

Review

BH3 Mimetics: Status of the Field and New Developments

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Abstract

Targeting apoptosis is an attractive approach in cancer therapy. The BH3-only proteins of the BCL-2 family (having only the BCL-2 homology domain BH3) can trigger apoptosis by binding to the prosurvival members of this family and neutralizing their functional activity (sequestration of the proapoptotic Bcl-2 family members). The "BH3 mimetic" concept has prompted the development of small molecules capable of mimicking BH3-only proteins and thus inducing apoptosis. The prototype BH3 mimetic ABT-737 selectively targets the three prosurvival proteins BCL-X_L, BCL-2, and BCL-W (but not MCL-1 or A1) and its oral derivative ABT-263 has proved promising in clinical trials. Some putative BH3 mimetics are also tested clinically while others are still being characterized. This article recapitulates the various known BH3 mimetics and presents the recent developments in the field. The latter include (i) the identification of molecular determinants responsible for the specific interactions between BH3 motifs and the binding grooves of prosurvival proteins and (ii) the characterization of new compounds and particularly BH3 mimetics that antagonize either selectively MCL-1 or BCL-2 or a broad range of prosurvival proteins. These data are critical advances toward the discovery of novel anticancer agents. *Mol Cancer Ther*; 12(9); 1691–700. ©2013 AACR.

Introduction

The apoptotic cell death program serves as a natural barrier to tumor development. Evading this program is a hallmark of cancer and constitutes an important mechanism in therapeutic resistance (1). This prompted the development of novel anticancer strategies targeting apoptosis (2) such as the inhibition of survival factors that are overexpressed in many malignancies (3, 4). Among the survival factors, those of the BCL-2 family have attracted much attention (5).

It is known that BCL-2 family proteins (sharing at least one of the four domains of BCL-2 homology: BH1–BH4) are key regulators of the balance between cell life and death. They control the mitochondrial membrane permeabilization allowing the liberation in the cytoplasm of the apoptogenic factors including cytochrome C, responsible for the cascade of caspase activation (6). The functional activity of the prosurvival members of the family (BCL-2, BCL-X_L, BCL-W, MCL-1, and A1) is to sequester the proapoptotic members BAX and BAK, which are the executioner molecules of the mitochondrial membrane permeabilization. All the prosurvival proteins can sequester BAX, whereas only BCL-X_L and MCL-1 can bind to BAK. This antiapoptotic activity is antagonized by the

BH3-only members (so-called because they only have the BH3 domain): the BH3-only proteins (NOXA, BAD and BIM for example) insert their α -helical BH3 domain into the hydrophobic groove of prosurvival proteins resulting (by displacement) in the release of sequestered BAX and BAK and their activation (indirect model of activation). These interactions are selective: NOXA binds only to MCL-1 and A1, and BAD binds only to BCL-2, BCL-X_L, and BCL-W, whereas BIM can bind to all five prosurvival proteins (6). There is also a model of direct activation in which certain "activator" BH3-only proteins (BIM, tBID) can directly engage and activate BAX and BAK. In this model, the "activator" proteins are sequestered by prosurvival proteins and the interaction between the latter and "sensitizer" BH3-only proteins (BAD, NOXA) induces the release (by competitive displacement) of the "activator" proteins (6). In either model, the binding of BH3-only proteins to prosurvival molecules results in BAX/BAK activation (5).

These data spawned the concept of the "BH3 mimetic" (7): a small molecule (either nonpeptide or derived from BH3 domains) that mimics the BH3-only proteins by inserting their BH3 domain into the hydrophobic groove of the prosurvival BCL-2 proteins, inhibiting their functional activity, and thus inducing apoptosis (5, 7). The BH3 mimetic concept is now considered as an attractive means to generate cancer drug candidates (1, 5, 8–10). Among the numerous generated compounds, ABT-737 (11) appears to be the prototype of an authentic BH3 mimetic: by binding with high affinity (in the nmol/L range) to BCL-2, BCL-X_L, and BCL-W, but not MCL-1 or A1, ABT-737 and its oral version ABT-263 (also called navitoclax) can induce BAX/BAK-dependent apoptosis *in vitro* and have antitumor effects in animal models (12,

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13). Until recently, most of the other known compounds did not fully meet the major criteria for defining a *bona fide* BH3 mimetic, i.e., high-affinity binding to prosurvival BCL-2 proteins and induction of BAX/BAK-dependent apoptosis as proposed by Lessene and colleagues (5). Showing off-target effects, these compounds were considered as putative BH3 mimetics (5, 9, 13–16). Given that MCL-1 has emerged as a critical prosurvival factor in a number of malignancies and in drug resistance (17), there is an urgent need to identify MCL-1-specific BH3 mimetics (18, 19). The development of small molecules capable of targeting all the main antiapoptotic BCL-2 proteins is also of clear therapeutic interest (8, 20–22).

This article gives an overview of the previous and new developments in BH3 mimetic research. It first summarizes the known true and putative BH3 mimetics and the further characterization of some of the latter. It describes important contributions to identify molecular determinants responsible for the specificity of interaction between α -helical BH3 motifs and the hydrophobic grooves of either MCL-1 or BCL-X_L. It then presents new compounds that have been proposed to function as BH3 mimetics, a direct "activator" of BAX and three new true BH3 mimetics: one is derived from the BH3 domain of BIM (a pan-BH3-only protein) and can antagonize a broad range of antiapoptotic proteins; MIM 1 selectively targets the binding pocket of MCL-1 and inhibits its activity; ABT-199, a second-generation derivative of ABT-737, is specific for BCL-2.

Previously Characterized BH3 Mimetics

A number of natural or synthetic small-molecule inhibitors of BCL-2 which were initially described (e.g., terphenyl, HA14-1, antimycin A, BH3-I, chelerythrine) showed either cytotoxicity or poor pharmacologic properties or failed to directly bind to the hydrophobic pocket of BCL-2 proteins with high affinity and/or induce BAX/BAK-dependent apoptosis (5, 7). Antagonists of several prosurvival BCL-2 proteins that have been further reported or have entered into clinical trials were extensively reviewed (4, 5, 8, 9, 16, 18). The chemical structures of these compounds are presented in Fig. 1, and their characteristics are recapitulated in Table 1 and briefly summarized below.

ABT-737 and ABT-263 (navitoclax)

As mentioned above, ABT-737 and its orally available derivative navitoclax (ABT-263; 11–13) are prototypic BH3 mimetics. They were considered for a time to be the sole true BH3 mimetics according to the criteria defined by Lessene and colleagues (5) because the other known compounds did not bind to the prosurvival proteins with a high enough affinity and/or killed BAX- and BAK-deficient cells as well as wild-type cells (5, 9, 13–15). The characteristics of navitoclax and ABT-737 largely overlap (except their pharmacokinetic properties). Notably, both

compounds mimic the BH3-only protein BAD by binding to BCL-2, BCL-X_L, and BCL-W but not Mcl-1 or A1 and are thus referred to as BAD-like BH3 mimetics. Induction of autophagy is not an off-target effect of ABT-737 but is due to the disruption of the interaction of BECLIN 1 with BCL-2 and BCL-X_L (23). ABT-737 and navitoclax show significant proapoptotic and antitumor effects only if MCL-1 is not or weakly expressed. The fact that these BH3 mimetics cannot bind to MCL-1 is one of the main causes of resistance particularly when cancer cells overexpress MCL-1 (9, 13). Promising data of phase I/II clinical trials with navitoclax have been recorded for various types of cancer (24–26).

Obatoclax

Also called GX15-070, this synthetic derivative of natural prodigiosins binds to all five prosurvival BCL-2 proteins albeit at low affinity (in the $\mu\text{mol/L}$ range; ref. 27). Of note, this affinity is as low as that of ABT-737 for MCL-1 and moreover, obatoclax kills BAX/BAK-deficient cells as potently as it kills wild-type cells (12, 14). Therefore, obatoclax does not meet the two criteria defining an authentic BH3 mimetic. In contrast, it seems rather to act through off-target mechanisms such as caspase-independent or autophagic cell death, endoplasmic reticulum stress response, and Noxa upregulation, the latter resulting in dissociation of MCL-1/BAK complex (14–16). This explains how this putative pan-BH3 mimetic can elicit apoptosis and overcome the resistance of malignant cells to ABT-737 that cannot bind MCL-1 (5, 9, 13, 15). A number of phase I/II trials in a variety of cancers have not proved convincing single-agent activity of obatoclax. Although it is well tolerated, dose escalation is limited by neurologic toxicity. Phase III trials with obatoclax in combination therapies are ongoing (28).

Gossypol family

Gossypol is a plant-derived polyphenolic aldehyde which has been used in the clinic for a long time before the discovery of its putative pan-BH3 mimetic properties [binding to BCL-2, BCL-X_L, and MCL-1 with modest (sub- $\mu\text{mol/L}$) affinities; refs. 8, 18, 29]. Its (-) enantiomer called AT-101 proved better proapoptotic and antitumor activities than gossypol *in vitro* and in animal models (8). However, this compound has shown only limited efficacy in clinical trials (9). Gossypol derivatives lacking the two aldehyde groups were designed to reduce toxicity: apogossypol and apogossypolone (ApoG2) are currently in preclinical evaluation, whereas the benzoylsulfonide derivative TW37 has already reached phase I/II trials (5, 8, 18, 30, 31). All these compounds have similar binding capacities (with minor differences regarding BCL-W or BCL-B) with moderate affinity, induce partially apoptosis in BAX/BAK-deficient cells, and show several off-target effects, indicating that they operate only partly as pan-BH3 mimetics (5, 9, 12–16). Interestingly, the apogossypol derivative BI-97C1 (also known as sabutoclax)

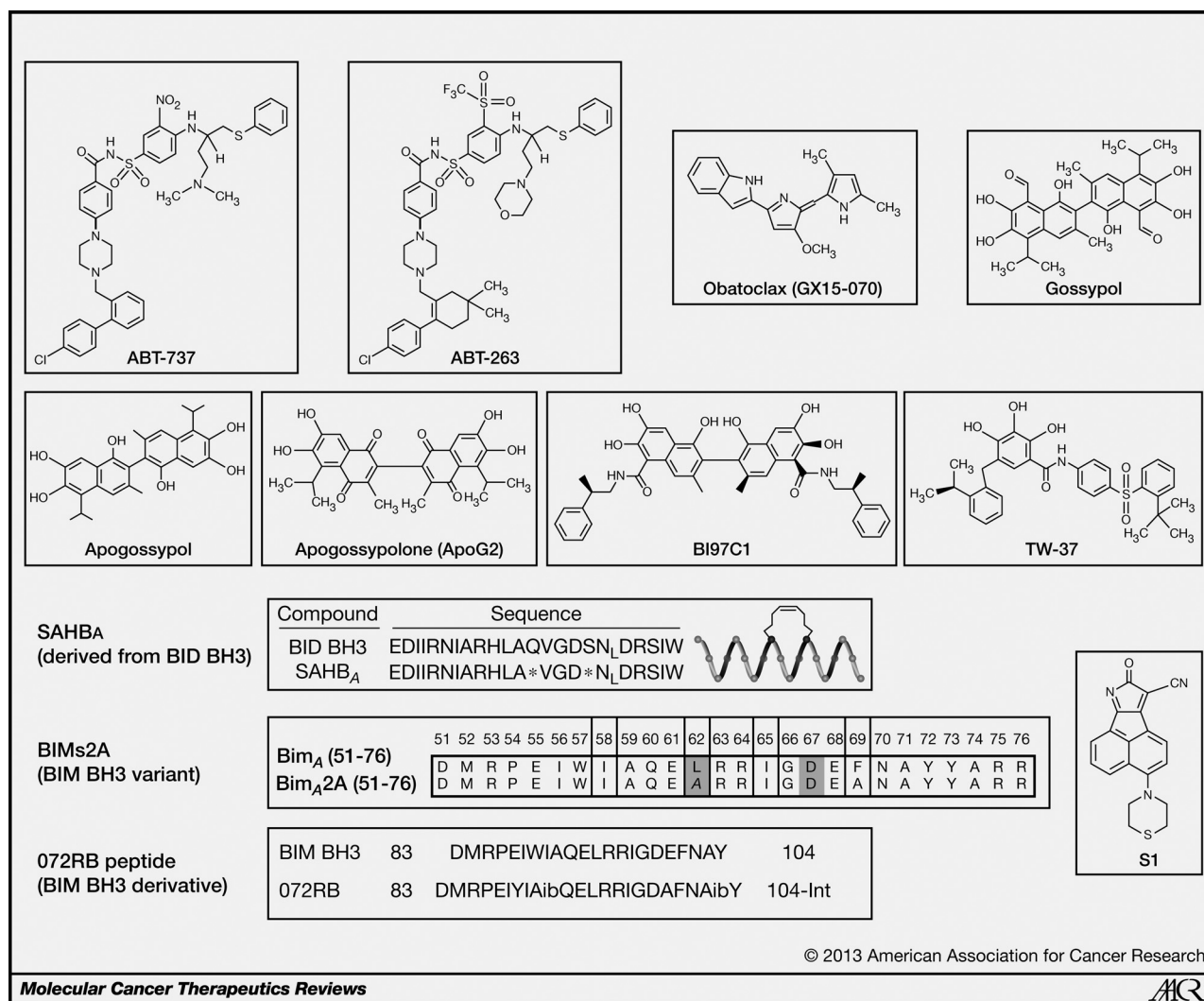


Figure 1. Chemical structures of previously characterized BH3 mimetics.

was reported to bind to prosurvival proteins (including A1) with higher affinities than the previous gossypol derivatives, to induce slight apoptosis in BAX/BAK-deficient cells and a few off-target effects (32).

Other BH3 mimetics

Walensky and colleagues originally generated a series of stabilized α -helix of BCL-2 domains (SAHB) designed to target prosurvival BCL-2 family members (33). These small molecules are hydrocarbon-stapled peptides derived from BH3 domains of BCL-2 proteins and are structurally stable, protease-resistant and cell-permeable. One of these compounds (SAHB_A) modeled on the BH3-only protein BID was found to bind to BCL-2 and BCL-X_L with moderate affinity and induce BAX/BAK-dependent apoptosis *in vitro* and antileukemic effects *in vivo* (33). However, no clinical data on this interesting SAHB with a BAD-like behavior has been reported. Two putative pan-BH3 mimetics were likewise characterized only in pre-

clinical studies: the synthetic heterocyclic compound S1 (34), which is described in the next section, and the peptide 072RB based on the BH3 region of BIM (35). Both were found capable of proapoptotic and antitumor effects (including *in vivo*) but did not fully meet the criteria for a true BH3 mimetic (15, 35).

Furthermore, two different laboratories have provided proof-of-concept that NOXA-like BH3 mimetics (specific for the crucial antiapoptotic protein MCL-1) can be designed: BIM₅2A is a variant of the BIM BH3 region (36) and MCL-1 SAHB is derived from the BH3 domain of MCL-1 itself (37). Ultrastructural analyses by X-ray crystallography indicated that both compounds selectively interact with the hydrophobic binding groove of MCL-1 and revealed the peptide determinants responsible for this interaction. Like NOXA, both BIM₅2A and MCL-1 SAHB are potent inhibitors of MCL-1-mediated antiapoptotic activity and show antitumor effects in hematologic cancer cell lines. BIM₅2A

Table 1. Previously characterized BH3 mimetics

Name	Origin	BH3 mimicry	BH3 mimetic	<i>In vivo</i> antitumor effects in animal models	Clinical trials
ABT-737	Small organic molecule	BAD-like	Authentic	Yes	No
ABT-263/navitoclax	ABT-737 derivative	BAD-like	Authentic	Yes	Yes
Obatoclax	Derivative of natural progridiosin	Pan-BH3-only	Putative/off-target effects	Yes	Yes
Gossypol	Plant-derived polyphenol	Pan-BH3-only	Partial/off-target effects	Yes	Yes
AT-101	Gossypol isomer	Pan-BH3-only	Partial/off-target effects	Yes	Yes
Apogossypol	Gossypol derivative	Pan-BH3-only	Partial/off-target effects	ND	No
Apogossypolone/ApoG2	AT-101 derivative	Pan-BH3-only	Partial/off-target effects	Yes	No
BI-97C1/sabutoclax	Apogossypol derivative	Pan-BH3-only	Partial/off-target effects	Yes	No
TW37	Gossypol analog	Pan-BH3-only	Partial/off-target effects	Yes	Yes
S1	Heterocyclic compound	Pan-BH3-only	Partial/off-target effects	Yes	No
072RB	BIM BH3-derived peptide	Pan-BH3-only	Putative	Yes	No
SAHB-A	BID BH3-derived SAHB	BAD-like	Putative	Yes	No
BIM _S 2A	BIM BH3 mutant	NOXA-like	Authentic	Yes	No
Mcl-1 SAHB	MCL-1 BH3-derived SAHB	NOXA-like	Authentic	ND	No

Abbreviations: BH3, BCL-2 homology domain 3; ND, not determined.

also shows antileukemic activity in animal models (Table 1).

Finally, Gavathiotis and colleagues have reported that a SAHB derived from the BIM BH3 α -helix can directly bind to BAX at an interaction site that is distinct from the canonical binding groove of prosurvival BCL-2 proteins (38). This interaction was further shown to induce the conformational change of the BAX activation (39). These data suggest that the BH3 mimetic concept might be used to design compounds having the ability to mimic "activator" BH3-only proteins according to the model of direct BAX activation.

Further Characterization of Putative BH3 Mimetics

S1

This small-molecule inhibitor was initially suggested to be a pan-BH3 mimetic because it binds to both BCL-2 and MCL-1 (albeit with modest affinity: in the sub μ mol/L range), disrupts BCL-2/BAX and MCL-1/BAK complexes, and triggers BAX/BAK-dependent apoptosis (34). A subsequent report showed that S1 does not act as a BH3 mimetic but instead induces the BH3-only protein NOXA (a specific antagonistic ligand for MCL-1) thus resulting in BAK release (15). Further characterization of S1 has been provided by recent data. One study described a dynamic change in the interactions of BIM with BCL-2 and MCL-1 leading to BAK release, suggesting that this mechanism might be involved in the effects of S1 in cells overexpressing MCL-1 (40). A second study indicated that autophagy has an important role in S1-triggered cell death through endoplasmic reticulum stress and disruption of the BCL-2/BECLIN 1 interaction (41).

Apogossypolone (ApoG2)

This gossypol derivative (lacking the two aldehyde groups) is a putative BH3 mimetic that binds to BCL-2, BCL-X_L, and MCL-1 with moderate affinity (sub μ mol/L range) and induces apoptosis in various tumor cells (30). Interestingly, a recent report showed that BAX and BAK are required for the apoptotic activity of ApoG2 (42), indicating that this compound acts at least partly through BH3 mimicry. This report also showed that ApoG2 can trigger the mitochondrial pathway for apoptosis in primary chronic lymphocytic leukemia (CLL) cells suggesting its potential value in CLL given that overexpression of several prosurvival BCL-2 family proteins (including MCL-1) has a crucial role in impaired apoptosis in CLL (16).

Obatoclax

Two studies have described new characteristics of this putative BH3 mimetic. The one reported that inhibition of the AKT/mTOR pathway is an additional off-target effect of obatoclax (43). The other has indicated that this compound can directly activate BAX (44), supporting the data of Gavathiotis and colleagues with the SAHB derived from BIM capable of binding and activating BAX (38, 39). This suggests that obatoclax, which does not bind prosurvival BCL-2 proteins with high affinity or induce BAX/BAK-dependent apoptosis, could however mimic an "activator" BH3-only protein.

Identification of New Putative BH3 Mimetics

Five new compounds were proposed to function as BH3 mimetics. Their chemical structures are shown in Fig. 2 and their characteristics are described below and listed in Table 2. *In vivo* antitumor activity has been only reported for BI-97D6.

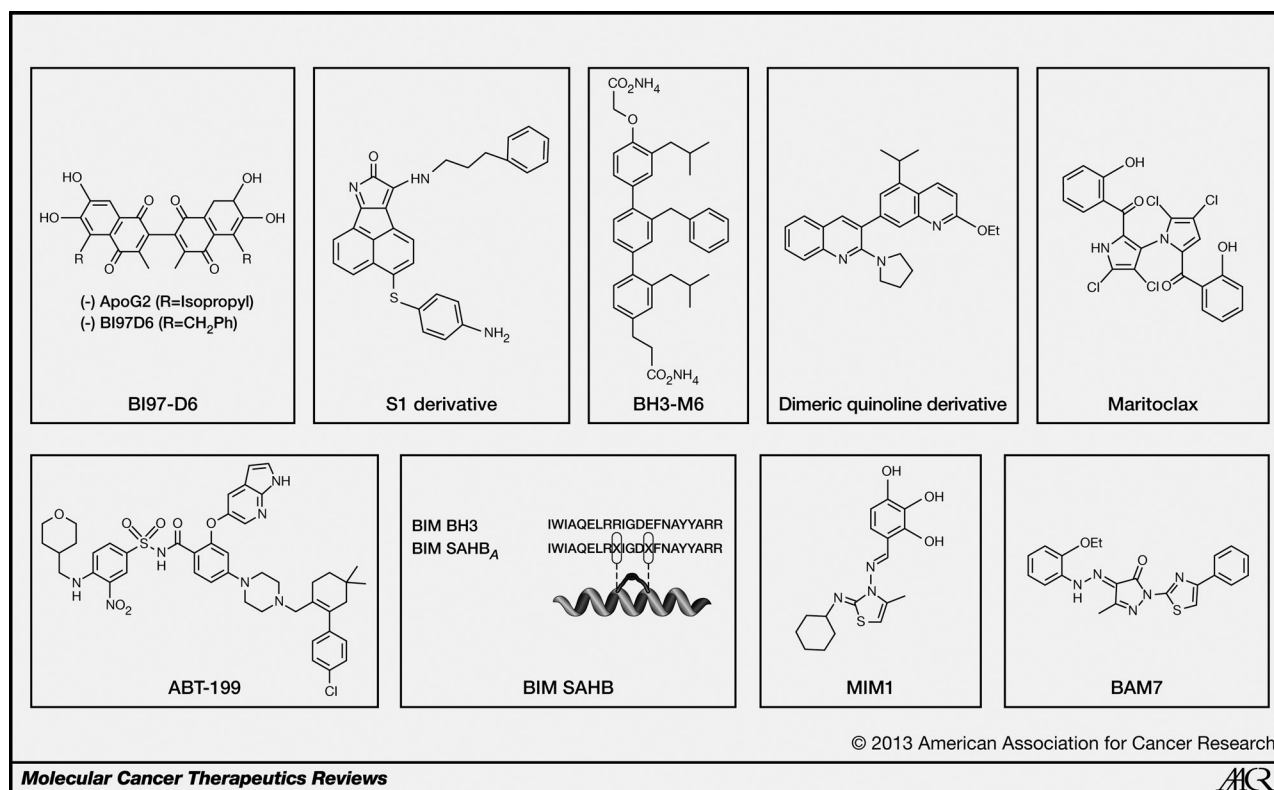


Figure 2. Chemical structures of the eight new compounds that have been proposed to function as BH3 mimetics and of the direct BAX activator molecule BAM7.

BI-97D6

BI-97D6 is the newer compound of the gossypol family: this (-) atropisomer of ApoG2 inhibits the binding of BH3 peptides to BCL-X_L, BCL-2, MCL-1, and A1 albeit with modest affinity (in the sub $\mu\text{mol/L}$ range). It displays *in vivo* effects in both BCL-2-transgenic and cancer xenograft mouse models (31). The observation that it shows only little cytotoxicity against BAX/BAK-deficient cells suggests a mechanism of action predominantly dependent on the BAX/BAK pathway. Though its real binding affinities

are not known, BI-97D6 may represent an interesting compound for BH3 mimetic research.

S1 Derivative

A series of substituted S1 has led to the discovery of a compound that binds to the grooves of BCL-2, BCL-X_L, and MCL-1 with an affinity 9- to 35-fold higher (in the 10–20 nmol/L range) and induces a better proapoptotic activity in tumor cell lines than the parental S1 does (45). This S1 derivative might be considered to be an

Table 2. New putative BH3 mimetics

Name	Origin	Targets	Binding affinity	BAX/BAK-dependent apoptosis	Antitumor effects in preclinical models
BI-97D6	ApoG2 atropisomer	BCL-2, BCL-X _L , MCL-1, A1	Modest (sub $\mu\text{mol/L}$)	Predominant	Yes
S1 derivative	Substituted S1	BCL-2, BCL-X _L , MCL-1	Moderate (10 nmol/L)	ND	No
BH3-M6	Terphenyl derivative	BCL-2, BCL-X _L , MCL-1	ND	BAX-independent BAK-dependent	No
Polyquinolines	Quinoline derivatives	BCL-2, BCL-X _L , MCL-1, A1	Low ($\mu\text{mol/L}$)	Yes	No
Maritoclax	Isolated from marine streptomycetes	MCL-1	ND	ND	No

Abbreviation: ND, not determined.

authentic pan-BH3 mimetic provided that it has kept the ability of the parent S1 to induce the BAX/BAK pathway.

BH3-M6

BH3-M6 is a synthetic terphenyl scaffold with functional groups that mimic the nature and spatial configuration of the key amino acids in the BH3 helix (20). Computational docking studies suggested that the compound can bind BCL-X_L, BCL-2, and MCL-1. Experiments in cell-free systems and intact human cancer cells showed that BH3-M6 prevents the interaction of the three prosurvival proteins with the BH3-only proteins BIM and BAD and the proapoptotic multi-BH domain proteins BAX and BAK. These effects result in release of proapoptotic proteins, liberation of cytochrome C, caspase activation, and thus apoptosis induction. It was concluded that BH3-M6 is a pan-BCL-2 family inhibitor (20). However, the binding affinities have not been determined and BH3-M6-promoted apoptosis is BAX-dependent but BAK-independent. Therefore, BH3-M6 does not meet the main criteria for BH3 mimicry (high-binding affinity and BAX/BAK-dependent apoptosis; ref. 5).

Polyquinoline derivatives

Several synthetic polyquinoline derivatives (especially dimeric derivatives of quinoline) can bind to BCL-X_L, BCL-2, MCL-1, and A1 albeit with low affinity (in the μmol/L range; ref. 46). These compounds inhibit the interaction of BIM with the prosurvival proteins and induce BAX/BAK-dependent apoptosis in malignant human lymphoid cells (but not peripheral blood mononuclear cells from healthy donors). Although quinoline derivatives represent a new class of pan-BCL-2 inhibitors, they may not be considered as true BH3 mimetics because of their low binding affinities.

Marinopyrrole A (maritoclax)

It was reported that this natural product derived from a species of marine streptomycetes (47) binds to MCL-1, but not BCL-X_L, disrupts the MCL-1/BIM complex, and elicits apoptosis in leukemia/lymphoma cell lines that are MCL-1-dependent, but not BCL-2 or BCL-X_L-dependent (48). These effects were associated with proteasomal degradation of MCL-1. The authors claimed to have identified a novel type of selective MCL-1 inhibitor capable of binding to and inducing the degradation of MCL-1. However, both the binding affinities and involvement of the BAX/BAK pathway have to be determined to evaluate whether maritoclax is indeed a natural BH3 mimetic specific for MCL-1.

Advances in Determining the Specificity of Interaction between BH3 Motifs and the Hydrophobic Grooves of BCL-X_L and MCL-1 and Implications in the Design of BH3 Mimetics

As already mentioned, the identification of MCL-1-specific and pan-BH3 mimetics appears as a priority. To

help in designing such compounds or improving the activity of known agents, several recent studies have attempted to define the molecular determinants responsible for the interaction of BH3 sequences with the binding sites in the groove of either MCL-1 or BCL-X_L. The latter is indeed capable of sequestering both BAX and BAK (like MCL-1), whereas the other prosurvival proteins BCL-2, BCL-W, BCL-B, or A1 can sequester only BAX.

The approach of Delgado-Soler and colleagues was to investigate which residues of the BH3 domain of BIM contribute to the interactions with each of the five main prosurvival proteins (BCL-2, BCL-X_L, BCL-W, MCL-1, and A1; ref. 21). The direct visualization of different complexes in live cells identified a BIM_S-BAD BH3 mutant as a specific partner for BCL-X_L but not BCL-W and MCL-1; this approach could be extended to other mutants such as the MCL-1-specific BIM_S2A (49). By studying different BH3 domains' interaction with BCL-X_L, a rational design of BH3 mimetics as inhibitors of BCL-X_L was proposed (50). Furthermore, the synthesis of a series of benzoylureas enabled the analysis of the conformational constraints involved in the BH3 domain/BCL-X_L interaction (51). Boersma and colleagues showed that diverse α/β patterns of functional helix in the BIM BH3 domain play a role in recognizing BCL-X_L and MCL-1 (52). Regarding the strategy of designing hydrocarbon-stapled peptides of the BH3 helix (SAHB type), it was reported that the binding affinity depends on the staple's position along the peptide (53).

There is a significant interest in the observation that BIM BH3 domain mutants with two or three sequence changes can bind selectively to only BCL-X_L or MCL-1 (54). A similar result has been found with another BIM BH3 mutant (BIM_S2A) which is a specific ligand for MCL-1 but not BCL-X_L (36). By screening a library of rhodanine derivatives for inhibition of BCL-2 proteins, two isomers that bind differentially to BCL-X_L and MCL-1 were identified (55). Analysis of BCL-X_L and MCL-1 mutants revealed several amino acids in the hydrophobic grooves which are essential for binding. Moreover, the position of a methoxy group can determine (via steric repulsion) the isomer's orientation at the binding site. Nevertheless, the value of these data is limited by the low binding affinities (μmol/L range) of the compounds (55). Dutta and colleagues compared the sequence determinants of BH3-binding specificity for MCL-1 versus BCL-X_L (by screening of BH3 peptide libraries) and identified BH3 peptides that selectively interact with either MCL-1 or BCL-X_L (or both) with high affinity. By examining the effects of 170 point mutations in the BH3 region of BIM, these investigators characterized important sequence features and constructed a predictive model that explains much of the binding specificity (56). Together, these findings may be used to improve the binding affinity of either MCL-1-specific or pan-BH3 mimetics.

To describe new peptide sequences which interact with the BH3-binding groove of MCL-1, an original approach that is not based on traditional BH3 peptides was

Table 3. Characteristics of the new BH3 mimetics ABT-199, BIM SAHB, MIM 1, and BAM7, and comparison with ABT-737/263

Compound	Type	Approach	BH3-Only mimicry	Targets	Binding	Activity	Apoptosis	Observations
ABT-737/263	Small molecule capable of binding to the hydrophobic groove of BCL-X _L	High-throughput screening by SAR-NMR	BAD-like	BCL-2, BCL-X _L , BCL-W	High affinity	Displacement of BAD and BIM from BCL-2	BAX/BAK-dependent only when MCL-1 is absent or neutralized	<i>In vivo</i> antitumor effects; Phase II clinical trials
ABT-199	ABT-263 derivative capable of binding to the hydrophobic groove of BCL-2	Design based on complex X-ray crystal structure	-	BCL-2	High affinity	Dissociation of BIM/BCL-2 complex	BAX/BAK-dependent in BCL-2-dependent tumor cells	<i>In vivo</i> antitumor effects; Phase I clinical trial
BIM SAHB	Derivative of the BIM BH3 helix (amino acids 146–166)	SAHB	BIM-like (pan-BH3-only)	BCL-X _L , BCL-W, MCL-1, A1	High affinity	Disruption of BAX/BCL-X _L and BAK/MCL-1 complexes	BAX/BAK-dependent even in ABT-737-resistant cells, but not in normal cells	<i>In vivo</i> antitumor effects; BH3 sequence-specific effects
MIM 1	Small molecule displacing the binding of MCL-1-SAHB to the hydrophobic groove of MCL-1 ^a	Competitive binding high-throughput screening	NOXA-like	MCL-1	Computational docking studies ^a	Inhibition of MCL-1-suppressed BAX activation ^b	BAX/BAK-dependent in MCL-1-dependent but not BCL-X _L -dependent cells	-
BAM 7	Small molecule displacing the binding of a BIM BH3 helix to the BAX trigger site	Competitive binding high-throughput screening	Activator-like	BAX	NMR analysis	BAX activation	BAX-dependent apoptosis in BAK-deficient cells	-

Abbreviations: BH3, BCL-2 homology domain 3; SAR-NMR, structure/activity relationship by nuclear magnetic resonance.

^aMCL-1 SAHB interacts with the binding groove of MCL-1, as shown by X-ray crystallography structural studies (ref. 37).

^bMCL-1 SAHB disrupts BAK/MCL-1 complex (ref. 37).

developed (57). Sabutoclax (BI-97C1), a BH3 mimetic of the gossypol family capable of antagonizing notably MCL-1 (32), was used as a competing agent to screen a randomized 12-residue phage display library. This approach revealed not only a number of α -helical peptides with canonical BH3 domain sequences but also two unique non-BH3 peptide motifs. These motifs exhibit characteristics of α -helices, the presence of two conserved amino acids required to bind MCL-1, and a reverse organization of the conserved residues when compared with canonical BH3 sequences. The so-called reverse BH3 (rBH3) peptides bind to MCL-1 selectively over BCL-X_L. Interestingly, a short peptide derived from an rBH3 motif shows binding to MCL-1 comparable to that seen for a 12-residue peptide from the BH3-only protein NOXA (that can only bind to MCL-1). A more recent study has described small molecules that bind to two different sites on MCL-1 with high affinity and specificity (58). Structural analysis of the complexes by X-ray crystallography has provided detailed molecular information for the development of MCL-1 antagonists. Furthermore, a patent review cites several compounds which were disclosed for selectively inhibiting MCL-1 but without biologic validation (59). Finally, Muppidi and colleagues have reported the first crystal structure of a NOXA-derived peptide in complex with its target MCL-1 (60). After remodeling, they obtained a pair of cross-linked peptides with greatly increased helicity, cell permeability, protein stability and cell-killing activity in MCL-1-overexpressing leukemia cells, thus describing a rational design of peptide-based selective MCL-1 inhibitors. All these data represent crucial advances to the discovery of NOXA-like BH3 mimetics capable of specifically antagonizing MCL-1.

Characterization of Novel BH3 Mimetics

Three new BH3 mimetics and a direct BAX activator molecule have been recently described. Their chemical structures are shown in Fig. 2 and their characteristics are presented below and recapitulated in Table 3.

ABT-199: a highly specific inhibitor of BCL-2

This compound is a synthetic derivative of navitoclax (ABT-263) which was developed for improving the clinical use of the latter (61). A dose-limiting thrombocytopenia is indeed a side-effect rapidly observed upon treatment of patients with navitoclax. Thrombocytopenia is not caused by decreased platelet production but by an on-target inhibition of BCL-X_L activity whose physiologic function is to protect platelets from apoptosis as they age (62). The navitoclax derivative has been designed to avoid this side-effect on the basis of the X-ray crystal structure of the BCL-2/small-molecule complex. ABT-199 binds only to BCL-2 with high affinity but not to BCL-X_L and induces BAX/BAK-dependent apoptosis in a number of hematologic tumor cell lines. While sparing human platelets, this true BH3 mimetic also promotes regression of BCL-2-dependent human hematologic malignancies in animal

models (61). In addition, a single administration of ABT-199 reduces tumor burden in the first 3 patients with CLL having entered a clinical trial (61). Actually, a phase I study of ABT-199 as a single agent in CLL and non-Hodgkin lymphoma is already ongoing (<http://clinicaltrials.gov/NCT01328626>). Preliminary data of the study on patients with non-Hodgkin lymphoma ($n=17$) indicate that ABT-199 is well-tolerated, does not provoke thrombocytopenia or any dose-limiting toxicities, and shows promising antitumor activity (63).

BIM SAHB: a BCL-X_L and MCL-1 antagonist with great therapeutic potential

The study conducted by LaBelle and colleagues has recently characterized the pharmacologic potential of a SAHB modeled on the BH3 helix of BIM (capable of engaging the five main prosurvival BCL-2 proteins; ref. 22). The BIM SAHB indeed binds to BCL-X_L, BCL-W, MCL-1, and A1 with nanomolar affinity, blocks the sequestration of BAX by BCL-X_L and BAK by MCL-1, and directly triggers BAX/BAK-dependent mitochondrial cytochrome C release: it therefore displays the hallmarks of a true pan-BH3 mimetic (Table 3). The BIM SAHB colocalizes with mitochondria, activates caspases-3 and -7, reactivates cell death in leukemia/lymphoma cells that are resistant to ABT-737 (but not in non-malignant cells), and exhibits tumor suppressor activity in a xenograft animal model of human acute myeloid leukemia. These *in vitro* and *in vivo* effects are dependent on the BH3 sequence. The selective activation of cell death in the aberrant lymphoid infiltrates of mice reconstituted with BIM-deficient bone marrow and the absence of side-effects confirm the therapeutic potential of the BIM BH3 replacement strategy with this new true BH3 mimetic targeting a broad range of prosurvival BCL-2 proteins (BIM-like activity; ref. 22).

MIM 1: a novel, small-molecule MCL-1-specific inhibitor

In parallel, Stewart and colleagues conducted a high-throughput screen for small molecules that displaced MCL-1 SAHB (that they have previously identified; ref 37) from its binding partner MCL-1: more than 70,000 structurally modified small molecules were screened in a competitive fluorescence polarization binding assay (64). Various subsequent screens yielded 28 compounds which were then tested for apoptosis induction in liposome- and cell-based assays. The most active molecule MIM 1 (for MCL-1 inhibitor molecule 1) has a thiazol core substituted with methyl, cyclohexylimino, and benzene-triol groups. MIM 1 proved to be a highly specific MCL-1 inhibitor: by targeting the hydrophobic pocket of MCL-1, it blocks MCL-1-mediated suppression of the proapoptotic BAX and induces BAX/BAK apoptosis in MCL-1-dependent but not BCL-X_L-dependent leukemia cells. Although several aspects have yet to be refined (65), MIM 1 seems to be the prototype of a novel class of MCL-1-specific BH3 mimetic agents for specifically treating malignancies in which MCL-1 has a crucial role.

BAM7: a direct BAX activator

Gavathiotis and colleagues previously reported that a SAHB derived from BIM can bind to BAX at a site that directly triggers its activation (38,39). By *in silico* screening libraries of small molecules for displacing their SAHB from its ligand BAX, Gavathiotis and colleagues have recently identified a BAX activator molecule, BAM7, which interacts selectively with the BAX trigger site but not with the canonical BH3-binding groove of prosurvival BCL-2 proteins. This compound (a pirazolone core substituted with different groups) induces BAX activation and BAX-mediated apoptosis in BAK-deficient cells (66). These data provide proof-of-concept that "activator" BH3 mimetics can be designed.

Conclusions

The findings that the small molecule BAM7 induces apoptosis through the direct binding and activation of BAX and that the putative BH3 mimetic obatoclax can directly activate BAX are of significant interest. They indicate that compounds that do not meet the criteria defining a true BH3 mimetic can yet behave as such in the context of the direct activation model, opening a new avenue of research with the development of "activator" BH3 mimetics. This raises the question whether putative BH3 mimetics other than obatoclax might be capable of directly activating BAX. The possibility that direct activators of BAK (the other executioner molecule of the mitochondrial membrane permeabilization) can be designed also deserves to be explored.

Regarding inhibitors of prosurvival BCL-2 protein activity, eight new small molecules were recently identified, one of which is specific for BCL-2, whereas two are selective for MCL-1 and the five others target several antiapoptotic proteins. The compounds BI-97D6, BH3-M6, polyquinolines, maritoclax, and the S1 derivative do not presently meet the criteria that define an authentic BH3 mimetic. Unless further demonstrations that these compounds can act as true BH3 mimetics, they should not be used as such to avoid misleading off-target effects. Data concerning molecular determinants (including peptide sequence organization) responsible for the specificity of interaction between BH3 motifs and the hydrophobic grooves of BCL-X_L and MCL-1 are likely to be crucial for improving the binding affinity of BH3 mimetic candidates and thus their biologic efficiency and therapeutic potential. The discovery of three authentic BH3 mimetics is the icing on the cake because they represent three novel types of BH3 mimetics. One, ABT-199, is an antagonistic ligand specific for BCL-2 and the final results of ongoing clinical trials are eagerly awaited. The two others have been identified from the SAHB approach: MIM 1 is highly specific for MCL-1 and BIM SAHB has nearly all the features of a pan-BH3 mimetic.

These data as a whole are critical advances toward the development of several new kinds of BH3 mimetic anticancer agents that would constitute the ultimate accolade in research on BH3 mimetics.

Disclosure of Potential Conflicts of Interest

The author discloses no potential conflict of interest.

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