative information about target affinities, modes of action, preclinical and clinical anticancer efficacies of both discovered and rationally designed SMIs directed against antiapoptotic Bcl-2 proteins. Major issues such as off-target effects and acquired or intrinsic drug resistance are discussed. Furthermore, authors put forward remarks pertaining to the choice of drugs to combine with Bcl-2 antagonists in the clinical trials and routes to be followed in the future development of these SMIs.

HA14-1

HA14-1 was identified in a computer screening based on the predicted structure of Bcl-2 as a small molecule (409 Da) nonpeptidic ligand of Bcl-2 hydrophobic surface groove [22]. HA14-1 has been shown to bind Bcl-2 with an IC_{50} value of approximately 9 μM in competition with fluorescent labeled Bak-BH3 (Table **1**). This Bcl-2-specific SMI proved to effectively induce apoptosis as a single agent at submicromolar range in several human hematopoietic cell lines (Table **2**). Besides, HA14-1 was reported to synergize with a number of approved and experimental anticancer agents including DNA damage and apoptosis inducers, immunosuppressants, inhibitors of mitosis, tyrosine kinases or proteasome in various hematological and solid cancers (Table **3**).

Mechanistically, HA14-1 was shown to induce mitochondrial Bax translocation, loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and to activate Apaf-1, caspases 9 and -3 in several human cell lines *in vitro* [22-24]. Chen and co-workers found that Bax-deficient cells were more resistant to HA14-1 further indicating the involvement of mitochondrial apoptotic pathway [23]. However, caspases were reported not to be required for HA14-1-driven apoptosis in Bcl-2 expressing hematologic cells [23, 25]. HA14-1 has also been shown to induce prosurvival autophagy together with apoptosis in murine leukemia cells. The autophagy-related morphological changes were reported to precede apoptotic nuclear morphology [26].

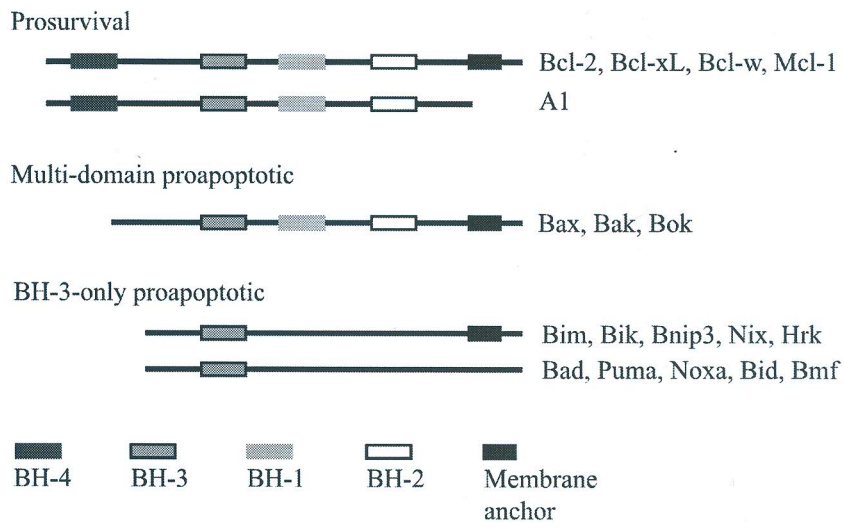

Fig. (1). Structures of Bcl-2 family proteins. The diagram shows the structures of three subgroups of anti- and proapoptotic members of Bcl-2 family roughly based on Bcl-2 homology domains that different group of proteins harbour. The sizes of the proteins are only approximations.

Table 1. Affinities of BH3 Mimetics for Prosurvival Bcl-2 Proteins

SMI	nM Affinities (Kd/Ki/Km)					References
	Bcl-2	Bcl-xL	Bcl-w	Mcl-1	A1	
HA14-1	9000*					[22]
BH3I-1		2400				[33]
BH3I-2'		3300				[33]
2-MeAA	2500					[8]
(-)-Gossypol	320	480		180		[34]
TW-37	290	1100		260		[34]
Apogossypolone	35	660		25		[7]
BI-97CI	320*	110		200*	620*	[35]
Chelerythrine		1500*				[36]
YC137	1300	>100000				[37]
Obatoclax	~500	~500	~500	~500		[38]
ABT-737	≤1	≤1	≤1	>1000	>1000	[2]
S1	310			58		[39]

*IC_{50} values obtained from competitive binding assays based on fluorescent polarization. For a comparative analysis of antiapoptotic Bcl-2 protein SMIs based on IC_{50} values, see reference [40]
Abbreviations: 2-MeAA, 2-methoxy-antimycin A3.

The evidence that indicates lack of caspase requirement for drug activity clearly associates HA14-1 with unintended cell death mechanisms. In addition, this SMI faces several purity, stability and toxicity issues. HA14-1 is a mixture of two stereoisomers and has been shown to have only 15 minute *in vitro* stability [27, 28]. A major issue that HA 14-1 encounters as a drug candidate is its potential toxicity to mitochondria of healthy cells. Perhaps, this may not be considered as an unanticipated drawback since all Bcl-2 ligands working on mitochondrial membranes may potentially exert severe mitochondrial toxicities such as interfering with energy coupling and respiration. Accordingly, other Bcl-2 ligands including antimycin A, chelerythrine and BH3I-2' were also implicated in dose-dependent energy uncoupling and respiratory inhibition [10]. Several *in vitro* studies verify that HA 14-1 induces significant radical oxygen species (ROS) production, energy uncoupling and respiratory inhibition [10, 29]. Induction of ROS formation subsequent to rapid HA 14-1 degradation was proposed to potentially be responsible for observed mitochondrio-toxicity [28].

HA 14-1 research has recently shifted to efforts to isolate MOM permeabilization activity from respiratory toxicity and to synthesize stable, ROS-free analogs. SV30 was developed as a more stable analoge of HA 14-1 and further possesses the advantage of purity as a single stereoisomer [30]. SV30 was reported to trigger apoptosis in a similar fashion with HA 14-1, at least partly *via* mitochondrial apoptotic pathway [27]. Similar single agent potencies were recorded for both precursor HA 14-1 and SV30 with IC_{50} values changing between 27 and 35 μM. Moreover, SV30 was reported to significantly synergize with paclitaxel, etoposide or beam radiation in rat glioma cells [27].

Among other HA14-1 derivatives with decreased mitochondrio-toxicity are EM20-25 and sHA14-1 [10, 29]. Both derivatives were shown to successfully induce *in vitro* apoptotic activity in human lymphoma/leukemia cells with IC_{50} values in the range of 20-27 μM. Moreover, EM20-25 and sHA14-1 reportedly potentiate toxicities of several experimental and approved apoptotic agents [10, 29, 31]. Although action mechanisms of these HA14-1 relatives are not well established as of the parental compound itself, sHA14-1 was implicated in off-targeting at ER membrane. This stable HA14-1 analoge was reported to cause calcium release from ER and trigger apoptosis in a manner similar to specific SERCA inhibitor thapsigargin prior to activation of mitochondrial apoptotic pathway [28, 29]. The investigation reveals that sHA14-1 could inhibit SERCAs located on ER membrane directly, independent of Bcl-2 regulation. Similarly EM20-25 was demonstrated not to act exclusively on intended mechanism of MOM permeabilization as Bax/Bak or caspase-9 activities were not required for apoptotic induction *in vitro* [32].

Novel derivatives of Bcl-2/xL-specific SMI HA 14-1 seem to present significant improvement on purity, stability and mitochondrio-toxicity issues that were associated with the parental compound, However, before reaching the clinical trials, more in-depth mechanistic interrogation and improvement on off-target hitting of non-Bcl-2 molecules such as SERCAs appear to be required.

ANTIMYCIN A

Antimycin A is an antifungal agent of bacterial origin which has long been known to block electron transport chain through binding cytochrome b-c1 complex. This molecule has been suspected to have additional interactions that influence mitochondrial membrane channels in the 1990s and was ultimately shown to compete with BH3 peptides to bind Bcl-2 and to interfere with pore forming ability of Bcl-xL in synthetic liposomes [8]. Molecular docking simulations indicate that this BH3 mimetic occupies the hydrophobic surface groove of antiapoptotic Bcl-2 proteins.

The 2-methoxy-antimycin A_3 is a derivation of antimycin A that is thought to lack respiration inhibitory action of its forerunner

Table 2. Preclinical Activities of BH3 Mimetics

SMI	IC_{50} ranges (μM)		Cancer models, references	
	Hematologic	Solid	Single agent activity	Little or no activity
HA14-1 and derivatives	5-30	20-50	Lymphoma, leukemia, myeloma, glioma, ovarian, prostate [10, 24, 25, 27, 29, 47-49]	Ovarian [48]
BH3Is	10-100	30-100	Leukemia, cervical, glioma [33, 50]	
2-MeAA		10-30	Lung, mesothelioma*, esophageal [42]	Breast [45]
(-)-Gossypol	1-20	1,5-10	Lymphoma1, leukemia2, myeloma3, prostate*, colon, HNSCC* [51-60]	
TW-37	0,17-0,32	0,2-2	Lymphoma*, leukemia, pancreatic*, head and neck*, prostate [21, 34, 61-64]	
ApoG2 and derivatives	0,1-18	0,6-31	Lymphoma*, leukemia, prostate, lung, pancreatic, liver, nasopharyngeal [7, 63, 65-69]	
BI-97CI	0,49	0,13-0,56	Lymphoma*, prostate*, lung [35]	
Chelerythrine	10	5-10	Leukemia, liver, cardiac, neuroblastoma [36, 70-72]	
YC137		0,5	Breast [37]	
Obatoclax	0,1-10	0,26-15,4	Lymphoma, leukemia, myeloma, lung, mammary carc*, colon*, cervical*, prostate* [73-80]	Bile duct and melanoma [81, 82]
ABT-737/263	0,001-15	0,01->100	Lymphoma*, leukemia*, myeloma, prostate, lung*, pancreatic, ovarian*, colorectal, GI [2, 11, 13, 43, 44, 83-101]	renal, bladder, head and neck [13, 97, 98]
S1		0,06-10	Breast, liver*, cervical [39, 102, 103]	

*Observed in *in vivo* mouse models in addition to *in vitro* set-ups.
Abbreviations: ApoG2. apogossypolone; 2-MeAA, 2-methoxy-antimycin A3; HNSCC, head and neck squamous cell carcinoma; GI, gastrointestinal.

although it was speculated that this compound may still have residual minor inhibitory action on electron transport [8, 41]. Although antimycin A_3 was demonstrated to bind Bcl-2 with a low micromolar affinity which is not comparable to nanomolar affinities of newer BH3 agonists, it exerted significant *in vivo* antitumor activity in mice models of mesothelioma [42]. Besides, the required 2-methoxy-antimycin A_3 dose for *in vivo* single agent activity was reported to be as low as 2 mg/kg/day, which is at least ten times of magnitude less than the standart *in vivo* doses of subnanomolar affinity BH3 mimetic ABT-737 [2, 43, 44]. Together, these outcomes may reflect more favorable pharmacokinetics and pharmacodynamics for 2-methoxy-antimycin A_3 could compensate for its relatively low target-affinity. The 2-methoxy-antimycin A_3 was reported to exert *in vitro* single agent activity against a couple of human epithelial and mesenchymal cancer cell lines in an IC_{50} range of 10-30 μM [42]. Moreover, 2-methoxy-antimycin A_3 was shown to potentiate *in vitro* cytotoxicities of antimitotic chemotherapeutics in breast cancer and TRAIL in prostate cancer cells [45, 46]. *In vivo* investigation of 2-methoxy-antimycin A_3 as combined with cisplatin has provided further support to rationalization of 2-methoxy-antimycin A_3 use as a complementary anticancer drug of therapeutical value [42].

GOSSYPOL AND DERIVATIVES

Gossypol is a racemic mixture of polyphenolic enantiomers (+)-gossypol and (-)-gossypol derived from cottonseed. This compound has long been studied and clinically evaluated for both antitumor and male contraceptive properties [104]. Recently, structure based database screening and fluorescent polarization based competition assays identified gossypol as an SMI of Bcl-2/Bcl-xL/Mcl-1 with nanomolar affinities [34, 56, 104]. Although both optical isomers show similar binding affinities for Bcl-xL and Bcl-2, (-)-gossypol was found to be more potent inducer of apoptosis in human cell cultures presumably because of serum influence [105]. Natural BH3 mimetic (-)-gossypol exerts *in vitro* and *in vivo* single agent growth inhibitory/apoptotic activity on human hematologic tumors and solid cancers including prostate, colon, and head and neck cancers with IC_{50} values in 1-20 μM range (Table **2**). (-)-Gossypol has been intensively studied on *in vitro* and *in vivo* human lymphoma models and reported to potentiate cytotoxic effects of various therapeutic and experimental compounds including protein synthesis and proteasome inhibitors, DNA damaging drugs, monoclonal antibodies and corticosteroids (Table **3**).

(-)-Gossypol was reported to initiate apoptotic response through mitochondrial depolarization, cytochrome c release, apoptosis-inducing factor (AIF) cleavage, activations of apical caspases 9, 8 and effector caspases 3,6, and 7 [7, 51, 54-57]. Involvement of mitochondrial and death receptor pathways were reported to be sequential as the first apical caspase activated was caspase-9. Although (-)-gossypol was reported not to influence cellular antiapoptotic Bcl-2 protein levels in Mohammad and co-worker's investigation on lymphoma cells, others revealed that (-)-gossypol may critically decrease the expressions of Bcl-2, Bcl-xL, and Mcl-1 as indicated in various cultured human cancer cells [52, 54, 56, 57]. The direct experimental support to assumed action mechanism of pan-Bcl-2 SMI (-)-gossypol comes from several coimmuno-precipitations that reveal the disruption of heterodimerization between pro- and antiapoptotic Bcl-2 proteins including interactions of Bcl-2/Bcl-xL with proapoptotic Bax and Bad [105].

Utilization of molecular (-)-gossypol-Bcl-2 interaction models led to structure based design of a new class of antiapoptotic Bcl-2 protein-directed SMIs [34]. One of these molecules, TW-37 binds Bcl-2/xL and Mcl-1 at nanomolar and low micromolar affinities similar to (-)-gossypol (Table **1**). However, TW-37 proved more potent in growth inhibition and apoptosis induction on *in vitro* models of several hematological malignancies and solid cancers with IC_{50} values in the range of 0,17-2 μM (Table **2**). In addition to

synergizing with cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone (CHOP) chemotherapy in lymphoma cells and mouse xenografts, TW-37 was also reported to enhance cytotoxicities of DNA damaging drugs on solid tumors (Table **3**).

TW-37 was further demonstrated to dose-dependently induce apoptosis through mitochondrial depolarization and activation of caspases 9 and 3 in human endothelial cells and B-cell tumors [61, 106]. Mechanistic *in vitro* investigations confirm that TW-37 is a potent pan-Bcl-2 inhibitor that disrupts interactions of proapoptotic Bax and truncated Bid (tBid) with prosurvival Mcl-1, Bcl-2 and Bcl-xL as revealed by coimmunoprecipitation data [62]. Heterodimer formations between Bim and prosurvival Mcl-1 and Bcl-xL were also shown to be hindered by this derivative [61].

TW-37 was also demonstrated to inhibit proangiogenic and prometastatic activities of Bcl-2 in addition to mitochondrial apoptotic pathway activation in human endothelial and pancreatic cancer cells. Such functionally separate effects were reported to be mediated by attenuation of NF-κB activity and suppression of angiogenic chemokine expression. Zeitlin and others demonstrated that subapoptotic concentrations of TW-37 were sufficient to inhibit VEGF-induced sprouting of endothelial cells in collagen [106]. *In vitro* inhibition of invasion and angiogenesis through use of TW-37 was further verified in cell migration and tube formation assays conducted on human endothelial cells [21]. The *in vivo* evidence to inhibition of tumor angiogenesis by TW-37 without serious systemic toxicity in mouse xenograft models provides further support to potential therapeutic value of BH3 mimetics as anticancer drugs with antiangiogenic/antimetastatic capacity [21].

Recently, removal of two reactive aldehyde groups that are considered to be responsible for the major clinical side effects such as emesis and diarrhea from (-)-gossypol gave rise to a new pan-Bcl-2 antagonist that is much more potent than its predecessors [7]. Namely apogossypolone binds Bcl-2/xL and Mcl-1 with low nanomolar affinities and was shown to induce apoptosis with IC_{50} values in the range of 0,1-31 μM on various *in vitro* and *in vivo* models of human cancers (Table **1**,**2**). Furthermore, apogossypolone synergized with DNA damaging chemotherapeutics gemcitabine and doxorubicin in human pancreatic and liver cancer cells and mouse xenograft models of these cancers, respectively [67, 68]. Antitumor efficacy of the CHOP regimen was also observed to be enhanced with apogossypolone addition in lymphoma mouse xenografts [65]. Mechanism of proapoptotic action of apogossypolone includes mitochondrial cytochrome c release together with caspase-9, -8 and -3 activations [7, 65-67, 107]. Similar to its forerunner, apogossypolone was reported to downregulate Bcl-2/xL and Mcl-1 at protein level while interfering with heterodimer formation among pro- and antiapoptotic members of Bcl-2 family [67, 68].

To further improve the potency and mechanism-based action, substitution of 5,5' isopropyl groups with more druggable ketone, alkyl and amide groups yielded very promising novel gossypol derivatives as initially identified by molecular docking computer screens. An optically pure apogossypol derivative named BI-97C1 displays increased affinity for Bcl-2 proteins compared to its precursors gossypol and apogossypol [35]. BI-97C1 was shown to inhibit binding of BH3 peptides to Bcl-xL, Bcl-2, Mcl-1 and A1 with IC_{50} values of 0.31, 0.32, 0.20 and 0.62 μM, respectively and reported to exert tens of magnitudes higher toxicity against human prostate, lung and lymphoma cells compared to its parental compounds (Table **1**,**2**). Likewise, BI-97C1 reportedly shows *in vivo* antitumor activity in Bcl-2 overexpressing human prostate cancer and Mcl-1-dependent B-cell xenografted mouse models. Mechanistic analysis indicates that BI-97C1 exerts less cytotoxicity against Bax/Bak-deficient cells compared to gossypol and apogossypol further confirming the specificity of the compound [35].

Another gossypol derivative worth mentioning is the compound 6f which was synthesized from apogossypolone in a similar fashion to derivation of BI-97C1 from apogossypol [69]. Indeed, 6f was shown to exert decreased affinity against its targets compared to apogossypolone. The 6f binds Bcl-xL, Bcl-2, and Mcl-1 with IC_{50} values of 3.10, 3.12, and 2.05 μM, respectively. However, *in vitro* and *in vivo* efficacy of 6f was reported to be better than apogossypolone on human solid and liquid cancer cells and a nude mice model of leukemia [69].

Similar to HA14-1, gossypol and derivatives have also been implicated in triggering protective form of autophagy in various human epithelial cancer cells [108]. Inhibition of autophagy has been reported to promote gossypol induced apoptosis in these cells. In this regard, clinical use of autophagic inhibitors such as stable wortmannin derivatives, 3-methyladenine or chloroquine together with Bcl-2 antagonists might serve to potentiate apoptosis once it is confirmed that the autophagy induced is of prosurvival type.

Being pan-Bcl-2 SMIs, gossypol and derivatives possess the advantage of targeting more anti,apoptotic Bcl-2 molecules than more specific Bcl-2 hitters that target only Bcl-2/xL/w subset. Recent pre-clinical and clinical evidence indicate that downregulation/inactivation of all prosurvival Bcl-2 proteins at once offers a greater advantage than targeting them individually [109]. Redundant functioning among these effectors is evident as preclinical data indicate that when Bcl-2/xL/w are specifically targeted, resistant cancer cells display intrinsically high Mcl-1 expression or they make a cellular switch to Mcl-1 activity for maintaining antiapoptosis function. Elevated potency, cancer cell-specificity, and improved mechanism-based cytotoxicity make the novel derivatives such as apopgossypolone, 6f, and BI97C1 even more promising than their parental compound gossypol.

OBATOCLAX

Obatoclax was discovered as a polypyrrole pan-Bcl-2 SMI utilizing a high throughput protein-protein interaction screening of natural compound libraries [38]. This polypyrrole binds to prosurvival Bcl-2, Bcl-xL and Mcl-1 with nanomolar affinities and was reported to exert *in vitro* and *in vivo* single agent apoptogenic activity on various human malignancy models (Table **1**, **2**). Although obatoclax was found to be minimally cytotoxic to human cholangiocarcinoma and melanoma cells, it proved apoptotic even at submicromolar concentrations against *in vitro* models of several human hematological malignancies, lung, colon, prostate and cervical carcinomas, and mouse mammary carcinoma. Additionally, a vast number of *in vitro* studies on various cancer models demonstrate that this SMI synergizes cytotoxically with approved/experimental anticancer agents and therapies (Table **3**).

In vitro and *in vivo* analysis indicates that obatoclax cytotoxicity mainly involves mitochondrial apoptotic pathway. Activation and mitochondrial translocation of Bax/Bak, mitochondrial depolarization, cytochrome c release, subsequent activations of caspases 9 and 3, all of which are hallmarks of mitochondrial apoptosis, were reported to precede apoptosis [15, 73, 76, 77, 80, 142]. Remarkably, molecular interrogation demonstrated that obatoclax is capable of direct Bax activation in a cell-free system [142]. Meanwhile, only Bim, tBid and Puma among several BH3-only proteins have been shown to directly activate Bax/Bak to initiate MOM permeabilization [9]. Moreover, obatoclax did not cause cytochrome c release from isolated mitochondria of sensitive cholangiocarcinoma cells indicating that obatoclax mediated mitochondrial cytotoxicity requires further cytosolic components [142].

Studies on human cancer cells reveal that obatoclax-induced apoptosis is preceded by liberation of Bak from Mcl-1/Bcl-xL, and Bim from Bcl-2/Mcl-1 [73, 75, 78, 81, 124]. In addition to cellular

Table 3. Anti-Tumoral Synergies Between BH3 Mimetics and Anticancer Agents

SMI	Synergistic agents	Cancer model, references
HA14-1 and derivatives	Cytarabine, dexamethasone, doxorubicin, Fas ligand, bortezomib, MG-132, staurosporine, chlorambucil, fludarabine, VSV oncolysis	Hematologic [10, 24, 29, 31, 47, 110]
	Radiotherapy, etoposide, paclitaxel, cisplatin, genistein	Other [27, 48, 49, 111-113]
BH3Is	TRAIL, radiation, doxorubicin, bortezomib, TRA-8	[50, 114-117]
2-MeAA	Cisplatin, docetaxel, paclitaxel, TRAIL	Solid tumors [42, 45, 46]
Gossypol and derivatives	Cyclophosphamid*, rituximab*, carfilzomib, etoposide, doxorubicin, 4-HC, CHOP*, radiotherapy, dexamethasone	Hematologic [51, 53, 56, 62, 65, 118]
	Radiation*, anti-androgen therapy, gemcitabine, cisplatin*, doxorubicin *	Other [54, 59, 60, 63, 64, 67, 119]
Chelerythrine	No data	
YC137	Miriplatin, antiestrogen treatment	Solid tumors [120, 121]
Obatoclax	Bortezomib, melphalan, dexamethasone, VSV oncolysis*, rituximab, cisplatin, doxorubicin, vincristine, ABT-737, cytarabine, MGCD0103, vorinostat	Hematologic [73-75, 77, 122, 123]
	Gefitinib, cisplatin	Lung [78]
	Bortezomib, tunicamycin, thapsigargin	Melanoma [80, 82]
	5FU/carboplatin, TRAIL	Other [19, 81, 124]
ABT-737/263	Cytarabine, doxorubicin, etoposide*, radiation, bortezomib*, carfilzomib, vincristine*, VAP cyclophosphamide*, vorinostat, rituximab*, R-CHOP*, rapamycin*, L-ASP*, dexamethasone*, imatinib, homoharringtonine, fludarabine, topotecan*, melphalan	Hematologic [43, 83, 85, 86, 88, 90-92, 125-129]
	Carboplatin/etoposide, actinomycin D, gemcitabine, etoposide*, paclitaxel, erlotinib*	Lung [93, 94, 130-133]
	Dacarbazine fotemustine imiquimod, SB202190, ARC	Melanoma [134-136]
	CPT-11, celecoxib, PE-based immunotoxins, oxaliplatin	Colorectal [14, 100, 137, 138]
	Docetaxel, carboplatin*, paclitaxel, K5I	Ovarian [44, 133, 139]
	ARC, GDC-0941*	Breast [136, 140]
	Cisplatin, etoposide	Head and neck [98]
	Gemcitabine, ARC, imatinib, etoposide, vinblastine, paclitaxel, K5I, TRAIL, sorafenib	Other [97, 99, 101, 131, 136, 139, 141]
S1	No data	

*Observed in *in vivo* mouse models in addition to *in vitro* set-ups.
Abbreviations: 2-MeAA, 2-methoxy-antimycin A3; 4-HC, 4-hydroxycyclophosphamide; CHOP, cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone; VSV, vesicular stomatitis virus; 5FU, fluorouracil; VAP, vincristine-doxorubicin-prednisone; R-CHOP, rituximab-CHOP; L-ASP, L-asparaginase; CPT-11, irinotecan; K5I, kinesin-5 inhibitor.

systems, obatoclax was also shown to disrupt Mcl-1-Bak interaction on intact MOM in a cell-free set-up [80]. However, along with caspase-dependent apoptotic pathways, caspase-independent cell death pathways were also associated with obatoclax induced cytotoxicity as indicated with indifference of apoptotic sensitivity to caspase-inhibition in primary tumors extracted from B-cell lymphoma patients [74].

Innovative experimental designs might prove useful in cell death research when it comes to in-depth analysis and confirmation of drug action. Utilization of yeast as a model organism to interrogate the interactions of obatoclax with prosurvival Bcl-2 proteins is one of those strategies [80]. Yeasts lack any Bcl-2 homologs and are less complicated in terms of intracellular survival/death signaling to the contrary of mammalian cells that possess a number of delicately interconnected pathways. Ectopic expression of proapoptotic Bax/Bak inhibits yeast growth. Obatoclax was shown to counter the resistance to growth inhibition that forced expression of Bcl-2/w/xL or Mcl-1 conferred to yeast cells. Furthermore, expression of any prosurvival Bcl-2 protein did not render the yeast cells sensitive to obatoclax in the absence of Bax/Bak expression. In concert with these findings, obatoclax induced apoptosis was shown to significantly resisted by Bax/Bak deficient mouse kidney cells [80]. Moreover, in agreement with the concept that BH3 mimetics are aimed to kill cells that are already primed to death, obatoclax was demonstrated to induce apoptosis in mantle cell lymphoma cells that were defective in DNA damage monitoring and cell cycle regulation. Normal peripheral blood mononuclear cells were reported to be spared by obatoclax [73]. On the contrary, a couple of investigations point out that obatoclax may also induce Bax/Bak-independent cell death mechanisms in human cancer cells [15, 75, 142]. Obatoclax has also been shown to induce cytotoxic form of autophagy as the sole cell killing machinery [74]. Brem and colleagues have shown that Beclin-1 knockdown inhibits obatoclax mediated cytotoxicity while caspase inhibition do not influence the cell death in human lymphoma cells. Further interrogation of such an action mechanism may prove invaluable to design alternative anticancer strategies using Bcl-2 antagonists to interfere with Beclin-1-Bcl-2 interaction.

In brief, mechanistic studies apparently confirm the potential of obatoclax to achieve mechanism-based toxicity. The few cases that

associate Bax/Bak-independent apoptotic action might be related with secondary activities to MOM permeabilization or interference with Bcl-2 binding of non-Bcl-2 proteins such as Beclin-1 rather than off-target hitting.

ABT-737

ABT-737 was developed by a rational drug design approach utilizing NMR-based screening and synthesis [2]. Exploiting the predicted structure BH3 docking groove of Bcl-xL, small compounds that target separate regions of the surface groove were brought together covalently to design ABT-737. The subnanomolar affinites of ABT-737 for Bcl-2/xL/w which were a few orders of magnitude lower than any previously reported Bcl-2 antagonist significantly improved the specificity.

The majority of the experimental evidence confirms that ABT-737 kills cancer cells solely *via* the intended mechanism. ABT-737 was shown to strictly require Bax/Bak to induce cell killing in mouse embryonic fibroblast (MEF) and human cancer cells as expected from a genuine BH3 mimetic [109, 125, 143]. A comparative study reports that sensitivities of Bax/Bak-deficient MEFs to HA14-1, BH3I-1, antimycin A, chelerythrine, and (-)-gossypol are similar to wild-type (wt) cells while ABT-737 is cytotoxic only to wt-MEF cells [125]. Similarly study of Vogler and co-workers indicate that ABT-737 mediated apoptosis is completely inhibited in Bax/Bak or caspase-9-deficient cells while Bax/Bak or caspase-9 expression is not required for cell death induced by HA 14-1, gossypol, apogossypol, obatoclax, and EM20-25 [32].

Such Bax/Bak-independent toxicities of SMIs that were tested against ABT-737 indicate that they might not behave solely as BH3 mimetics but might have additional cellular targets which could be explained by their relatively lower affinities for target Bcl-2 proteins. Furthermore, ABT-737 was demonstrated to induce cytochrome c from isolated mitochondria of only cancerous cells and not from healthy counterparts. Buron and co-workers report that in addition to leading Bax-dependent cytochrome c release, ABT-737 also shows tumor-specific mitochondrio-toxicity as opposed to other tested antiapoptotic Bcl-2 protein SMIs including HA14-1, EM20-25, YC-137, chelerythrine, gossypol, and TW-37 [109]. This tumor-specific activation of intrinsic apoptotic pathway further adds to the therapeutic potential of this BH3 mimetic. Additionally, ABT-737 was indicated not to directly trigger cytochrome c from mitochondria, but instead to decrease the cellular apoptotic threshold which makes it a suitable drug to combine with conventional anticancer therapies [125].

Among *in vitro* and *in vivo* cancer models that ABT-737 displays single agent efficacy are hematological malignancies and solid tumors of lung, prostate, pancreas, colon, ovaries and GI tract (Table **2**). ABT-737 has proven effective with low nanomolar IC_{50} values against several lymphomas, leukemias, myelomas, and small cell lung carcinoma (SCLC) which are known for extraordinarily high Bcl-2, Bcl-xL and Mcl-1 expression [2, 11, 83-96]. Furthemore, ABT-737 has been shown to synergize with a huge array of both experimental and approved cytotoxic agents on *in vitro* and *in vivo* hematological and solid cancer models (Table **3**).

Similar to HA14-1 and gossypol, ABT-737 was also associated with prosurvival autophagy. Human colon cancer cells were indicated to respond to anticancer drug celecoxib both by apoptosis and autophagy both of which were potentiated through addition of ABT-737 [14]. Genetic and pharmacological inhibition of autophagy confirmed the prosurvival nature of the process by enhancing apoptosis induced by celecoxib and ABT-737 together. Moreover, critical involvements of Bcl-2 and Bcl-xL in autophagy induction as well as apoptotic process were verified through use of silencing and ectopic expression. However, although such findings appear to rationalize the therapeutic strategy of combining autophagy inhibitors with Bcl-2 antagonists, presence of data that indicate obatoclax mediated activation of prodeath autophagy rather than the prosurvival type complicates the situation [14].

Putative anticancer agents do not always work for cell killing, but instead may promote growth inhibition or senescence. SMIs of antiapoptotic Bcl-2 proteins have not been exceptions to this [57, 64, 68, 107, 144, 145]. Although apoptosis was not shown to necessarily follow growth inhibition, raising the drug concentration or addition of a combinatory cytotoxic anticancer agent were shown to execute apoptosis in several investigations. Likewise, ABT-737 was demonstrated to induce p53-dependent senescence in solid tumors without subsequent apoptosis [13]. Further molecular interrogation revealed that ABT-737 caused a certain level of cellular ROS accumulation that was sufficient to induce marginal DNA damage, p53 activation and low-level caspase activity which was insufficient to initiate apoptotic process.

Despite the fact that it misses Mcl-1/A1, subnanomolar affinity of ABT-737 for Bcl-2/xL/w appears to help this SMI kill predominantly by the intended mechanism. Having shown to solely trigger Bax/Bak-dependent apoptosis and specifically kill tumor cells while sparing normal ones ABT-737 stands as one of the most promising BH3 mimetics in anticancer therapy. However, ABT-737 is not orally bioavailable; a major limitation which could limit chronic single agent therapy and dose flexibility in combination regimens. But, the introduction of the orally bioavailable analogue ABT-263 seems to has overcome such clinical limitations [90].

OTHER BH3 MIMETICS

BH3Is and chelerythrine were introduced in early 2000s as low micromolar affinity inhibitors of Bcl-xL [33, 36]. These SMIs were shown to induce intrinsic apoptotic pathway in various mammalian cancer cells (Table 2). Moreover, BH3Is were reported to sensitize cancer cells to chemo-/radiotherapy through activation of intrinsic apoptotic pathway (Table 3). However, BH3I series and chelerythrine were implicated in severe mitochondrial toxicity and/or off-targeting which significantly hinder their therapeutical potential as BH3 mimetics [10, 28, 146]. Furthermore, chelerythrine which las long been known as a PKC inhibitor, was also shown to induce both Bax-Bak and ROS-independent apoptosis suggesting nonmechanism based action on cardiac derived cells [72].

YC137 is another BH3 mimetic that was demonstrated to have low micromolar affinity for Bcl-2 [37]. YC137 was reported to exert single agent activity through activation of intrinsic apoptotic pathway against breast cancer cells. In addition, YC137 was shown to sensitize several solid tumors to DNA damaging chemotherapeutics and antiestrogen treatment (Table **3**).

S1 was introduced recently as a promising SMI that binds both Bcl-2 and Mcl-1 with low nanomolar affinities [102]. This compound which may potentially target both prosurvival Bcl-2 subsets, was shown to trigger apoptosis at nanomolar to low micromolar IC_{50} values in breast, liver, and cervical cancer cell lines (Table **2**). Further mechanistic analysis indicates that S1 works through intrinsic apoptotic pathway, does not activate Bax directly and exerts significantly lower toxicity to Bax/Bak double deficient cells compared to wild-type cells as anticipated from a genuine BH3 mimetic [39].

SOMETHING NOT TO BE MISSED: TARGETING MCL-1

Despite all prosurvival Bcl-2 family members exert their antiapoptotic function in a similar manner through engagement of their proapoptotic counterparts, structurally they could be divided into two relatively divergent subsets. While Bcl-2, Bcl-xL and Bcl-

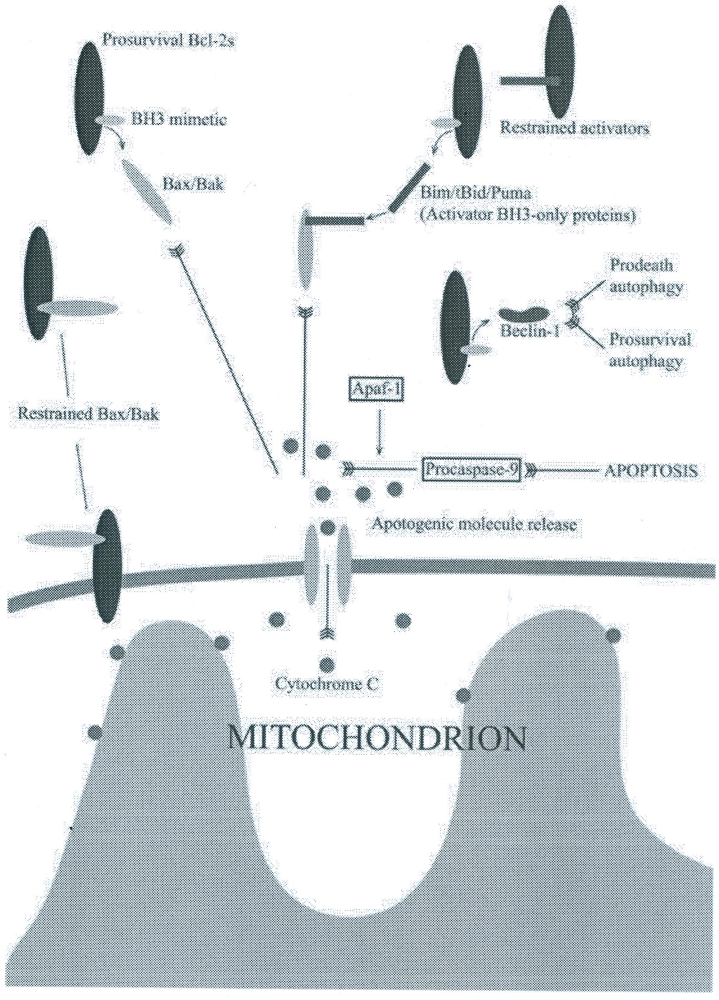

Fig. (2). Assumed/demonstrated action mechanisms of BH3 mimetics in cancer cells. See text for details.

w are structurally close to each other based on the accessibility of their hydrophobic groove by proapoptotic Bcl-2 proteins, Mcl-1 and A1 present structural characteristics that fall between the liganded and unliganded conformers of Bcl-2 [147]. The relatively narrow hydrophobic groove of Mcl-1 seems to hurdle the access of Bcl-2/Bcl-xL-specific SMIs such as ABT-737 and HA14-1 [39]. Moreover, molecular investigations have shown that BH3-only proteins have binding preferences among their prosurvival target Bcl-2s. Although Bim and Puma are promiscuous binders with no selectivity at all, Bad, Bmf, and Noxa appear to have strictly restricted Bcl-2 targets [148] (Fig. **3**). However, whilst having slightly different affinities for BH3-only molecules, plenty of evidence indicates that prosurvival Bcl-2s are capable of redundant operation [48, 100, 139].

Recently, Mcl-1 mediated resistance emerged as one of the major obstacles as several anticancer chemotherapeutics and Bcl-2/xL/w-specific inhibitors failed to succeed because of intrinsic or drug-induced Mcl-1 expression. Although elevated Bcl-2/xL/w expression has proven invaluable as an indicator of ABT-737 sensitivity, a multitude of evidence implicates Mcl-1 in ABT-737 and HA14-1 resistance. Success of ABT-737 to induce apoptosis was shown to correlate negatively with Mcl-1 expression in different hematological and epithelial cancer models. Konopleva and colleagues demonstrated that acute myeloid leukemia (AML) cells with high intrinsic Mcl-1 expression proved resistant to ABT-737 and this resistance was overcome by suppression of Mcl-1 expression [85]. Similarly, resistance to ABT-737 was reported to correlate with Mcl-1 expression in a panel of SCLC and leukemia/lymphoma cell lines [149]. Downregulation of Mcl-1 using shRNA proved to nullify drug resistance in cervical and breast cancer cells [125, 140]. Furthermore, stabilization of Mcl-1 at protein level was shown to cause failure of ABT-737 to suppress hepatoma xenograft tumors in mice [141].

On the other hand, sensitivity to SMIs with restricted binding profiles may not be simply determined by expression profiling of antiapoptotic Bcl-2s. Mason and colleaugues' investigation failed to discern any simple relationship between expressions of Bcl-2/xL/w or Mcl-1 and ABT-737 sensitivity in primary chronic lymphocytic leukemia cells [88]. In such cases, endogeneous expression of Mcl-1/A1-specific Noxa or A1 itself may prove helpful determinants of sensitivity. Accordingly, either intrinsic or enforced Noxa expression was reported to correlate with ABT-737 sensitivity confirming the critical status of Mcl-1 in a number of *in vitro* solid and hematological cancer models [149]. Proapoptotic BH3-only protein Noxa is known to exclusively bind/neutralize Mcl-1/A1 among prosurvival Bcl-2 proteins [9, 125, 148] (Fig. **3**). Enforced Noxa expression that sensitized MEFs and myelomonocytic cells to ABT-737 was presumed to inactivate Mcl-1 since A1, the other target of Noxa, was absent from many cell types that van Delft and others utilized [125]. Likewise, RNA interference studies on

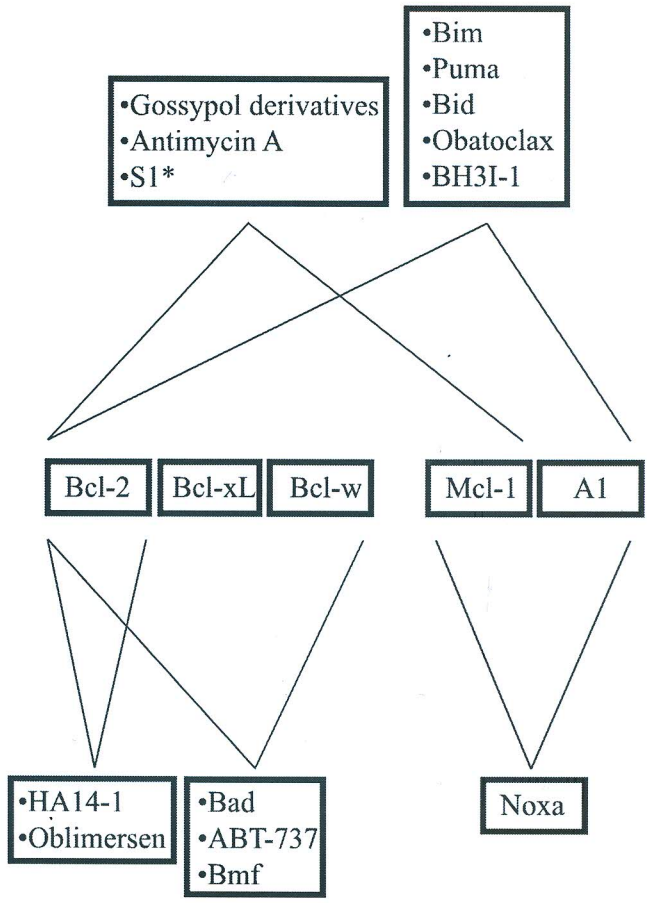

Fig. (3). Differential targeting of prosurvival Bcl-2s by BH3 mimetics and BH3-only proapoptotic proteins.

*Bcl-2 antagonist S1 was shown to bind both Bcl-2 and Mcl-1 which are structurally divergent. However, its binding to other prosurvival Bcl-2 proteins are not demonstrated yet [102]. BH3 only proteins Hrk and Bik exert preference for Bcl-xL, Bcl-w, and A1 [148].

lymphoma and renal carcinoma cells revealed the critical role of A1 expression in ABT-737 resistance. Manipulative downregulation of A1 as well as its close functional partner Mcl-1 was reported to reverse ABT-737 resistance [97].

Mechanistic analysis of the synergies among ABT-737 and various chemotherapeutics or experimental apoptotic agents may contribute to rational selection of anticancer agents to combine with Bcl-2/xL/w-specific BH3 mimetics. (Table **4**). Numerous agents that damage DNA, interefere with mitosis, DNA/protein synthesis, or inhibit tyrosine kinase activity were shown to potentiate ABT-737 cytotoxicity through critical Mcl-1 downregulation or neutralization. One such synergy was reported to exist between bortezomib and ABT-737. Bortezomib is a proteasome inhibitor approved as a drug against mantle cell lymphoma and multiple myeloma. Inhibition of proteasome degradation impacts cellular Mcl-1 more dramatically than Bcl-2/xL in short term due to quick turnover rates of the molecule. Mcl-1 is unique among other pro-survival Bcl-2s with its rapid (~17 min) steady-state turnover rate which is a result of constitutive ubiquitination by ubiquitin ligases [148, 150]. Massive accumulation of Mcl-1 was reported to be responsible from bortezomib resistance in various cancers. Similarly ABT-737 is barely efficient as a single agent in a number of cancers due to high-level expression of Mcl-1. However, bortezomib-ABT-737 combination reportedly succeeded in human colorectal carcinoma cells by inhibition of elevated Mcl-1 activity which is attributed to inactivation of bortezomib-elevated cellular Mcl-1 by Bim/Bak that were released from Bcl-2/xL by ABT-737 [100]. Engagement of Bcl-2/xL/w with ABT-737 also releases some BH3-only proapoptotic Bcl-2 proteins to bind their normally less-preferred targets Mcl-1/A1 (Fig. **3**). For instance, Bmf which has a binding preference for Bcl-2 over Mcl-1 was reported to make a shift from Bcl-2-binding to Mcl-1-engagement in Bcl-2-overexpressing lymphoma cells treated with a HDAC inhibitor plus ABT-737 [128]. Vorinostat-mediated Bmf upregulation and competition of ABT-737 for Bcl-2 binding was reported to cause this switch in binding behaviour of Bmf. In this regard, combining Bcl-2/xL/w-specific SMIs with chemotherapeutics that increase BH3-only proteins which broadly bind all prosurvival Bcl-2s may be a rational solution to overcome drug resistance.

To the contrary of Bcl-2/xL/w-specific Bcl-2 SMIs, broad Bcl-2 antagonists proved successful in killing several cellular and animal models of human cancers with Mcl-1-conferred drug resistance [140, 151]. Administration of (-)-gossypol was demonstrated to induce time and dose-dependent apoptosis in primary CLL B-cells *via* downregulation of Mcl-1 which was shown to be the critical survival protein against fludarabine induced apoptosis in these cells [52]. Residual malignant cells that remain in association with accessory cells in their microenvironment following surgery or first course of chemo-/radiotherapy are the major cause of relapses. Balakrishnan and co-workers showed that Mcl-1 was upregulated when CLL B-cells were cocultured with bone marrow stromal cells that protected them from both spontaneous and fludarabine

Table 4. Bases of Anti-Tumoral Synergy with ABT-737

Synergistic agent	General action mechanism*	Critical synergistic mechanism†	Cancer models, references
Cytarabine, etoposide, cisplatin, gamma-irradiation, carboplatin/etoposide, gemcitabine	DNA damage	Mcl-1 downregulation (transcriptional or post-translational)	Hematologic [43, 86, 125, 143] Lung [93, 130, 131, 139, 143] HNSCC [98] Breast [136, 140] Melanoma [136] Liver [141] Pancreatic [143] Prostate [131] Other [131, 136, 139]
ARC, actinomycin D, homoharringtonine	Protein synthesis inhibition		
Paclitaxel, K5I	Mitotic arrest		
Sorafenib	Tyrosine kinase inhibition		
GDC-0941	PI3-kinase inhibiton		
L-asparaginase	Asparagine hydrolysis		
Etoposide, oxaliplatin, cisplatin, CPT-11, dacarbazine, fotemustine, carboplatin/etoposide	DNA damage	Noxa upregulation and subsequent Mcl-1 or A1 neutralization	Lymphoma [90, 126, 128] Myeloma [90] Colorectal [100, 138] Melanoma [134] Lung [93, 130] Other [97]
Actinomycin D	Protein synthesis inhibition		
Vinblastine, paclitaxel	Mitotic arrest		
Bortezomib, MG-132	Proteasome inhibition		
Imiquimod	Immune activation		
Vorinostat	HDAC inhibition		
Etoposide	DNA damage	Puma upregulation	Lymphoma [126] Melanoma [135] Lung [93]
SB202190	p38 inhibition		
Bortezomib	Proteasome inhibition		
Vorinostat	HDAC inhibition	Transcriptional Bmf and Bim upregulation	Lymphoma [128]
Topotecan	DNA damage	Activation of p53	Leukemia [43]
Imatinib	Tyrosine kinase inhibition	KIT/PDGFRA survival signaling inhibition	Gastrointestinal [101]
Rapamycin	Inhibition of mTOR	STAT5-mediated AKT/mTOR targeting	Myeloproliferative [129]
		Cell cycle arrest	Lymphoma [145]
PE-based immunotoxins	Protein synthesis inhibition	ER toxicity	Colon [137]
TRAIL	Apoptotic induction	Extrinsic apoptotic pathway activation	Pancreatic [99]

*Widely known anti-tumor mechanism of the synergistic drug.
†Specific action mechanism of the synergistic drug that was shown to cause synergy with ABT-737.
Abbreviations: CPT-11, irinotecan; K5I, kinesin-5 inhibitor; HDAC, histone deacetylase; ER, endoplasmic reticulum; HNSCC, head and neck squamous cell carcinoma.

triggered apoptosis. (-)-Gossypol administration was also able to induce apoptosis and reverse the critical Mcl-1 accumulation beating the microenvironment mediated resistance against fludarabine induced apoptosis [52]. Furthermore, apogossypolone was reported to upregulate Noxa while downregulating Mcl-1 together with other prosurvival molecules Bcl-2 and Bcl-xL in killing liver cancer cells [67]. Similarly, potent apogossypol derivative BI-97C1 was reported to exert single agent antitumor activity against prostate cancer mouse xenografts and cytotoxity against human lymphoma cells which rely on Mcl-1/A1 for survival.[35]. Synergy between obatoclax and death ligand TRAIL is another evidence implicating pan-Bcl-2 inhibitors in overriding Mcl-1 resistance. Huang and others demonstrated that obatoclax contributed to this synergistic cytotoxicity *via* inhibition of Mcl-1 sequestration that neutralizes proapoptotic Bak/Bim in human cholangiocarcinoma and pancreatic cancer cells [81, 124].

CLINICAL TRIALS

An alternative anticancer strategy to interrupt elevated Bcl-2 oncogene activity is antisense mediated downregulation which proved promising during pre-clinical investigations. The antisense oligodeoxyribonucleotides (ODNs) are designed to specifically bind mRNAs on the basis of base-complementarity and to subsequently turn the relevant genes off by preventing their translation. In this regard, oblimersen, a Bcl-2 antisense ODN appeared as the first antisense drug to reach advanced clinical trials in oncology [152]. These trials in several hematological malignancies and solid tumors have yielded mixed results [153, 154] (Table **5**). The basic issue that oblimersen has faced is inefficient drug delivery as indicated by limited Bcl-2 downregulation at target tumors. The antisense ODNs are large polyanions that cannot easily diffuse into the cells and they are subject to rapid systemic clearance. Because of short plasma half-life oblimersen is administered as prolonged continuous intravenous infusions which is fairly inconvenient for patients. Furthermore, some positive effects of oblimersen in clinical trials were at least partly attributed to immune stimulation by their two CpG motifs [154]. Although novel *in vitro* and *in vivo* approaches to oblimersen delivery such as administration within lipid nanoparticles or human serum albumin coated liposomes produced progress in plasma half-life and tumor accumulation of ODNs, future approval of oblimersen remains uncertain [153, 154].

The initial phase I/II clinical study reports pertaining to the efficacy of (-)-gossypol in heavily pretreated patients with prostate and lung cancers have recently been available to the scientific society (Table **6**). Although AT-101 (R-(-)-gossypol acetic acid) was well tolerated in both non-small cell lung carcinoma (NSCLC)

Table 5. Clinical Trials of Oblimersen

Condition, references	N	CMB	Clinical activity						
			OR %	CR %	PR %	SD %	Relative improvement	Other*	Major drug related toxicities
Relapsed/ refractory B-cell NHL phase II [155]	42	RTX	42	23	19	28	NA		Well tolerated
Advanced breast cancer phase I/II [156]	30	DXN + DTX	70	0	70		NA	Very little Bcl-2 downregulation	Well tolerated
CRPC phase I/II [157]	111	DTX	54	30	24	43	No (Vs. DTX-only)	Increased toxicity (Vs. DTX-only)	Thrombocytopenia Thrombosis
Advanced skin cancer phase II [158]	12	None	0	0	0	25	NA	Antitumor activity in one patient	Lymphopenia Cytopenia
Advanced melanoma, phase III [159]	771	DTIC	14	3	11		Increased PFS, OR, DR and CR (Vs. DTIC-only)		Neutropenia Thrombocytopenia
Relapsed/ refractory myeloma phase III [160]	224	DEX					No improvement in TTP or ORR (Vs. DEX-only)		Well tolerated
Relapsed/ refractory CLL phase III [161]	121	FDB + CYC	, 17				Increased CR and DR (Vs. FC-only)		Well tolerated
Advanced upper GI cancer phase I [152]	15	5-FU + CSP	NA	NA	NA	NA	NA	Antitumor activity	Neutropenia

*Any clinical activity observed at any proportion of patient.
Abbreviations: N, enrollment, CMB, combined with; OR, overall response; CR, complete response; PR, partial response; SD, stable disease; NA, not applicable; NHL, non-Hodgkin lymphoma; CRPC, castration resistant prostate cancer; CLL, chronic lymphocytic leukemia; RTX, rituximab; DXN, doxorubicin; DTX, docetaxel; DTIC, dacarbazine; DEX, dexamethasone, FDB, fludarabine; CYC, cycloheximide; 5-FU, 5-fluoro-uridine; CSP, cisplatin; PFS, progression free survival; OS, overall survival; TTP, time to progression.

and SCLC patients with minor hematological toxicities, gastro-intestinal toxicities were reported as the major toxicities that may limit dosage flexibility, especially in prostate cancer patients [162-164]. AT-101 was safely combined with a reduced topotecan dose and a promising 10% partial response was reported in cancer patients with relapsed SCLC. Moreover, partial response rate was reported as 17% in drug sensitive cohort. However, lack of a topotecan-only group in this study makes it difficult to judge on the contribution of AT-101 to the synergistic efficacy in chemosensitive cohort [162]. Another gossypol clinical trial has been conducted on patients with recurrent chemosensitive extensive stage SCLC. Recently introduced outcomes of this phase II study are not very encouraging with absence of any response to single agent AT-101 therapy among 14 evaluable patients [165]. Similarly, phase II clinical trial data obtained from advanced and metastatic NSCLC patients indicate that no improvement was achieved by addition of AT-101 to docetaxel therapy in terms of overall survival [163]. Phase I/II clinical trials evaluating (-)-gossypol efficacy either as a single agent or in combination with approved anticancer drugs including docetaxel and cisplatin are ongoing on patients with advanced or metastatic solid tumors.

Another broad Bcl-2 antagonist obatoclax has been being assessed in patients with advanced hematological malignancies and SCLC either alone or as combined with topotecan (Table **6**). Early outcomes of phase I/II trials point to a few partial or even complete responses. Together with a 20% partial resonse rate, clinical anticancer activity was observed in 2 out of 5 pretreated advanced non-Hodgkin's lymphoma (NHL) patients [166]. Pretreated chronic lymphocytic leukemia (CLL) and myelofibrosis patients suffering from thrombocytopenia and anemia were reported to achieve improvement in their platelet and hemoglobin counts during obatoclax monotherapy [151, 167]. Although obatoclax was well tolerated overall, grade 1/2 central nervous system (CNS) toxicities including ataxia, euphoria, and confusion were reported to be the major infusional toxicities. The exact mechanism of CNS toxicity is not understood yet, but protective effects of Bcl-2 expression against neuronal apoptosis and alteration of synaptic transmission were suggested as the possible causes [151]. Ongoing phase I/II clinical trials evaluating obatoclax efficacy are primarily focused on relapsed or refractory leukemia, myeloma, lymphoma and SCLC. Obatoclax has been being combined with chemotherapeutics such as rituximab, doxorubicin, vincristine, carboplatin, etoposide and topotecan for assessment.

The preliminary clinical data pertaining to dose safety, pharmacokinetics, pharmacodynamics, and preliminary efficacy of ABT-263, the orally bioavailable form of ABT-737, have been

Table 6. Clinical Trials of Antiapoptotic Bcl-2 Protein SMIs

SMI, condition, references	N	CMB	Clinical activity						
			OR %	CR %	PR %	SD %	Relative improvement	Other*	Major drug related toxicities
AT-101 CRPC Phase I/II [164]	23	None	0	0	0	11	NA	PSA decline (11% of patients)	GI toxicity
AT-101 SCLC Phase I/II [162]	30	TPT	10	0	10	50	NA	17% PR in sensitive relapsed cohort	Minor hematologic toxicities
AT-101 NSCLC Phase II [163]	105	DTX					No improvement in PFS or OS (Vs. DTX-only)		Well tolerated
AT-101 CRPC Phase II [169]	220	DTX + PDN					No improvement in PSA decline		
AT-101 SCLC Phase II [165]	14	None	0	0	0	21			GI toxicity Anorexia
OBATOCLAX CLL Phase I [167]	26	None	4	0	4		NA	Improvement in hemoglobin and platelet counts	Somnolence Ataxia Confusion
OBATOCLAX Hematologic malignancies Phase I [170]	44	None	2	2	0		NA	• Hematologic improvement • 4% CR in AML patients	Well tolerated with minor neurologic symptoms
OBATOCLAX SCLC Phase I [171]	8	TPT	25	0	25	50	NA	Phase 2 ongoing	Somnolence Ataxia
OBATOCLAX SCLC Phase II [172]	9	TPT	0	0	0	56	No (Vs. historical TPT efficacy)		Thrombocytopenia Neutropenia Ataxia Anemia
OBATOCLAX Solid tumors and lymphoma Phase I [166]	35	None	3	0	3	20	NA	• Clinical activity against NHL • 20% PR in NHL	Neurologic symptoms
OBATOCLAX Myelofibrosis Phase II [151]	22	None	0	0	0		NA	Improvement in hemoglobin and platelet counts	Ataxia Fatigue Euphoria
ABT-263 SCLC and other solid tumors Phase I [173]	47	None	2	0	2	17	NA	3% PR and 28% SD in SCLC and carcinoid patients	Thrombocytopenia
ABT-263 Lymphoid malignancies Phase I [168]	55	None	22	0	22		NA	• Reduction in tumor size in 46% of patients • 50% PR in CLL/SLL patients	Thrombocytopenia Neutropenia

*Any clinical activity observed at any proportion of patient

Abbreviations: N, enrollment; CMB, combined with; OR, overall response; CR, complete response; PR, partial response; SD, stable disease; NA, not applicable; CRPC, castration resistant prostate cancer; PSA, prostate specific antigen; GI, gastrointestinal; SCLC, small cell lung cancer; TPT, topotecan; NSCLC, non-small lung cancer; DTX, docetaxel; PFS, progression free survival; OS, overall survival; PDN, prednisone; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin's lymphoma; SLL, small lymphocytic leukemia.

quite promising (Table **6**). ABT-263 proved to have clinical single agent antitumor activity in 46% of the patients with several relapsed or refractory lymphoid malignancies which are known to exhibit high cellular levels of antiapoptotic Bcl-2 proteins [168]. Furthermore, half of the CLL/SLL patients were reported to partially respond to ABT-263 monotherapy. ABT-263 was reported to be safe and well tolerated in patients with solid tumors and 3% partial response rate was observed among SCLC and pulmonary carcinoid patients along with 28% rate of stable disease [168]. Although the initial reports point at thrombocytopenia and T-cell lymphopenia as the major toxicities that limit dosing flexibility, the doses that were shown to be effective in preclinical animal models

were reported to be safely achieved by daily dosing schedule thank to the favorable pharmacokinetics of the drug. Undesired pharmacodynamic activity of ABT-263 against platelets and lymphocytes are attributed to intravascular apoptotic events regulated through Bcl-xL and Bcl-2, respectively. In this view, acute thrombocytopenia and lymphopenia upon administration of the drug could be assessed as evidence of intravascular inactivation of these two guardian proteins. Currently, phase II clinical trials of ABT-263 on relapsed or refractory leukemia/lymphoma patients are ongoing to evaluate the SMI either as single agent or in combination with bendamustine and rituximab. Likewise, phase I trials are being conducted on patients with various solid tumors to determine safety profile, maximum tolerated dose and pharmacokinetics of the drug. Remarkably, a phase II multi-center trial is currently recruiting previously untreated CLL patients to assess the efficacy of ABT-263 in combination with rituximab. Considering that the previous clinical trials of BH3 mimetics were conducted on heavily pretreated patients, the outcomes of the trial may indicate the efficacy at the initial stages of the disease.

Noteworthy, over interpretation should avoided when assessing the outcomes of clinical trials. Because, almost all these trials are conducted on heavily pretreated patients with relapsed, refractory, metastatic or advanced stage cancers. Clinical response of an already deteriorated condition might not completely reflect the potential of Bcl-2 antagonists at the initial stages of cancers. Furthermore, data from combination therapy trials lacking chemotherapeutic-only group or an historical monotherapy success information hinder the evaluation of specific contribution of the tested SMIs.

CONCLUDING REMARKS

One of the major obstacles in anticancer therapy is to achieve sufficient cytotoxicity for tumor killing while maintaining a low patient drug load. In this regard, use of SMIs of antiapoptotic Bcl-2 proteins could be conceived as a milestone in the area because of their relatively low toxicities compared to conventional chemo-/radiotherapies. This stems from unusual action mechanism of these SMIs. Antiapoptotic Bcl-2 protein SMIs are aimed to interfere with protein-protein interactions different from the conventional drugs that trigger direct DNA damage, interfere with DNA/protein synthesis and mitosis or inhibit certain tyrosine kinases. Other than single agent activity against cancer cells that are primed to death they have the potential to decrease patient drug load by synergizing with established chemo- and radiotherapies in both hematological and solid cancers.

Perhaps the most important concern about BH3 mimetics is off-target hitting and nonmechanism based toxicity which could increase toxicity to normal cells. While BH3I-1 and sHA14-1 were shown to have off-target molecules, Bcl-2 ligands such as HA 14-1, chelerythrine and BH3I-2 were implicated in mitochondrio-toxicity that leads to energy uncoupling and respiratory inhibition. Hence, elimination of toxicity to mitochondria of normal cells and increasing the target specificity are among the research priorities. In this regard, Bad-like Bcl-2 antagonist ABT-737 and its orally bioavailable derivative ABT-263 stand among the most extensively studied BH3 mimetics at preclinical level, especially for their action mechanism specificity. ABT-737 targets Bcl-2, Bcl-xL and Bcl-w with subnanomolar affinities, but misses structurally divergent Mcl-1 and A1. Current knowledge indicates that ABT-737 kills solely *via* the intended mechanism, i.e., intrinsic apoptotic pathway and exerts tumor specificity. However, Mcl-1/A1-mediated tumor resistance has been observed as a result of the restricted target range of this SMI. This limitation which was raised by redundant operation capability of antiapoptotic Bcl-2s could be bypassed by combining ABT-263 with established anticancer regimens that specifically downregulate Mcl-1/A1 or upregulate potent Mcl-1/A1 antagonist Noxa as well as promiscuous Bcl-2 binders Bim/Puma.

A couple of studies implicate broad Bcl-2 antagonists obatoclax and gossypol in Bax/Bak independent toxicity. However, although increased target range may possibly decrease target specificity, there is no direct evidence that gossypol and obatoclax have off-target molecules. In addition, some, if not all, nonmechanism based toxicities attributed to these SMIs may be due to inhibition of interactions of Bcl-2s with non-Bcl-2 molecules such as BH3 containing autophagic initiator Beclin-1 or MIM channels. It is established that aiming all of the antiapoptotic Bcl-2s at once could be of more clinical value than subset-specific targeting because of the plasticity of antiapoptotic gene expression in tumors and functional redundancy among antiapoptotic Bcl-2 proteins. In this view, recent gossypol derivatives apogossypolone, BI-97C1 and compound 6f that were demonstrated to have superior *in vitro/in vivo* efficacies compared to their parental compound appear to have potential to join gossypol, obatoclax, and ABT-263 for clinical trials. Furthermore, ongoing development of novel broad Bcl-2 antagonists are likely to bring about even more potent SMIs that might bind their targets with low nanomolar affinities. Accordingly, they are supposed to exert less off-target effects and kill more dependently on Bax/Bak.

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