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Direct Activation of Bax Protein for Cancer Therapy

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Abstract

Bax, a central cell death regulator, is an indispensable gateway to mitochondrial dysfunction and a major pro-apoptotic member of the Bcl-2 family proteins that control apoptosis in normal and cancer cells. Dysfunction of apoptosis renders the cancer cell resistant to treatment as well as promotes tumorigenesis. Bax activation induces mitochondrial membrane permeabilization, thereby leading to the release of apoptotic factor cytochrome *c* and consequently cancer cell death. A number of drugs in clinical use are known to indirectly activate Bax. Intriguingly, recent efforts demonstrate that Bax can serve as a promising direct target for small-molecule drug discovery. Several direct Bax activators have been identified to hold promise for cancer therapy with the advantages of specificity and the potential of overcoming chemo- and radioresistance. Further investigation of this new class of drug candidates will be needed to advance them into the clinic as a novel means to treat cancer.

Keywords

Bcl-2 family proteins; apoptosis; Bax activators; drug discovery; cancer therapy

1. INTRODUCTION

Cell death[1] has numerous vital roles in sculpting tissues and optimizing functions (e.g. in the immune or central nervous systems) during normal human body development.[1,2] Apoptosis, a.k.a. programmed cell death, is a major death process of cells that is critical for elimination of unwanted, damaged or infected cells and is associated with diverse biological processes including cell development, differentiation and proliferation.[3] Insufficient apoptosis may promote cancer and auto immune diseases, while excessive cell death may augment ischemic conditions and drive neurodegeneration.[4] The recognition that apoptosis is crucially involved in the regulation of tumor formation and also critically determines treatment response is one of the most important advances in cancer research in recent years.[5-7] The intrinsic and extrinsic signaling pathways are two principal processes leading to apoptosis.[8] The intrinsic pathway, also termed as the mitochondrial pathway, is triggered by diverse cytotoxic stimuli including oncogenic stress, chemotherapeutic agents as well as

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CONFLICT OF INTEREST

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metabolic stress. These stimuli activate related Bcl-2 family proteins leading to mitochondrial outer membrane permeabilization (MOMP).[9] Upon disruption of the outer mitochondrial membrane, a set of apoptotic proteins are released including cytochrome *c* and Smac/DIABLO.[3,10,11] Cytosolic cytochrome *c* recruits apoptosis protease-activating factor 1 (Apaf-1) and procaspase 9 to generate "apoptosome" which activates caspase 9 leading to processing of caspase 3.[12-14,10] Caspases are executive proteins of apoptosis. The extrinsic pathway, a.k.a. death receptor pathway, is activated by the interactions between death receptors and their cognate ligands of the tumor necrosis factor (TNF) family. Death ligand stimulation brings about oligomerization of the receptors and recruitment of the adaptor protein FADD and caspase 8, resulting in the formation of a death-inducing signaling complex (DISC). Autoactivation of caspase 8 activates other effector caspases including caspase 3, 6 and 7.[15] These two pathways converge on effector caspases and subsequently on other proteases and nucleases that drive cell death. Mitochondrial signaling is critical for normal cellular homeostasis and participates in the pathogenesis of various diseases[12,16] including cancer,[17] diabetes mellitus,[18] obesity,[19] and neurodegenerative disorders[20,18] such as Parkinson's disease.[21,20] Thus, abnormalities in mitochondrial signaling represent an actively pursued research frontier of the biomedical enterprise.

The mitochondrial signaling is primarily regulated by the interactions of B-cell lymphoma 2 (Bcl-2) family proteins.[22] Based on their different structures and functions, the Bcl-2 family is classified into two groups (Fig. 1a): anti-apoptotic proteins (e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1) and pro-apoptotic proteins. Pro-apoptotic proteins are further divided into two sub-classes: multi-domain proteins (e.g. Bax and Bak) and BH3-only proteins (e.g. Bid, Bim, Puma, Bad, Noxa, Bik, Bmf and Hrk) according to the presence of Bcl-2 homology domains (BH1-4 domains).[22] Multi-domain pro-apoptotic proteins Bax and Bak are essential executive proteins responsible for MOMP and a requisite gateway to mitochondrial dysfunction as well as cell death.[23,16] Cells lacking both *Bax* and *Bak* have proven to be completely resistant to truncated Bid (t-Bid)-induced cytochrome *c* release and apoptosis.[23] Bax likely can be inhibited by all the anti-apoptotic proteins,[24] whereas Bak is inhibited predominantly by Bcl-x_L, Mcl-1 and A1[25] (Fig. 1b). Bcl-2 anti-apoptotic (or pro-survival) family members sequester BH3-only proteins or neutralize Bax and Bak, thus preventing the allosteric activation of Bax and a subsequent mitochondrial program of apoptosis.[26,27] The BH3-only proteins are classified as "activators" and "sensitizers" based on the direct activation model, one of the three generally accepted apoptotic models.[28,29] Activator-type BH3-proteins such as Bid,[30,24] Bim[31] and Puma[32,33,24] function by the direct physical binding and activation of Bax to induce apoptosis, while sensitizer-type BH3-proteins such as Bad, Noxa and Bik engage pro-survival proteins to free up activators and induce Bax activation-mediated apoptosis.[28,34] Homeostasis is maintained by controlling the ratio of active pro- and anti-apoptotic proteins along with tissue-specific patterns of expression.[35]

Currently, three models are available as to how Bcl-2 family proteins control apoptosis - the direct activation model, the displacement model and the unified model.[4,36,37] The direct activation model[27] (Fig. 2a) contends that activator type BH3-only proteins directly associate with Bax/Bak[4] and cause the downstream series of events leading to cell death.[38,39,20,54] The sensitizer type BH3-only proteins bind to the anti-apoptotic proteins to

liberate activators, thereby facilitating Bax activation and MOMP.[28] The sole function of anti-apoptotic proteins is to sequester BH3-only proteins instead of Bax or Bak.[40] The displacement model (Fig. 2b) concludes that BH3-only proteins cause apoptosis through Bax or Bak indirectly by neutralizing the relevant anti-apoptotic proteins, thereby enabling the activation of Bax and Bak to proceed.[24,41,42,163] Bax and Bak are always active and anti-apoptotic proteins constitutively bind them to prevent MOMP in this mode. The sole function of BH3-only proteins is to displace Bax and Bak from pro-survival proteins, rather than bind Bax or Bak. The unified model[43] (Fig. 2c) builds on the embedded together model which combines features of both aforementioned models. The unified model assigns dual functions to pro-survival proteins, sequestering not only the activator type BH3-only proteins but also the active forms of Bax and Bak. The embedded together model[44] similar to the unified model emphasizes that binding to membranes is essential for interactions between Bcl-2 family members.[45,46]

Overexpression of anti-apoptotic Bcl-2 or Bcl-x_L exists in a large number of human cancers,[47] and inactivating mutations of pro-apoptotic proteins occurs in numerous cancers leading to an uncontrolled growth of tumors.[48-50] Moreover, overexpression of anti-apoptotic Bcl-2 and its close relatives is a major component of chemoresistance.[51] Bcl-2 family proteins are critical checkpoints of apoptotic cell death,[52,35] and targeting various Bcl-2 family members is thus one of the most promising therapeutic strategies for dysfunctional apoptosis related diseases including cancer,[4,53-59,36] autoimmune diseases and[35] neurodegenerative disorders.[60,43] In this review, we summarize the current understanding of the structures and physiological functions of Bax protein, and focus on recent advances in the direct targeting of Bax for cancer therapy. Several newly emerged direct activators of Bax including peptides and non-peptide small molecules, and their potential as novel cancer therapeutics are highlighted.

2. STRUCTURE OF BAX

Bax, a tumor suppressor,[61] was first identified as a heterodimer with Bcl-2 in 1993.[62] It is a 21 kD protein of 192 amino acids possessing 9 α-helices and its three-dimensional structure was resolved by nuclear magnetic resonance (NMR) in 2000.[63] Just like other three dimensional structures of Bcl-2 family proteins,[35] Bax exhibits a similar tertiary structure (Fig. 3). Helices α5 (Hα5) and Hα6 constitute the core of the protein and are embedded within the other 7 helices which are amphipathic and keep their hydrophilic residues exposed to the exterior.[64] Hα5 and Hα6 are recognized as the putative mitochondria pore-forming domain[65] and transmembrane domain.[66-68] *N*-terminal Hα1 containing a mitochondrial addressing signal is believed to be essential for the translocation of Bax to mitochondria.[69-71] In addition, Hα1 is the interaction site for BH3-only proteins t-Bid and Puma.[72] Hα1 controls the engagement of the α9 helix into the dimerization pocket formed by BH1, BH2 and BH3, rendering Bax as a monomer in cytosol. Once Hα1 is attacked by BH3-only proteins (e.g. tBid, Bim and Puma) and exposed, Hα9 will disengage from the hydrophobic groove, leading to mitochondrial insertion.[39] There is a long and unstructured loop between Hα1 and Hα2, which is a common feature of Bcl-2 family members. The amino acid sequence of this region is highly variable, and its function remains to be further elucidated. Hα2 comprises the BH3 domain which is requisite for the heterodimerization

with other Bcl-2 family members.[73] It has been determined by mutational analyses that Bax/Bcl-2 heterodimerization requires the BH1, BH2 and BH3 domains of Bcl-2 but only the BH3 domain of Bax.[74,75] Hα2, Hα3, Hα4 and Hα5 form a hydrophobic groove of the protein which is the canonical BH3-binding site. Hα9 is bound to the Bax hydrophobic groove[63] and thought to participate in the conformational stability.[76] Bax is predominantly an inactive monomer in the cytosol of healthy cells or loosely attached to the mitochondrial, nuclear, or endoplastic reticulum membrane.[70,77] The hydrophobic side chains (e.g. Ser55, Thr56, Leu59, Leu63, Ile66 and Leu70) of the BH3 helix point inward toward Hα5/Hα6 and are covered by Hα9. During apoptosis, Bax translocates to the mitochondria in the fully activated form whose Hα9 disengages from the binding groove and Hα2 rotates about its axis to expose the hydrophobic side chains of the BH3 domain.[64]

Some critical amino acid residues have also been identified. Cys62 within Hα2 close to the BH3 domain and Cys126 between the Hα5 and Hα6 are both exposed and potentially form a disulfide bridge for homo- or heterodimerization.[78,77,61] Ser184, at the end of C-terminus, and Thr167, between Hα8 and Hα9, are identified as two important phosphorylation sites. Phosphorylation of Ser184 by protein kinase C zeta,[79] or AKT[80] neutralizes Bax, while its dephosphorylation by protein phosphatase 2A actives Bax.[81] Mutation or deletion of Ser184 also influences Bax localization.[82,83] Pro168 and Pro13 are also found to be important for Bax activation and localization to mitochondria.[84, 85]

3. Function of Bax and its associated signaling

Bax is a unique entry point for intrinsic apoptotic signaling (Fig. 4). The intrinsic pathway is initiated by various stimuli including DNA damage, cytokine deprivation and cytotoxic stress.[86-88,61] Under these stresses, BH3-only proteins activate Bax via direct or indirect means according to the direct activation model. The direct mode is characterized by activators (e.g. Bim, t-Bid) that bind and activate Bax, leading to MOMP. The indirect mode is manifested by sensitizers (e.g. Bad, Noxa) that inhibit anti-apoptotic protein's ability to bind activators and thereby induce subsequent Bax-mediated apoptosis. Bax function is tightly regulated through a series of changes including conformational switching (inactive to active conformation),[89] trafficking (from cytosol to mitochondria),[90] and aggregation status changes (from monomer to dimer and multimer).[91] Oligomerized Bax facilitates mitochondrial membrane permeabilization and promotes the formation of pores which enables the release of cytochrome *c* and Smac/DIABLO from the intermembrane space into cytosol.[10,92-94] Together with apoptotic protease activating factor 1 (Apaf-1), dATP and procaspase-9, cytochrome *c* forms a complex "apoptosome", which activates caspase 9 followed by downstream activation of other executioner caspases and ultimately results in cell death.[12]

Bax also participates in the extrinsic apoptotic pathway that is mediated by transmembrane death receptors.[21] Bax reinforces the extrinsic pathway when caspase 8 cleaves Bid to generate the activated t-Bid (Fig. 4).[95,22] It is also revealed that Bax-deficient human colon carcinoma cells are resistant to death-receptor ligands, while Bax-expressing sister clones are sensitive,[96] indicating that Bax is essential for death receptor-mediated apoptosis. Moreover, it has been reported that sensitization of melanoma for TNF-related apoptosis-

inducing ligand (TRAIL)-induced apoptosis appears to be particularly dependent on Bax.[97] Knockdown of Bax prevents release of Smac from the mitochondria and thereby blocking TRAIL-induced apoptosis.[97,98] Suppressed Bax activity is one of the major reasons of TRAIL resistance in melanoma.[11,99,100]

Bax is also involved in the endoplasmic reticulum (ER) signaling pathway which plays a decisive role in many cellular events especially in cell death via crosstalk with mitochondrial pathways.[101,102] Bax not only increases ER Ca^{2+} load and enhances Ca^{2+} release,[103,104] but also modulates the unfolded-protein response (UPR) by directly interacting with inositol-requiring 1α (IRE1α).[48] Bax is also believed to regulate mitochondrial dynamics in healthy cells and to be required for normal fusion of mitochondria into elongated tubules.[105]

Bax is expressed in essentially all organs,[106] indicating that it may be a regulator of apoptosis in various cell types.[107,108] Bax-deficient mice display selective expansion of cell population,[62] selective hyperplasias, and resistance to certain apoptotic stimuli.[109] Although mice lacking Bax are viable with limited phenotypic abnormalities, *Bax*$^{-/-}$*Bak*$^{-/-}$mice show various developmental defects.[110]

Down-regulation and mutation of Bax plays an important role in tumor resistance to apoptosis.[111] Reduced *Bax* expression was found to be associated with Cisplatin resistance in ovarian carcinoma cells.[112] The *Bax* gene is down-regulated in tumor colorectal cancer cell lines acquiring resistance to 5-FU compared to wild type HT-29 cells, suggesting that Bax down-regulation may serve as a key factor during both colorectal carcinogenesis and cell resistance to 5-FU.[113] Down-regulation of *Bax* also plays an important role in *Zoledronat*-resistant lung cancer cell lines.[114] Decreased Bax/Bcl-2 ratio and caspase activity serves as the main mechanism of temozolomide (TMZ)-induced chemoresistance in U87MG cells[115] and paclitaxel resistant MCF-7 cells.[116] Inhibition of Bax conformational change by Akt also contributes to chemoresistance.[117,118] Loss of function mutation of Bax was reported to be found in hematopoietic malignancies[31] and results in TRAIL resistance in mitochondria dependent type II cancer cells.[119] Acquired point mutation of Bax G179E confers resistance to ABT-299 by abrogating Bax translocation to mitochondria. In addition, G179E Bax mutation also induces partial cross-resistance to other antineoplastic drugs.[120] Phosphorylation (Ser184) of Bax inactivates its pro-apoptotic function by maintaining Bax in the cytoplasm and heterodimerizing with anti-apoptotic Bcl-2 proteins, and thus contributes to increased survival and chemoresistance of human lung cancer cells.[83,121,118] Interestingly, Bax dephosphorylation (T172, T174 and T186) of wild-type p53-induced phosphatase 1 (Wip1) suppresses Bax-mediated apoptosis in response to γ-irradiation in prostate cancer cells and the effect can be reversed by a Wip1 inhibitor.[122,121] Taken together, abnormalities regarding Bax including down-regulation, inactivated mutation, phosphorylation and dephosphorylation affect the ratio of Bax/Bcl-2,[62,123] and confer resistance to cell death as well as overexpression of Bcl-2.[124] Therefore, Bax activators may be used to promote pro-apoptotic activity with the potential to overcome resistance, and serve as a surrogate in lieu of enhanced of Bax expression as a means to augment apoptotic stimuli and decrease tumor enlargement.[125]

Given the critical role of Bax in apoptosis, it is not surprising that various anticancer agents that induce apoptosis of cancer cells involve the participation of the pro-apoptotic protein Bax. Examples include Hsp90 inhibitor 17-AAG[126] and histone deacetylase (HDAC) inhibitors.[127] Hsp90 inhibitors promote p53-dependent apoptosis through Puma and Bax.[128] Cells lacking Bax and Bak prevent apoptosis mediated by HDAC inhibitors.[129] Human colorectal cancer cells that lack functional Bax genes are partially resistant to the apoptotic effects of chemotherapeutic agent 5-fluofouracil, and completely abolish the apoptotic response to the chemopreventive agent sulindac and other nonsteroidal antiinflammatory drugs (NSAIDs).[130] Selected drugs which exert their effect with respect to Bax activation are listed in TABLE 1.

4. Small molecules as direct Bax activators

While a number of anticancer drugs in the clinic induce Bax activation indirectly to facilitate apoptosis, none of them directly activates Bax. Accumulating evidence suggests that direct activation of Bax can be viewed as a novel and specific approach for cancer therapy. First, Bax is often differentially expressed in cancer cell lines versus normal cells. It is reported that human lung cancer cells expressing higher levels of total Bax also contain relatively higher levels of pBax (e.g. H292 and H1975).[143] Approximately 21% of human hematopoietic malignancies possess loss-of-function mutations of Bax, perhaps most commonly in the acute lymphoblastic leukemia subset.[49] Additionally, Bax has unique and critical sites that are not shared with other Bcl-2 family members, providing the molecular basis for an ideal targeted approach for cancer treatment with the desired outcome of a decreased side effect profile. Given that Bax plays a primary role in the intrinsic apoptosis pathway and participates in the extrinsic pathway, which is not the case for BH-3 only proteins as evidenced by their dependence on Bax/Bak,[23,144] small-molecule activation of Bax represents a novel approach to promote the pro-apoptotic function of this centrally acting protein. It has been shown that the expression of Bax appears to play an important role in suppressing cancer development,[80,145] and decreased Bax levels contribute to chemoresistance in a number of cancers including lung cancer, chronic lymphocytic leukemia (CLL), prostate cancer and others which may be ameliorated by small-molecule Bax activators.[62, 127] Intriguingly, several small-molecule activators of Bax have been identified to induce cell death in a Bax-dependent fashion via direct binding to Bax *in vitro* and *in vivo*. By leveraging the requisite conformational change of Bax from its inactive to active state via several binding sites and key amino acid residues, the potential to explore the rational development of Bax small-molecule activators may prove fruitful towards the discovery of novel cancer therapeutics. Given that many anticancer agents induce cancer cell death via Bax activation, direct activation of Bax may also provide powerful agents for novel drug combinations. Taken together, direct activation of Bax represents a promising approach for cancer therapy.

4.1 Peptides Bid $SAHB_A$ and Bim SAHB

Direct involvement by selected BH3 domains of BH3-only proteins has been implicated in initiating Bax activation.[38,146,147] Not only BH3-only proteins such as Bim, Bid and Puma, but also the tumor suppressor p53,[148] are found to act as direct activators of Bax, resulting in

cytochrome *c* release and apoptosis.[149,150] A convenient and quick option to access Bax activators is through BH3 domain mimetics. Given that traditional peptides typically have poor uptake and are often comprised by their loss of secondary structures, several new approaches have emerged including a chemical strategy termed hydrocarbon stapling.[151-154] The stabled Bid BH3 (**1**; Fig. 5a) mimetic Bid $SAHB_A$ (stabilized α helices of Bcl-2 domains, **2**) is the first peptide identified to directly associate with Bax.[147] The intramolecular all-hydrocarbon "staples"[139] are generated by inserting non-natural amino acids bearing olefin tethers into the BH3 sequence, followed by ruthenium-catalyzed olefin metathesis (Fig. 5a). The peptide displays dramatically stabilized Bid BH3 α helicity compared to the random coil in solution.[155] Fluorescein isothiocyanate (FITC)-derivatized Bid $SAHB_A$ demonstrates a direct binding interaction with full-length Bax, exhibiting an EC_{50} of 885 nM measured by a solution-phase fluorescence polarization assay (FPA) (Fig. 5b).[156,157] In contrast, Bid $SAHB_{A\ (L,D\text{-}A)}$ (**3**) mutant known to impair the biological activity of Bid BH3 with enhanced α helicity,[28] displays no binding activity at Bax or Bid BH3. At the same time, Bid $SAHB_A$ also displays binding activity for full-length Bcl-x_L with an EC_{50} of 230 nM. *In vitro* mitochondrial cytochrome *c* release assays were also performed using Bak-deficient mouse liver mitochondria. Dosing with Bid $SAHB_A$ and Bax resulted in the release of cytochrome *c* in a dose-dependent manner. Addition of Bcl-x_L inhibits Bid $SAHB_A$ induced Bax activation. Bid $SAHB_{A(L,D/A)}$ mutant and Bad $SAHB_A$ (**4**) reveal no significant cytochrome *c* release. These results suggest that direct binding interaction between Bid $SAHB_A$ and Bax is sufficient to activate Bax and the interaction is specific. Similarly, Bax coimmunoprecipitated with Bid $SAHB_A$, but not with mutant Bid $SAHB_A$s, indicating that cell-permeable Bid $SAHB_A$ can interact with Bax in cells. Bid $SAHB_A$ may ultimately be a valuable pro-apoptotic agent as it has bifunctional properties by directly engaging both pro-apoptotic and pro-survival multi-domain proteins.

Utilizing BH3 peptides including Bid BH3 as chemical probes, new progress has been made on the mechanism of Bax activation based on the crystal structures of BaxΔC21 with detergents and BH3 peptides.[158] Compared to the inactive form of Bax (Fig. 5c, left panel), Hα1-Hα5 are released from Hα6-Hα8 in the active form of BaxΔC21 (Fig. 5c, right panel) induced by the engagement of Bid BH3 into canonical hydrophobic groove of Bax and BH3 domain of Bax is dislodged. The freed Bax BH3 domain then competes with activator BH3-only proteins for binding Bax to form stable homodimers, which is the fundamental unit of the Bax oligomers. Several hydrophobic residues of Bid BH3 interacting with Bax are highlighted as h0 (resides Ile82 and Ile 83), h1 (Ile86), h2 (Leu90), h3 (Val93) and h4 (Met97). Mutations at h0 position (I82A/I83A) abolish its activity of Bax activation in all assays. An isoleucine or a glutamate in h0 favors activator function on Bax. The destabilizing cavity is formed at the Bax-Bid BH3 intersurface to promote the liberty of Bax BH3 domain and trigger Bax oligomerization. Nevertheless, no cavities can be formed when BH3 peptides interact with pro-survival proteins. These findings based on the crystal structures of Bax and BH3 peptide complexes provide new insight on the mechanism of Bax activation and make it feasible to design BH3 sequences targeting Bax or pro-survival proteins selectively.

Bim BH3 (**5**) mimetic Bim SAHB (**6**, Fig. 6a), obtained in a same fashion as Bid $SAHB_A$, has also been investigated. It shows 35-fold greater potency than Bid $SAHB_A$, with an EC_{50} value of 23.7 nM. The direct Bim SAHB binding to Bax was confirmed by using NMR spectroscopy techniques and a new triggered binding site (Fig. 6b, right site), at which Bim SABH binds Bax leading to a battery of events including its direct activation and Bax-mediated mitochondrial apoptosis, was revealed.[159] This Bax trigger site is defined by helices α1 and α6, on the opposite face of canonical BH3 binding site (Fig. 6b, left site) of anti-apoptotic proteins. However, more recent studies on the crystal structure of Bim BH3 peptide complexed with BaxΔ21 (Fig. 6c) did not address Bax activation at this site directly, while supporting the canonical binding site.[160] The apoptotic response of *Bax*$^{-/-}$ *Bak*$^{-/-}$ mouse embryonic fibroblasts (DKO MEFs) reconstituted with Bax or Bax (K21E) and Bim SAHB or Bim SAHB (R153D) was examined. Neither Bim SAHB bearing a single amino acid mutation within the core BH3 consensus sequence nor Bax mutagenesis at the α1-α6 interaction site induces time-dependent apoptosis. Specificity of Bim SAHB-induced Bax activation was further evidenced by the "staple scan" and mutagenesis studies. Different *in vitro* assays (oligomerization, 6A7 immunoprecipitation, liposomal and mitochondrial assays) that measure ligand-induced Bax activation indicate that Bim SAHB directly and dose dependently activates Bax. The identification of the novel binding sites to interact with and activate Bax, and the elucidations of the crystal structures of BH3 peptides complexed with BaxΔ21 represent exciting breakthroughs that support the direct targeting of Bax for the therapeutic modulation of apoptosis.

4.2 Non-peptide small molecule activators

Low bioavailability, poor membrane permeability and metabolic instability are the most common issues regarding the development of peptides as therapeutic agents.[161-163] Although the described "stapled" peptides have relatively increased stability, cell-permeability, and the ability to induce apoptosis via direct binding and activation of Bax, non-peptide small molecules are preferred given the capacity of medicinal chemistry campaigns to fine tune molecular architecture towards the optimization of desired drug-like traits. Recently, several screen campaigns have been carried out to discover non-peptide small molecules that can directly bind and activate Bax to induce the apoptosis of cancer cells based on the Bax trigger site and critical amino acids residues.

4.2.1 BAM7 and its analogue BTC-8—BAM7 is a non-peptide small-molecule direct activator of Bax which was identified in 2012 by Walensky et al.[164] Based upon the newly recognized triggered site of Bax by Bim SAHB, a diverse *in silico* screen of 750,000 small molecules was conducted using Glide 4.0. The top 100 hits were selected for experimental analysis using competitive FPA involving FITC-BIM SAHB and Bax. BAM7 (**7**; Fig. 7a), a pyrazolone core substituted with an ethoxyphenylhydrazono, methyl, and phenylthiazole moieties, was identified as the most effective small-molecule binder of Bax among the compounds tested in this series, with an IC_{50} value of 3.3 μM. NMR analysis of [^{15}N]Bax upon BAM7 titration shows that BAM7 and Bax interact at the very surface used by the BIM BH3 helix to trigger Bax activation. Different from *N*-terminal acetylated Bim SAHB (Ac-Bim SAHB), which can effectively compete with FITC-Bim SAHB for binding to the diversity of Bcl-2 family multi-domain protein members, BAM7 shows little or no

competitive binding interactions with other Bcl-2 family targets including C-terminal deleted Bcl-x_L (Bcl-x_LΔC), Mcl-1ΔNΔC and BakΔC even at 50 μM concentration (Fig. 7b). Thus, BAM7 exhibits a high selectivity for Bax. The interaction between BAM7 and Bax induces characteristic structural changes yielding functional Bax oligomerization and Bax-dependent cell death (Fig. 7c) according to structural, biochemical and cellular studies. *In vitro*, BAM7 only impairs the viability of $Bak^{-/-}$ MEFs which rely on Bax in a time- and dose-dependent manner, and BAM7 has no effect on $Bax^{-/-}$ or DKO MEFs. BAM7-treated $Bak^{-/-}$ MEFs display characteristic microscopic features of apoptosis including cellular shrinkage (Fig. 7d). These results suggest that directly targeting Bax with a non-peptide small-molecule activator is feasible to trigger its pro-apoptotic activity. BAM7 is a selective small molecular Bax activator that binds to the Bax trigger site, representing a new approach toward combating human cancer.

Recently, a structure-based lead optimization of BAM7 led to the discovery of BTC-8 (**8**; Fig. 7a), which induces MOMP with an EC_{50} of 700 nM, approximately one order of magnitude more potent than BAM7 in cultured HuH7 cells.[165] BTC-8 was obtained through replacement of thiazole with phenyl ring and introduction of exocyclic basic group. BTC-8 can induce translocation of Bax to mitochondria leading to the release of cytochrome *c*, activation of caspase 3 and formation of apoptotic nuclei. Moreover, it is selectively toxic for cancer cells (HuH7, NB4, SHSY-5Y and LLC1) and immortalized cells (MEF) while having little effects on healthy resting cells (healthy splenocytes). BTC-8 shows *in vivo* efficacy in a murine Lewis lung carcinoma mouse model at a low intraperitoneal injection dose of 1 mg/kg. After only 4 days of treatment, a significant tumor mass reduction was observed to reach 50% compared with control with no gross toxicity.[165]

4.2.2 Compound 106 (ZINC 14750348)—Structure-based drug design was used to discover agents capable of activating Bax, and compound **106** (**9**; Fig. 8a) was predicted to bind the Bax hydrophobic groove by a virtual screening approach.[166] A total of approximately 10 million small molecules in the ZINC drug-like database were screened in the classic carboxyl-terminal transmembrane helix binding site based on the NMR structure of Bax (PDB code: 1F16) to identify compounds that can bind Bax. Among 46 high-scoring molecules that can fit into the hydrophobic groove, compound **106** was found to exhibit the highest Bax- and dose-dependent cytotoxicity.

Compound **106**, a pyrazolo[4,3-*c*]pyridine core substituted with 3,4-dimethylbenzyl, 2-methoxybenzyl and (*R*)-1-(3-hydroxypiperidin-1-yl)ethanone moieties can fit well into the cavity in the Bax hydrophobic groove according to the virtual screening experiments. Compound **106** can trigger cell apoptosis in a Bax-dependent fashion. It selectively induces cell death of $Bak^{-/-}Bax^{-/-}$ MEFs expressing Bax rather than vector or Bak (Fig. 8b). Compound **106** activates Bax *in vitro* by altering the proteins conformation, inducing Bax insertion into mitochondria and subsequent cytochrome *c* release. Compound **106** kills various tumor cell lines including murine Lewis lung carcinoma (LLC) cells, A549 human non-small cell lung carcinoma cells, and PANC-1 human pancreatic carcinoma cells in a Bax- and dose-dependent fashion. Moreover, compound **106** inhibits lung tumor growth and induces tumor cell apoptosis *in vivo* on the female C57BL/6 mice implanted LLC cells at the

dose of 40 mg/kg/day by intraperitoneal injection. Compound **106** functions synergistically with carboplatin[167] or ABT-737[168] to induce human tumor cell death, indicating that Bax activators may serve as a component of combination chemotherapy regimens. Intriguingly, compound **106** is preferentially toxic to transformed MEFs cells (transformed by expression of the K-Ras and E1A oncogenes) compared to their normal counterparts with similar Bax expression (Fig. 8c). This finding suggests that tumor cells may respond more acutely than normal cells to stressful conditions such as Bax activation because of an overwhelmed antiapoptotic reserve, and Bax activators may thus have a superior selectivity for cancerous cells over normal cells.

4.2.3 SMBA COMPOUNDS—Recently, several new small molecular ligands as direct activators of Bax have been identified by our team.[143] Based on the previous finding that nicotine-induced Bax phosphorylation at serine 184 (S184) inactivates the pro-apoptotic function of Bax,[80] it was reasoned that the pocket around the S184 site is an attractive target for structure-based drug discovery. A total of about 300,000 molecules by using the NCI compound library were docked into the structural pocket around S184 residue with the DOCK program suite virtual screening software. Further investigation of selected hits on their apoptotic effects against human lung cancer A549 and H1299 cells led to the discovery of three structurally diverse lead compounds SMBA1 (**10**), SMBA2 (**11**) and SMBA3 (**12**) (Fig. 9a). All three compounds exhibit significant suppression effects on nicotine-induced Bax phosphorylation in A549 cells. The competition fluorescence polarization assay[169] demonstrates that SMBAs selectively bind to the Bax protein and display excellent binding affinities with K_i values of 43.3 ± 3.25 nM, 57.2 ± 7.29 nM and 54.1 ± 9.77 nM for SMBA1, SMBA2 and SMBA3, respectively, but fail to bind to other Bcl-2 family proteins such as Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, BFL-1/A1, Bid and Bak at the concentration of up to 0.5 μM (Fig. 9b). SMBAs selectively impair the viability of *Bak*$^{-/-}$ MEFs, but exhibit no effects on MEFs lacking Bax (*Bax*$^{-/-}$) or both Bax and Bak (*Bax*$^{-/-}$ *Bak*$^{-/-}$), indicating that Bax not Bak is the required essential target for SMBAs to induce apoptosis. Structural modeling with these chemical leads reveals that SMBAs can fit well into the Ser184 binding pocket. Further mechanistic investigation has validated that SMBAs indeed alter various apoptotic biomarkers and induce conformational changes of Bax, Bax oligomerization, mitochondrial insertion and cytochrome *c* release by blocking S184 phosphorylation.[143]

The anticancer activity of SMBA1 was further evaluated *in vivo* using nude mice bearing subcutaneous lung cancer xenograft derived from A549 cells. Significant antitumor efficacy was observed at the doses of 40 mg/kg and 60 mg/kg with treatment for 14 days. Body weight change of mice was monitored once every other day during treatment with increasing doses of SMBA1. No significant body weight loss and normal tissue toxicity *in vivo* were observed at all the tested doses, indicating that SMBA1 represents a new and safe class of anticancer agents. Intriguingly, SMBA1 shows target specificity, and almost displays no antitumor effect in Bax-deficient lung cancer xenograft derived from A549 expressing Bax siRNA at the effective dose of 40 mg/kg, demonstrating that Bax expression is essential for SMBA1 suppression of tumor growth *in vivo* (Fig. 9c).

5. Conclusions and future directions

Since its discovery in 1993, Bax has attracted an increasing amount of attention due to its central role in the regulation of apoptosis. Bax is critical to maintain homeostasis while it tends to be disordered in cancerous cells. Based on the solid foundation of cancer genetics and cell biology studies, direct binding and activation of Bax as a promising approach for cancer therapy is not only feasible, but also proven to be effective both *in vitro* and *in vivo*. Direct Bax activators have demonstrated a variety of advantages including potential superiority to overcome radio- and chemoresistance as well as to selectively induce apoptosis of cancer cells with low toxicity in normal cells.

To date, several classes of novel direct Bax activators including Bid $SAHB_A$, Bim SAHB, BAM7, BTC-8, compound **106** and SMBAs have been identified to effectively induce Bax-mediate apoptosis *in vitro* and *in vivo*. Peptides Bid $SAHB_A$ and Bim SAHB exhibit an improved capability of overcoming the traditional limitations of peptides such as poor cellular permeability, bioavailability, solubility and metabolic stability as well as triggering Bax-mediated apoptosis. Bid $SAHB_A$ treatment consistently suppresses leukemia growth *in vivo* on immunodeficient mice bearing established human leukemia xenografts.[155] However, it shows a relatively lower selectivity of Bax over other related Bcl-2 family members. Inspired by the designation of venetoclax, a Bcl-2 BH3 mimetic, as a breakthrough therapy for the treatment of 17p deletion relapsed-refractory CLL granted by FDA, these stapled peptides as BH3 mimetics of activator BH3-proteins may have great potential to be further optimized as unique chemical probes and peptide-based drugs.[170,171] Both BAM7/BTC-8 and SMBAs directly bind Bax with high selectivity over other Bcl-2 family members and induce Bax-dependent cell death in a genetically defined context. Intriguingly, BTC-8 is highly efficacious *in vivo* in a murine Lewis lung carcinoma mouse model even at a low dose of 1 mg/kg. SMBA1 displays nanomolar binding affinity to the unique Ser184 site and suppresses tumor growth in the lung tumor xenograft mouse model at the dose of 40 mg/kg with no overt toxicity. More extensive structure-activity relationship (SAR) studies are imperative to improve efficacy and drug-likeness to yield optimized drug candidates for human clinical trials. Given the tremendous market for novel anticancer agents, these target-specific molecules that directly activate Bax offer great potential and hold promise for cancer treatment.

Both challenges and opportunities remain regarding the development of direct Bax activators. First, only a very limited number of small molecules have been reported that directly bind and activate Bax leaving the door open for the discovery of small molecules with diverse scaffolds suitable for preclinical development, representing an endeavor that is urgently needed. With the assistance of modern drug discovery technologies and multidisciplinary approaches including high-throughput screening (HTS), structure-based drug design, and computer-aided drug research, identifying new chemical entities directly targeting Bax likely can be facilitated. The discovery of BTC-8 appears to be a good example of structure-based drug design. Due to the well established *in vitro* and *in vivo* assays, HTS is feasible to yield more potent and otherwise undiscovered chemical scaffolds as direct Bax activators. Meanwhile, virtual screening with classic drug discovery guidelines properly applied is also a wise option given the crystal structures of Bax and relevant

complexes as well as several different binding pockets are well characterized. Furthermore, HTS of fragments with relatively weaker binding and fragment-based drug design (FBDD)[172,173] may also facilitate the discovery and enhance the structural diversity of Bax activators. Given the importance of several specific amino acid residues of Bax (e.g. Ser184) and the role of the associated phosphorylation, developing appropriate phosphonate probes[174,175] might be very useful for elucidating the mechanisms of Bax activation and assisting the target-specific drug discovery.[175]

As discussed, the currently available structural studies on Bax and BH3 peptides complexed with BaxΔ21 have provided critical insights into the molecular mechanism of Bax activation and interactions between Bax and other Bcl-2 family members. If co-crystal structures of Bax and its non-peptide small molecular activator complexes are revealed, we envision that the rational drug design will be significantly facilitated to yield new insights for directly targeting Bax. It is the opinion of the authors that developing novel and efficient small molecules as specific and direct Bax activators will find an important place in both biopharmaceutical industry and academic settings, and open new avenues for understanding the fundamental mechanisms of Bax in multiple cellular contexts and eventually lead to the development of viable therapeutic regimens that may benefit cancer patients. Given the important role of Bax in apoptosis and drug resistance, combination therapies of Bax activators and chemotherapeutic drugs can also take prominence in the years to come.

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Abbreviations

Bcl-2	B-cell lymphoma 2
MOMP	mitochondrial outer membrane permeabilization

Smac/DIABLO	second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI
TNF	tumor necrosis factor
FADD	Fas-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
ER	endoplasmic reticulum
HDAC	histone deacetylase
CLL	chronic lymphocytic leukemia
SAHB	stabilized α helices of Bcl-2 domains
DISC	death-inducing signaling complex
BH	Bcl-2 homology domains
NMR	nuclear magnetic resonance
UPR	unfolded-protein response
IRE1α	inositol-requiring 1α
TMZ	temozolomide
Wip1	wild-type p53-induced phosphatase 1
NSAIDs	nonsteroidal antiinflammatory drugs
FITC	fluorescein isothiocyanate
FPA	fluorescence polarization assay
SAR	structure-activity relationship
HTS	high-throughput screening
FBDD	fragment-based drug design

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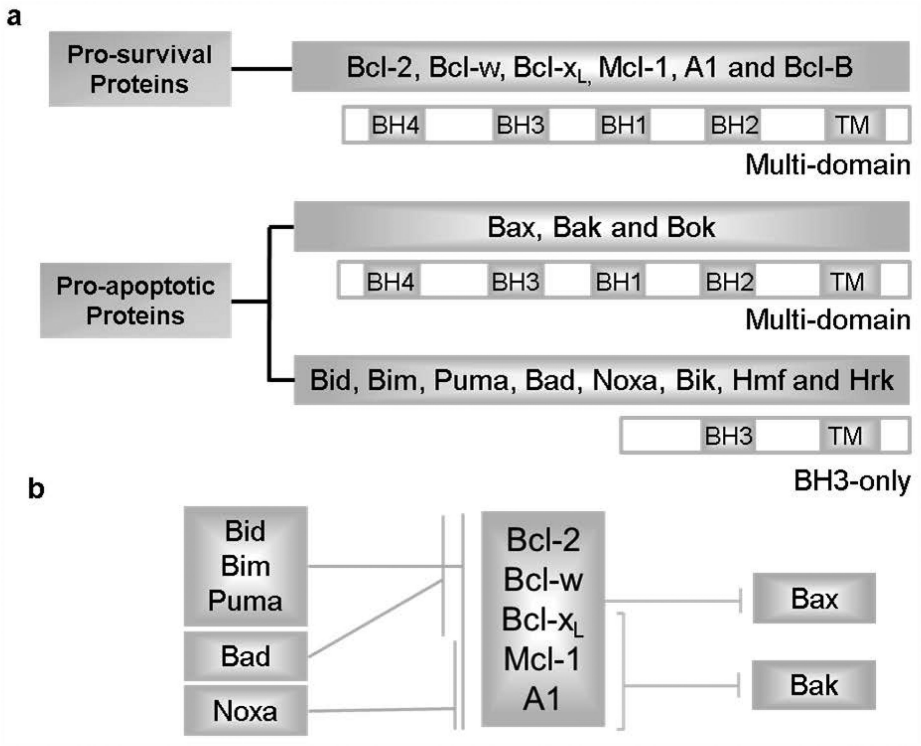


Figure 1. The Bcl-2 family proteins and their interactions

a, The Bcl-2 family is classified into pro-survival proteins (or anti-apoptotic proteins, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, etc.) and pro-apoptotic proteins due to their different functions. The pro-apoptotic proteins are further divided into two sub-classes: multi-domain proteins (e.g. Bax, Bak and Bok) that exhibit BH1-4 domains and BH3-only proteins that exhibit sole BH3 domain (e.g. Bim, Bid, Puma, Noxa, Bad, Hrk, Bmf and Bik). Based on the direct activation model, BH3-only proteins consist of “activators” (e.g. Bim, Bid and Puma) that are able to bind and activate Bax directly and “sensitizers” (e.g. Bad and Noxa) that act by releasing activators from pro-survival proteins. **b**, Bax may be inhibited by all pro-survival proteins, while Bak is inhibited mainly by Bcl-xL, Mcl-1 and A1. Some BH3-only proteins (e.g. Bim, Bid and Puma) are able to neutralize all pro-survival proteins, while some of them (e.g. Bad and Noxa) can only bind a limited subset.

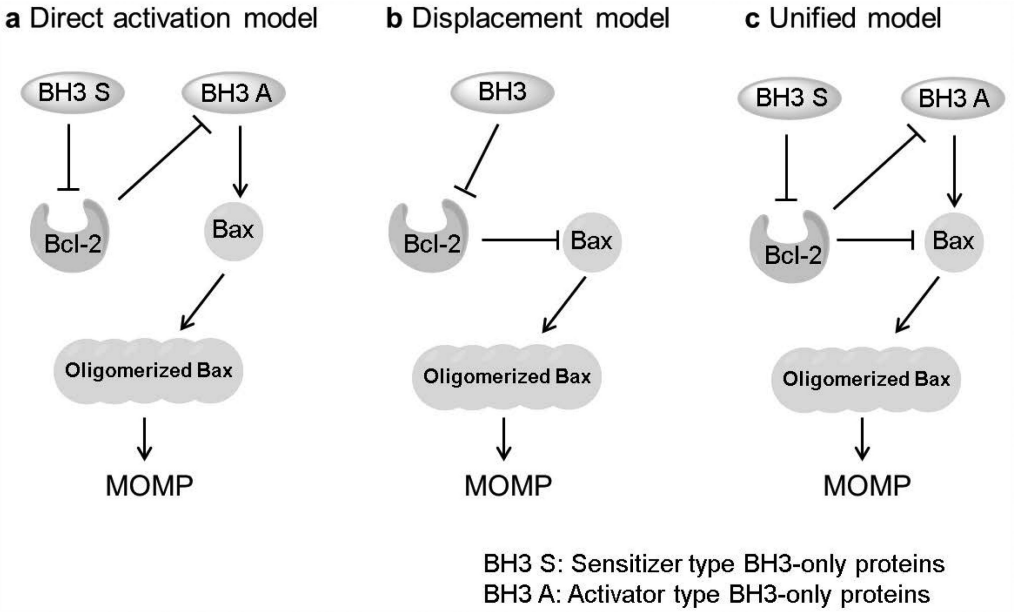


Figure 2. Different models of Bax activation-mediated mitochondrial outer membrane permeabilization (MOMP)

a, The direct activation model contends that activator type BH3-only proteins directly bind and activate Bax/Bak. **b**, The displacement model concludes that BH3-only proteins trigger apoptosis indirectly through Bax or Bak by neutralizing the relevant pro-survival proteins. **c**, The unified model assigns dual functions to anti-apoptotic proteins, sequestering not only the activator type BH3-only proteins but also the active forms of Bax and Bak.

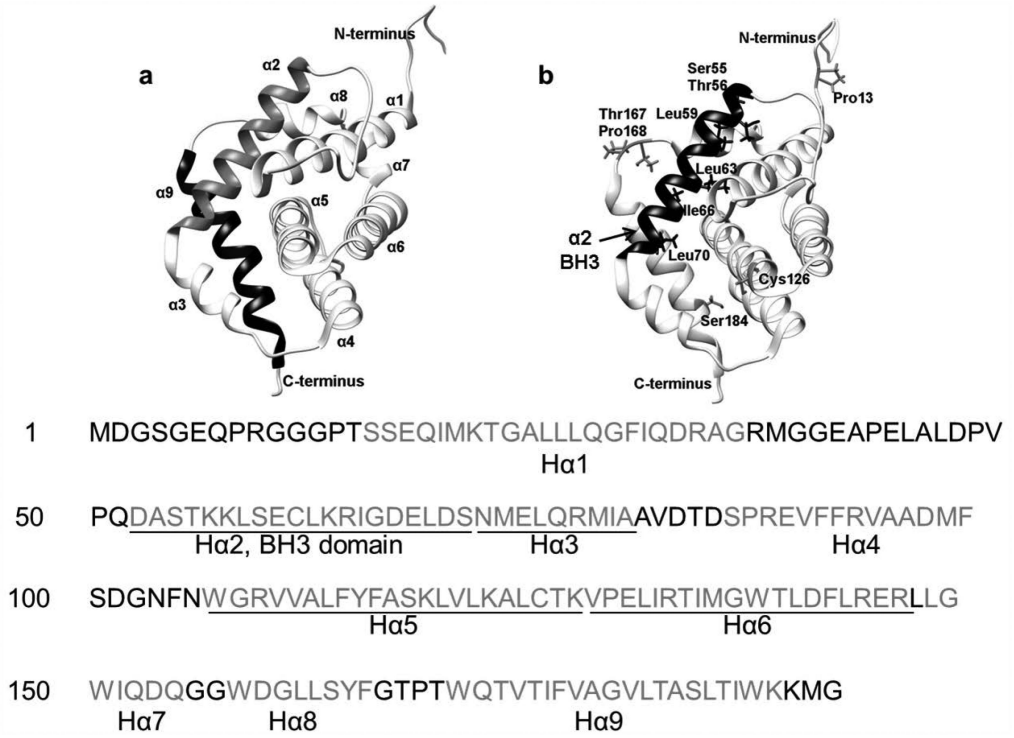


Figure 3. Structure of Bax

a, Ribbon representation of Bax (PDB: 1F16). The 9 α-helices are indicated separately. Hα2 comprises BH3 domain. Hα5 (helices α5) and Hα6 form the core of the protein and are embedded within the other 7 helices. **b**, Critical amino acid residues (e.g. Cys126, Thr167, Pro168, Pro13 and Ser184) are highlighted including hydrophobic residues on BH3 domain (e.g. Ser55, Thr56, Leu59, Leu63, Ile66 and Leu70).

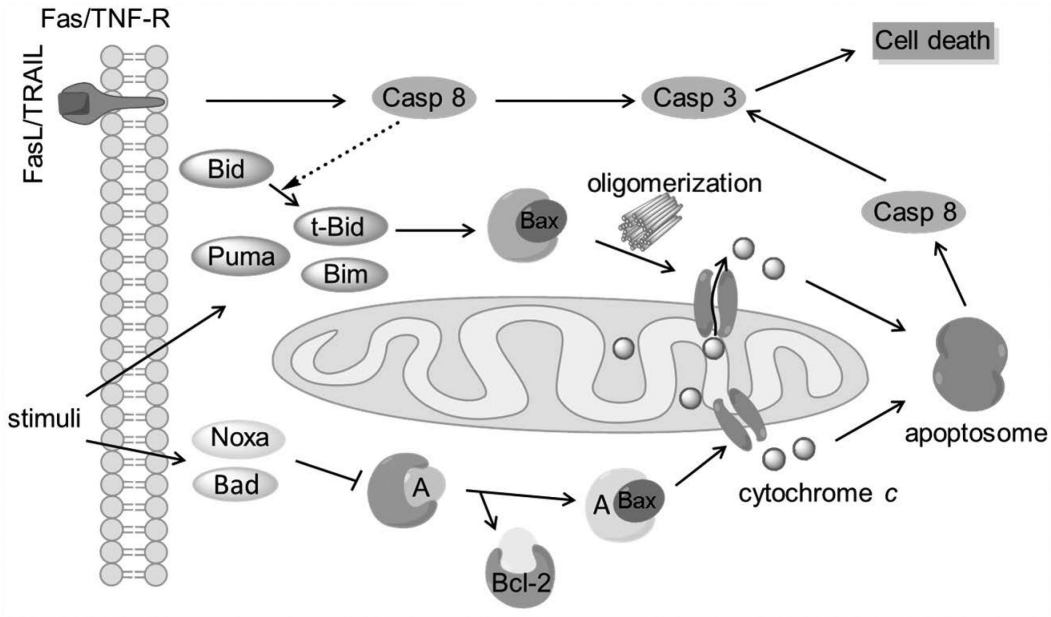


Figure 4. The apoptotic pathways mediated by interactions between Bax and other Bcl-2 family members based on the direct activation model

Once initiated by stimuli, BH3-only proteins activate Bax directly or indirectly. Oligomerized Bax leads to mitochondrial membrane permeabilization/pore formation and cytochrome *c* release. Cytochrome *c*, Apaf-1, dATP and procaspase-9 form the apoptosome which activates caspase 9 and other downstream executioner caspases, thereby ultimately leading to cell death. Casp: Caspase. A: Activator type BH3-only proteins.

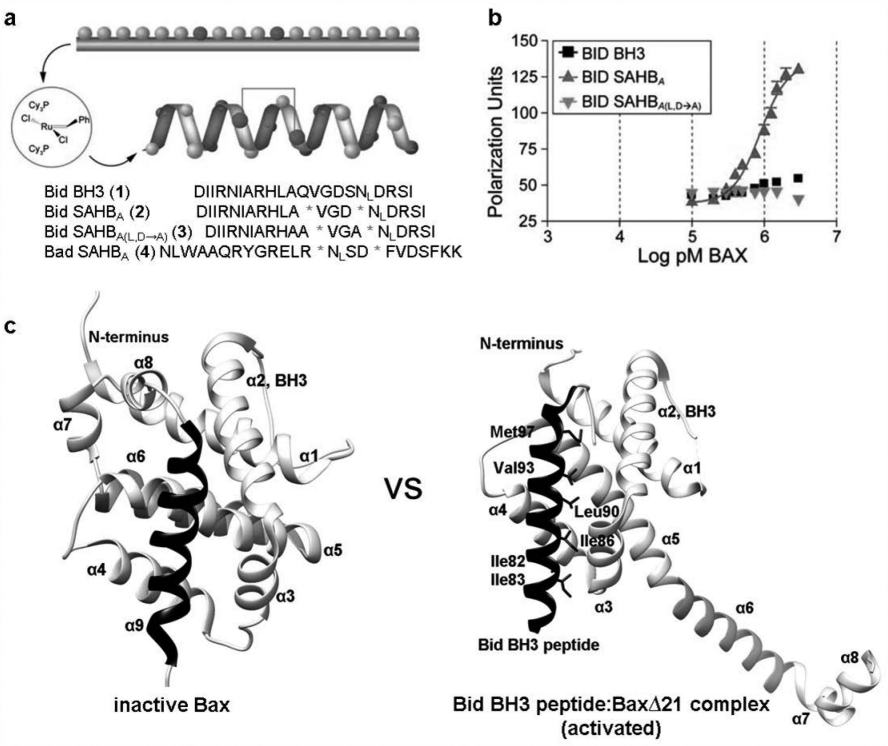


Figure 5. Bid SAHB$_A$ binds Bax and activates Bax directly

a, Structure and sequence of Bid SAHB$_A$. The native methionine of Bid BH3 was replaced with norleucine (N_L) in Bid SAHB$_A$ due to the incompatibility of sulfur with the metathesis reaction. **b**, Fluorescence polarization binding assays were carried out using FITC-labeled peptides (50 nM) and full-length Bax. Direct interaction between Bid SAHB$_A$ and Bax was observed. **c**, Comparison of the inactive form of Bax (PDB: 1F16) and activated form of BaxΔ21 (PDB: 4BD2). The crystal structure of Bid BH3 peptide (SESQEDIIRNIARHLAQVGDSMDRSIPPGL) complexed with BaxΔ21 shows that Hα1-Hα5 are released from Hα6-Hα8. Reproduced, with permission, from REF. 147© the Elsevier Inc. (2006).

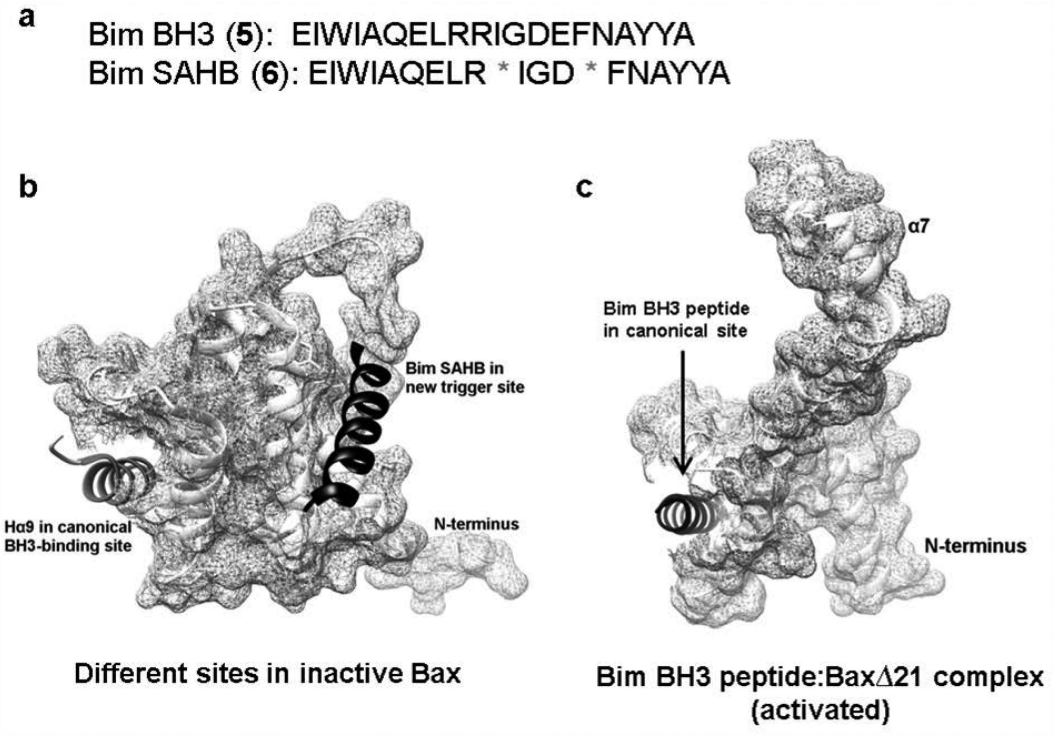


Figure 6. Different interaction sites of Bim BH3 peptides with Bax

a, Sequence of peptides Bim BH3 and Bim SAHB. **b**, Canonical BH3-binding site and the Bax trigger site located on the opposite sides of each other (PDB: 2K7W). **c**, The crystal structures of Bim BH3 peptide (RPEIWIAQELRRIGDEFNAYYA) complexed with Bax (PDB: 4ZIE) showing that the interaction site lies in the hydrophobic groove.

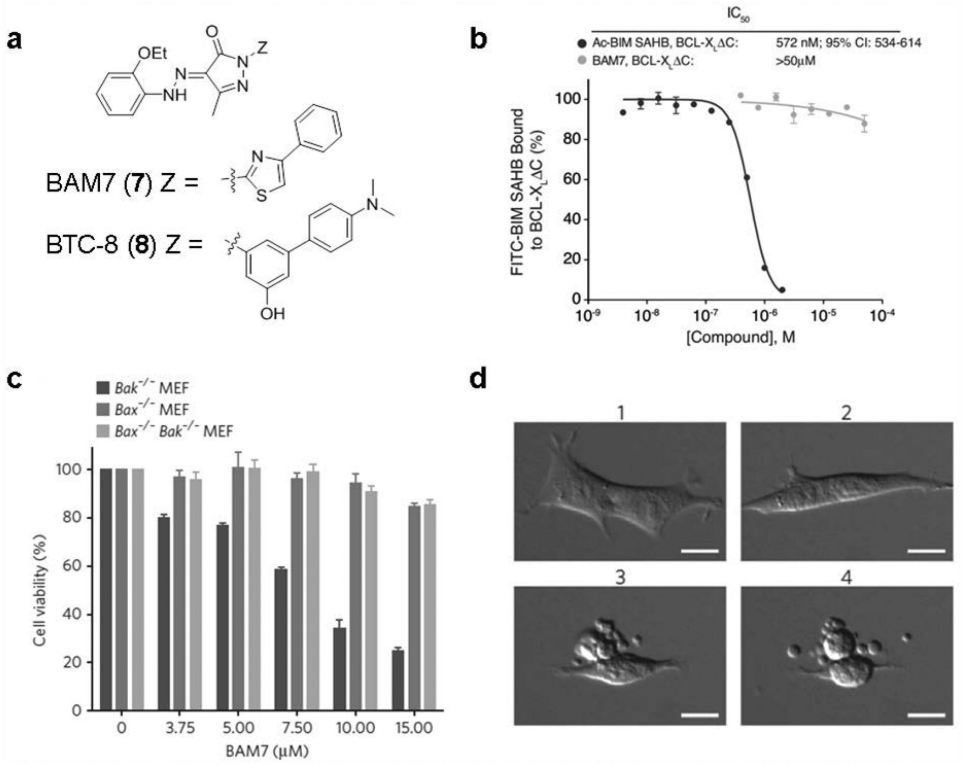


Figure 7. BAM7 specifically binds Bax and induces Bax-dependent cell death

a, The structure of BAM7 and its analog BTC-8. **b**, The specificity of BAM7 for the BH3 binding site on Bax was examined by competitive FPA employing FITC-BIM SAHB and Bcl-x_LΔC (Only the result of BCL-x_L is shown here). **c**, BAM7 selectively impairs the viability of *Bak*$^{-/-}$ MEFs but has no effect on MEFs lacking Bax (*Bax*$^{-/-}$) or both Bax and Bak (*Bax*$^{-/-}$ *Bak*$^{-/-}$). d. *Bak*$^{-/-}$ MEFs demonstrate the morphologic features of apoptosis in response to BAM7 treatment at the concentration of 15 μM. 1, 20 min; 2, 6 h; 3, 12 h; 4, 12.5 h. Scale bars, 15 μm. Reproduced, with permission, from REF. 164 © the Macmillan Publishers Limited (2012).

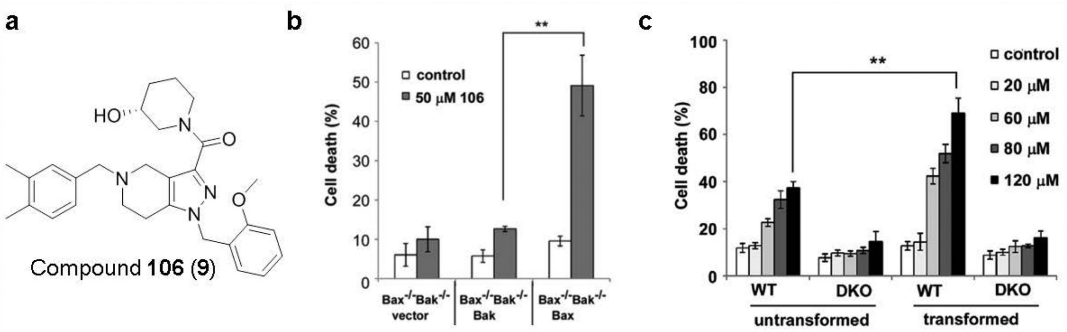


Figure 8. Compound 106 induces Bax-dependent apoptotic cell death

a, The chemical structure of compound **106** (ZINC 14750348). **b**, Compound **106** preferentially induces cell death of *Bak*$^{-/-}$*Bax*$^{-/-}$ MEFs expressing Bax at the concentration of 50 μM. **c**, The viability of the untransformed MEFs and their isogenic counterparts (transformed by expression of the K-Ras and E1A oncogenes) was determined 48 h following treatment with compound **106** at various concentrations (20-120 μM). Reproduced, with permission, from REF. 166 © 2014 by the American Society for Microbiology.

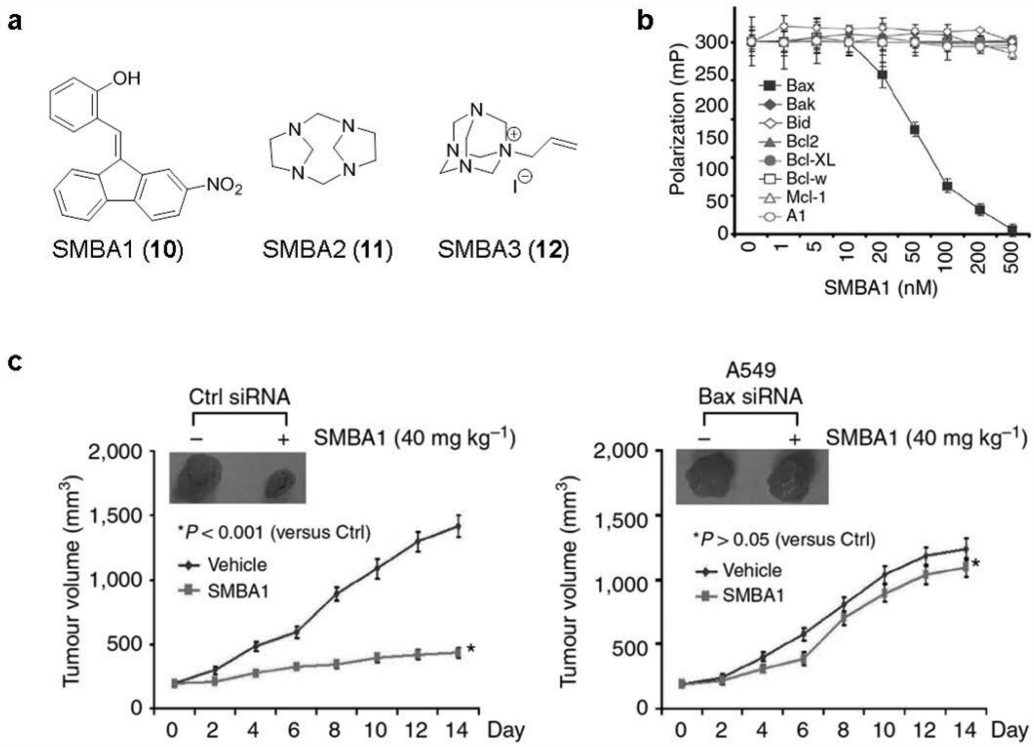
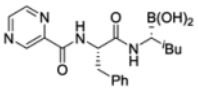
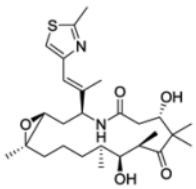
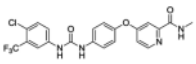
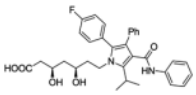
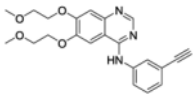
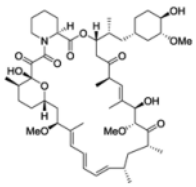
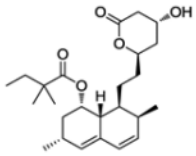
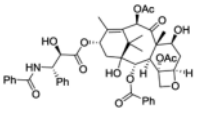
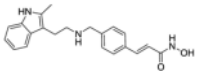


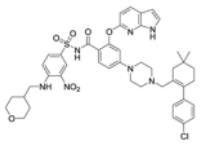
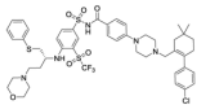
Figure 9. SMBA1 suppresses lung cancer *in vivo* by specifically targeting Bax

a, The chemical structures of SMBA1~3. **b**, Bax agonist SMBA1 binds selectively with Bax rather than other relevant Bcl-2 family members at the increasing concentration (0~500 nM). **c**, Mice with xenografts derived from A549 expressing Bax siRNA, or Ctrl siRNA were treated with SMBA1 (40 mg/kg) or vehicle for 14 days. Reproduced, with permission, from REF. 143 © the Macmillan Publishers Limited (2014).

Table 1

Selected drugs or drug candidates involving indirect activation of Bax

Name	Structure	Action mode	Status	Ref.
Bortezomib		Proteasome inhibitor	Launched	131-133
Ixabepilone		Tubulin modulator	Launched	134
Sorafenib		Flt3, Kit *etc*. tyrosine kinase inhibitor	Launched	135
Atorvastatin		HMG CoA reductase inhibitor	Launched	136
Erlotinib		EGFR inhibitor	Launched	137
Sirolimus		mTOR inhibitor	Launched	138
Simvastatin		Hypercholesterolemia	Launched[a]	139
Paclitaxel		Mitotic inhibitor	Launched	140
Panobinostat		Histone deacetylase inhibitor	Pre-registration	129

Name	Structure	Action mode	Status	Ref.
Venetoclax (ABT 199)		Bcl-2 inhibitor	Phase III (breakthrough therapy)	141
Navitoclax (ABT 263)		Bcl-2 inhibitor	Phase II	142

[a]Simvastatin was approved as a lipid lowering medication rather than an agent for cancer therapy. The recently reported anticancer effect of simvastatin is correlated to Bax activation.