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Small Molecule Allosteric Inhibitors of BAX

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Abstract

BAX is a critical effector of the mitochondrial cell death pathway in response to a diverse range of stimuli in physiological and disease contexts. Upon binding by BH3-only proteins, cytosolic BAX undergoes conformational activation and translocation, resulting in mitochondrial outer membrane permeabilization. Efforts to rationally target BAX and develop inhibitors have been elusive, despite the clear therapeutic potential of inhibiting BAX-mediated cell death in a host of diseases. Here, we describe a class of small molecule BAX inhibitors, termed BAIs, which bind directly to a previously unrecognized pocket and allosterically inhibit BAX activation. BAI-binding around the hydrophobic helix $\alpha 5$ using hydrophobic and hydrogen bonding interactions stabilizes key areas of the hydrophobic core. BAIs inhibit conformational events in BAX activation that prevent BAX mitochondrial translocation and oligomerization. Our data highlight a novel paradigm for effective and selective pharmacological targeting of BAX to enable rational development of inhibitors of BAX-mediated cell death.

Introduction

Programmed cell death is a physiological process in multicellular organisms that clears unhealthy and excess cells to ensure healthy development and tissue homeostasis¹. In

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Author Contributions

T.P.G performed NMR, biochemical and molecular modeling studies. D.E.R. performed cell-based studies, S.L. performed mass spectrometry studies. D.A. and R.N.K performed cell-based studies. E.G. conceived the study, designed experiments and wrote the manuscript, which was edited by all authors.

Competing Financial Interests

E.G., R.N.K., T.P.G, and D.A. are inventors on a patent application PCT/US2018/021644 submitted by Albert Einstein College of Medicine that covers compounds, compositions and methods for BAX inhibition for the treatment of diseases and disorders.

response to acute injury or chronic stress conditions, loss of cells through programmed cell death contributes to the pathogenesis of numerous diseases including myocardial infarction, stroke, toxicity from chemotherapy and radiation, and various neurological diseases²⁻⁴. Genetic and biochemical studies have revealed a crucial role for the BCL-2 family proteins in regulating apoptotic cell death⁵⁻⁶. The BCL-2 protein family has anti- and pro-apoptotic members that antagonistically regulate mitochondrial outer membrane permeabilization (MOMP) and mitochondrial dysfunction^{7,8}. Activation of pro-apoptotic BAX and/or BAK by BH3-only proteins is essential for induction of MOMP, whereas anti-apoptotic members inhibit pro-apoptotics to prevent MOMP^{7,8,9}. MOMP allows the cytoplasmic release of cytochrome *c*, Smac/Diablo and other pro-apoptotic factors from mitochondria, which enable the activation of the apoptotic signaling cascade¹⁰.

While BAK resides constitutively at the MOM, BAX is located predominantly in the cytoplasm as inactive monomers or dimers and must translocate to the MOM upon activation by BH3-only proteins^{11,12}. Once activated, BAX and BAK undergo homo- or hetero-oligomerization at the MOM to permeabilize the membrane¹³. Recent studies expanded the function of BAX to regulate necrosis through regulating the opening of the mitochondrial permeability transition pore^{14,15}. Interestingly, for this function, translocation but not oligomerization of BAX at the MOM is required. Additionally, despite the notion of redundancy for BAX and BAK activity, genetic deletion of BAX alone leads to profound protection from stress-induced cell death in various disease models¹⁶⁻¹⁹. These studies strongly suggest that BAX may be an attractive target for the pharmacological inhibition of cell death.

Despite significant advances in targeting BCL-2 proteins with small molecules to re-activate cell death in tumors^{20,21}, efforts to rationally target these proteins to prevent cell death, are elusive. Previous attempts led to small molecules that inhibit BAX/BAK channels and oligomerization and provided useful insights²²⁻²⁵. Lack of structural information of these inhibitors bound to BAX or BAK, however, has impeded their rational development. Moreover, BAX can still promote mitochondrial dysfunction without the requirement for oligomerization indicating that inhibition at an earlier step of BAX activation would be more effective^{14,15}.

Here, we focused on the rational inhibition of BAX using recent advances we have made in studying BAX structure and activation. Previous reports identified sites that regulate BAX activation by the BH3-only proteins, such as the N-terminal trigger site and the canonical groove, which becomes available upon C-terminal helix α 9 release (Fig. 1a,b)²⁶⁻²⁷. Additionally, distinct inhibitory binding sites on BAX that interact with peptides from the BH4 domain of BCL-2 and the cytomegalovirus protein vMIA were also identified (Fig. 1c)^{28,29}. We recently reported the first rational discovery of small molecules that bind the N-terminal trigger site and activate BAX to promote apoptosis^{30,31}. While we embarked on screening approaches for inhibitors of BAX, unexpectedly, we found select carbazole-based compounds that were previously reported to inhibit BAX/BAK mitochondrial-associated channels^{22,24}. These molecules enabled us to identify a novel binding site on BAX and a new allosteric mechanism of BAX inhibition resulting from binding at this site. Our findings may prove useful in delineating more precisely the mechanistic roles of BAX and provide a

rational approach to the development of BAX inhibitors for diseases mediated by BAX-dependent cell death.

Results

Discovery of direct BAX activation inhibitors

To identify direct BAX inhibitors we have performed screens of small molecules using the liposomal release assay. In this assay, compounds are evaluated for their ability to inhibit membrane permeabilization induced by tBID-mediated BAX activation. Inhibition in this assay can result from impairment of various steps in the BAX activation pathway, including conformational activation, translocation to the liposomal membrane, and oligomerization^{25,31,32}. Two carbazole-based compounds were evaluated that differ only with respect to the presence of an –OH or –F group in the piperazinyl-propyl extension from the carbazole ring. We named these BAX activation inhibitor 1 (BAI1) (**1**) and 2 (BAI2) (**2**) (Fig. 1d). BAI1 and BAI2 demonstrated inhibition of tBID-induced BAX-mediated membrane permeabilization in a dose dependent manner with an IC₅₀ of 3.3 μM and 4.6 μM, respectively (Fig. 1e, Supplementary Fig. 1a,b). To confirm direct activity of BAIs on BAX, we compared BAI1's activity in liposome release assays with tBID, BIM SAHB and the BAX activator BAM7, each one having different affinity for BAX (Supplementary Fig. 1c-e). The observed IC₅₀ for BAI1 is independent of either the activating molecule or its concentration confirming a direct activity on BAX (Supplementary Fig. 1g). In addition, BAI1 was able to inhibit heat-induced BAX activation with a comparable IC₅₀ (Supplementary Fig. 1f,g). This prompted us to evaluate direct binding of BAI1 to inactive BAX in solution using ligand-detected ¹H-Nuclear Magnetic Resonance (NMR) and microscale thermophoresis (MST) experiments. Upon titration with inactive BAX, proton peaks of the carbazole ring of BAI1, showed evidence of direct and specific binding by either standard 1D ¹H, STD and CPMG experiments (Fig. 1f). Moreover, titration of inactive BAX into BAI1 and monitoring the H3 peak of the carbazole ring by 1D ¹H NMR, showed dose-dependent chemical shift perturbation (CSP) and broadening of the peak, consistent with a 1:1 binding ratio (Supplementary Fig. 2). A dissociation constant for BAI1 and BAI2 with BAX of 15.0 ± 4 μM and 17.5 ± 4 μM, was determined by MST (Fig. 1g, Supplementary Fig. 3). Taken together, these data demonstrate that BAI1–2 directly bind to soluble BAX.

BAIs bind to inactive BAX at a novel allosteric site

Next, we performed 2D ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) NMR analysis of ¹⁵N-labelled BAX³³. Addition of BAI1 shifted select NMR cross-peaks while preserving the overall features of the NMR spectra, indicating fast exchange between the bound and unbound conformations of BAX, consistent with a K_d in the low micromolar range (Supplementary Fig. 4). CSP analysis of BAX with BAI1 exhibited significant chemical shifts in residues located at different regions in the protein. The highest changes were observed in select residues of the α3, α4, α5 and α6 (Fig. 2a). Although the observed CSPs were smaller than might be expected for an interaction of this nature, they are localized and are specific. Interestingly, small CSPs have been observed in previous NMR studies of BAX^{26,28–31,34}. To confirm the specificity of these CSPs we repeated the titration

using the analogue BAI3, which has ~7-fold lower activity compared to BAI1. We observed significantly reduced CSPs compared to BAI1 (Supplementary Fig. 5). Mapping the BAI1 CSPs onto the structure of inactive BAX revealed that residues undergoing significant shifts are localized at one specific surface of the protein centered where helices α 3, α 4, α 5 and α 6 converge (Fig. 2b). Distant from this cluster of residues, a few solvent-exposed residues in helix α 1, α 2 and α 1- α 2 loop also undergo significant CSPs. Interestingly, the region on the surface of BAX, where helices α 3, α 4, α 5 and α 6 converge, form an uncharacterized binding pocket (Fig. 2b).

To support the identification of this pocket, the structure of inactive BAX³³ (PDB ID:1F16) was analyzed with SiteMap (Schrödinger, LLC, 2017), a computational tool to evaluate the propensity of ligand binding pockets in protein structures. SiteMap suggested five binding pockets for the BAX structure including our newly identified pocket, termed BAI-site, and the BAX trigger site (Supplementary Fig. 6a). By comparing the sites identified by SiteMap with the residues showing significant CSP in our HSQC NMR titrations, we found that only the BAI-site overlaps with residues undergoing CSP (Supplementary Fig. 6b). Furthermore, we used hy-TEMPO, a soluble paramagnetic relaxation enhancement (PRE) probe, with the idea that such a simple molecule will bind non-specifically to the solvent exposed surface preferentially binding to pockets on the surface of BAX confirming the presence of the SiteMap-proposed binding pockets³⁵. hy-TEMPO induced PRE on the majority of the BAX solvent exposed residues, with the most significant PREs associated with the BAX trigger site and the BAI-site (Supplementary Fig. 6c-e). This confirmed the presence of a relatively deep solvent exposed cavity at the proposed interaction site, confirming the existence of the BAI-site on the BAX structure.

We next performed molecular docking of BAI1 using an induced fit docking approach guided by those residues showing significant CSPs. BAI1 was docked preferentially at the junction of the C-terminus of α 3, the α 3- α 4 loop, and residues of α 5 and α 6 (Fig. 2c), which is the same as that suggested by SiteMap (Supplementary Fig. 6e). To account for potential ambiguity in the NMR data, docking was repeated against a largely-extended surface of BAX that surrounds the BAI-site, yielding only the same docking poses within the BAI-site (Supplementary Fig. 7). BAI1 binds consistently with a binding pose that uses the carbazole ring buried near to the hydrophobic core of BAX within the BAI-site while the piperazinyl-propanol extension from the carbazole ring is more solvent exposed (Fig. 2d,e). Indeed, this is a reasonable binding mode as the hydrophobic carbazole ring with the two bromines is surrounded by several aliphatic and aromatic hydrophobic core residues including Val91, Ile136, Leu132, Trp139, Phe116, Val95, Leu185 and Leu120. BAI1 balances these hydrophobic interactions with two potential hydrogen bonds using the piperazinyl-propanol extension (Fig. 2d,e). The positively charged piperazine ring is predicted to form a hydrogen bond with the side chain of Asp84, and the hydroxyl of the propanol is predicted to form a hydrogen bond with the side chain of Lys123 and main chain carbonyl of Ile80 (Fig. 2d,e). Consistent with this binding mode, Ala81, Lys123 and Val83 cross-peaks undergo the most significant CSP upon titration of BAI1 (Fig. 2a). To further assess the lowest-energy docked mode of BAI1, we subjected BAX alone and BAX bound to BAI1 in two independent molecular dynamics (MD) simulations (Supplementary Fig. 8a,b). In both MD simulations, the BAI1 binding mode remained stable within the BAI-site with

an average RMSD of 0.65 \pm 0.31 Å and 0.34 \pm 0.1 Å compared to an average RMSD of 1.7 \pm 0.25 Å and 1.4 \pm 0.12 Å for the backbone atoms of the ordered protein regions (Supplementary Fig. 8c,d).

Moreover, we also analyzed BAI2 by HSQC-NMR analysis and docking. The CSP analysis highlighted residues concentrated in the BAI-site and a few sporadic CSPs in residues around the BAI-site (Supplementary Fig. 9a). Docking of BAI2 is consistent with BAI1 preserving all key interactions (Supplementary Fig. 9b-d). The fluorine atom however, could not form a hydrogen bond with Ile80 or Lys123 but could form a halogen bond with the carbonyl of Ile80.

Lastly, to further confirm the BAI-site, we generated two BAX mutants. The first mutant D84K/D86K was designed to remove hydrogen bonding with the piperazine ring (Fig 2d,e, Supplementary Fig 10a)²⁹. The second mutant V83W/L120W was designed to partially fill the proposed binding pocket with tryptophan side chains to potentially mimic BAI binding (Supplementary Fig. 10b,c). Neither BAX mutant showed any effect on retention time in size exclusion chromatography analysis or the ability of BAX to bind to BIM BH3, suggesting no effect on BAX folding or on the activation site (Supplementary Fig. 11). Both mutants caused significant reduction in the observed CSPs in 2D NMR based titrations of BAI1, confirming BAI1's interaction with the pocket (Supplementary Fig. 12). Consistently, both mutants resulted in a significant reduction in the inhibition of BAX by BAI1 in response to activation by BH3-only proteins or heat activation at 42 °C (Supplementary Fig. 13a-c). Notably, V83W/L120W mutant showed significant loss in response to BH3-mediated activation in liposome release assays, suggesting a BAI-mimicry inhibition effect as anticipated (Supplementary Fig. 13d,e). Taken together, our results position BAIs at a novel binding site on BAX that is implicated in BAX inhibition.

Structure-activity relationships (SAR) of BAIs

Next, we evaluated the contribution of each functional group of the BAI scaffold to the binding and inhibition of BAX. We used ligand-observed CSP calculated by 1D ¹H-NMR of the aromatic peaks of BAIs to measure the binding interaction (Supplementary Fig. 14a,b). We also used the liposomal release assay to measure BAX inhibition (Supplementary Fig. 14c). Supplementary Table 1 shows structures of BAI compounds and their corresponding ligand-observed CSP and IC₅₀ values in BAX-mediated membrane permeabilization. Loss of each bromine group from the carbazole ring in BAI3 (**3**) and BAI4 (**4**) reduces BAX binding and inhibition, and BAI3 with unsubstituted carbazole has the worse binding and inhibition activity. The smaller chlorine group in BAI5 (**5**) compared to the bromine group in BAI4 resulted in less binding, but slightly better BAX inhibition. The morpholino group in BAI6 (**6**) resulted in reduced inhibition compared to the piperazine group of BAI1; and when the piperazine group is replaced with one or two propyl groups, as in BAI7 (**7**) and BAI8 (**8**), binding and inhibition were reduced. This SAR is likely explained by the morpholino ring possessing no hydrogen bond donor and the one or two propyl groups not aligning for hydrogen bonding as well as the piperazine ring. Interestingly, we found that the carbazole ring can be replaced with the phenothiazine group in BAI9 (**9**) and BAI10 (**10**) with a modest change in activity. This suggests that additional substituted tricyclic or

bicyclic ring systems could fit into the BAI-site. Overall, the SAR is consistent with the hydrophobicity and shape complementarity of the di-substituted carbazole-based compounds and potential hydrogen-bonding interactions of the piperazine ring. Positive correlation of binding affinity to the BAI-site and inhibition of BAX can inform future rational design strategy for development of BAX inhibitors (Supplementary Table 1 and Fig. 14d).

BAIs allosterically inhibit BAX activation in solution

While the C-terminal canonical pocket is buried from its interaction with $\alpha 9$, the BAX trigger site is available for binding in the inactive BAX structure^{26,36} (Fig. 1a,b). The BAI site is geographically distinct from the N-terminal trigger site and the C-terminal canonical pocket. Therefore, we hypothesized that BAIs inhibit BAX through a non-competitive inhibition mechanism. Two other known peptides from BCL-2 and ν MIA proteins have been shown to bind and inhibit BAX^{28,29} (Fig. 1c). Interestingly, by comparing the structural models of the BCL-2 and ν MIA peptides bound to BAX, we found that the ν MIA peptide binds at the back surface of the BAI-site (Supplementary Fig. 15a). Indeed, some peripheral residues of the BAI-site that do not interact with BAI interact with the ν MIA peptide (Supplementary Fig. 15a-c)²⁹. Although, the mechanism of how ν MIA peptide inhibits BAX is not fully understood, it has been proposed that it can promote BAX translocation and block BAX oligomerization by binding at this region near the C-terminal helix of BAX²⁹. We used the program DYNAFIT (BioKin) to simulate the competition of BAI1 with ν MIA and tested this using a fluorescence polarization assay with the ν MIA peptide and BAX. We found that addition of BAI1 had no effect on the affinity of the ν MIA peptide to BAX, consistent with our structural analysis (Supplementary Fig. 15d). Therefore, the binding site and mechanism of BAI-mediated BAX inhibition appears to be distinct from that of ν MIA.

Since BAIs bind to inactive BAX, we evaluated how BAI1 affects BH3-mediated BAX activation. BH3 binding to BAX induces conformational changes that transform the inactive soluble monomer into an oligomer^{36,37}. Early conformational changes upon BAX activation involve the opening of the $\alpha 1$ - $\alpha 2$ loop from the trigger site, the exposure of the $\alpha 2$ (BH3 domain) and the mobilization of $\alpha 9$ from the canonical pocket³⁶. Using ¹⁵N-¹H HSQC-NMR analysis of ¹⁵N-labelled full-length BAX, we monitored BAX activation overtime upon addition of BIM SAHB (Fig. 3). The addition of BIM SAHB leads to activation of the inactive BAX as demonstrated by the broadening and reduction of the BAX monomer cross-peaks overtime (Fig. 3a,b). Within a few hours, the majority of BAX monomer cross-peaks disappeared below the detection limit (Fig. 3c). Only peaks corresponding to the flexible N-terminus of oligomerized BAX can be detected in the activated state. In contrast, in the presence of BAI1, BIM SAHB-induced BAX activation is strongly inhibited with ~ 80% of the BAX remaining monomeric at 24 hours after BIM SAHB addition (Fig. 3d-f). We monitored BAI1-inhibition over an extended time frame and, even after 12 days, 50% of the BAX monomer was present in the NMR sample (Fig. 3f). Next, we calculated CSPs of BAX spectra in the presence of both BIM SAHB and BAI1 (Supplementary Fig. 16a). Significant CSPs were observed in both the BIM SAHB-binding site (trigger site) and the BAI-site (Supplementary Fig. 16b,c), suggesting that both BIM SAHB and BAI1 are bound to BAX, and that BAI1 effectively blocks the conformational activation of BAX induced by BIM SAHB. BAI1 was not able to fully inhibit BIM SAHB-induced activation of the BAI-site

mutants D84K/D86K and V83W/L120W as monitored by ^{15}N - ^1H HSQC-NMR analysis, consistent with their loss of binding and activity (Supplementary Fig. 17). To confirm these results with an alternative method, we used size-exclusion chromatography. BIM SAHB induced complete BAX oligomerization of the BAX monomer, whereas BAI1 strongly inhibited the effect of BIM SAHB and preserved BAX as a monomer after 24 hours (Supplementary Fig. 18). Taken together, these data demonstrate that BAI1 is a potent inhibitor of BAX conformational activation and oligomerization in solution, not by direct competition with the activator, but rather through an allosteric mechanism that favors the inactive BAX.

Mechanism of BAI-induced BAX inhibition

To further interrogate the mechanism of BAI-induced BAX inhibition, we performed hydrogen deuterium exchange mass spectrometry (HXMS) to probe the direct and indirect effects on the protein structure upon BAI1 binding. We calculated the difference in the accessibility of deuterium on inactive BAX unbound and bound to BAI1 (Supplementary Fig. 19a-c). HXMS analysis showed protection in different regions of the BAX structure upon BAI1 binding, which included residues associated with the BAI-site (α 3- α 4, α 5) but also pronounced effects in residues of α 1, α 1- α 2 loop, α 3, α 7, α 8 and α 9 that are associated with the BAX trigger site, and regions undergoing conformational changes upon BAX activation (Supplementary Fig. 19c,d). BAI1 increased deuterium incorporation only in residues of α 6 and α 7. Next, we measured PRE effects using the soluble PRE probe, hy-TEMPO, to account for changes in hy-TEMPO access to the BAX surface in the presence or absence of BAI1. The observed differences in PRE effects demonstrated protection from hy-TEMPO access to the BAX structure induced by BAI1 within the BAI-site (α 3- α 4, α 5), but also in α 3, α 6, α 7, α 8 and α 9 regions that undergo conformational changes upon BAX activation (Fig. 4a). Only a few isolated residues showed significantly increased access to hy-TEMPO binding due to BAI1 binding (Fig. 4a). Mapping of the PRE effects on the structure of BAX revealed that BAI1 binding leads to significant protection of several regions around the BAI-site and particularly within the hydrophobic core residues of helix α 5, which is key to maintaining the inactive BAX structure (Fig. 4b). These local and global effects of BAI1 probed by HXMS and PRE analysis are remarkable for a small molecule and suggest that the inhibitory effect of BAI1 is mediated by direct and allosteric stabilization of key areas of the BAX structure, resulting in suppression of conformational activation.

As BAI1 suppresses conformational changes induced by BH3-only proteins, we investigated biochemical evidence for inhibiting BAX activation, dimerization and MOMP. Indeed, we found that BAI1 inhibited tBID-induced BAX dimers and oligomers formed in the presence of liposomal membranes as detected by BMH crosslinking and SDS-PAGE analysis (Fig. 4c, 4d). Moreover, BAI1 inhibited BAX membrane association and translocation induced either by tBID ($\text{IC}_{50} = 5 \pm 1 \mu\text{M}$) or BIM SAHB ($\text{IC}_{50} = 2 \pm 1 \mu\text{M}$) in a dose dependent manner (Fig. 4e, Supplementary Fig. 20) and with a similar IC_{50} to BAX-mediated liposomal release (Supplementary Table 1). These data are consistent with BAI1 inhibiting the conformational change of the BH3 domain (α 2) and the membrane anchoring C-terminal helix α 9 that are required for dimerization and translocation, respectively^{27,36}. One of the earliest conformational changes upon BH3-mediated BAX activation, is the exposure of the BH3

residues recognized by the anti-BH3 domain antibody^{27,36}. In a pull-down assay with anti-BH3 domain antibody, BIM SAHB induced the exposure of the BH3 domain from BAX, and this was suppressed by BAI1 (Fig. 4f, 4g, Supplementary Fig. 21). Since BAI1 inhibits the early conformational changes seen in BAX activation, we investigated whether BAX can stabilize BAX. We measured the temperature-dependent activation of BAX using a translocation assay (Fig. 4h). For BAX alone we measured an activation temperature of ~50 °C, almost 15 °C lower than the reported melting temperature for BAX³⁸. The addition of BAI1 caused significant stabilization of BAX with a 10 °C increase in the activation temperature. Taken together, our biochemical and structural data are in agreement with an allosteric mechanism of inhibition of conformational activation by BAI1.

BAI1 selectively inhibits BAX-dependent cell death

Based on BAI1 mechanism, we reasoned that BAI1 should selectively inhibit BAX-mediated apoptotic cell death. To test this, we used mouse embryonic fibroblasts (MEF) and promoted apoptosis with TNF α + cyclohexamide stimulus, which induces activation of BAX and BAK. Titration of BAI1 effectively inhibited TNF α + cyclohexamide mediated-apoptosis monitored by caspase 3/7 activation in a dose-dependent manner (IC₅₀ = 1.8 μ M) (Fig. 5a). To test selectivity for BAX, we compared wild type MEFs with mutant MEFs lacking *Bax* (BAX KO; *Bax*^{-/-}) or *Bak* (BAK KO; *Bak*^{-/-}). While BAI1 inhibited TNF α + cyclohexamide mediated-apoptosis in wild type and BAK KO MEFs (Fig. 5b,c), it had no effect in BAX KO MEFs (Fig. 5d). Next, we evaluated the effect of BAI1 on BAX-induced mitochondrial depolarization mediated by BIM BH3 peptide. Using MEFs deficient for both *Bax* and *Bak* (*Bax*^{-/-}; *Bak*^{-/-}) that had been reconstituted with human *Bax*, we found that BAI1 inhibited dose-dependently BIM BH3-induced mitochondrial depolarization. (Fig. 5e). Moreover, BAI1 inhibited cell death in OCI-AML3 cells induced by BTSA1, a direct BAX activator through interaction with the trigger site (Fig. 5f). Taken together, these studies demonstrate the selectivity of BAI1 to inhibit BAX activation and BAX-mediated cell death.

Discussion

Our studies highlight small-molecule BAX inhibitors that target a novel binding site on inactive BAX to inhibit BAX activation. This binding site has features ideal for a drugable pocket to facilitate potent and selective targeting of BAX, including a deep hydrophobic groove that is surrounded by polar and charged residues. This pocket is distinct from the already identified binding sites on BAX that interact with BH3-only peptides (to activate BAX) or the inhibitory peptides from the BCL-2 and ν MIA proteins²⁶⁻²⁹. Our data are consistent with BAIs operating through a mechanism of inhibition that stabilizes interactions within the hydrophobic core of BAX, thereby inhibiting the induction of conformational changes, including the mobilization of both the BH3 domain and the C-terminal helix α 9 from the hydrophobic core, which are critical to BAX dimerization/oligomerization and mitochondrial translocation^{5,37}. Binding of BAI1 to BAX does not inhibit the binding of the BH3 activators to the BAX trigger site, but changes the consequences of the binding leading to these conformational changes. The BAI site is distal from both known BH3 activation sites and the sites where the BH3 domain and α 9 are localized on the BAX structure,

therefore we consider the mechanism of BAI1 to be that of an allosteric inhibitor of BAX activation.

BAI1 and BAI2 were first identified from a screen using a BAX-mediated cytochrome *c* release assay as inhibitors of BAX- and BAK-associated channels, and it was suggested that they may promote disassembly of pre-formed BAX/BAK channels^{22,24}. Although inhibition of channel activity by BAI1 and BAI2 is not excluded by our work, the direct effects of these small molecules on BAX had not been evaluated. Nevertheless, our studies suggest that additional carbazole-based compounds, phenothiazine-based compounds, and potentially other compounds with fused or unfused ring systems can also bind to the BAI-site of inactive BAX and inhibit BAX activation.

In contrast to BAIs, a fragment from an NMR-based screen was recently shown to bind adjacent to the BAI-site and sensitized BH3-mediated BAX activation³⁴. This fragment competed with the binding of the vMIA peptide, while allosterically mobilizing the α 1- α 2 loop and the BAX BH3 domain (α 2) adjacent to the trigger site (α 1/ α 6). Such opposite binding effects of this fragment compared to BAIs, despite their adjacent binding location, suggest a remarkable plasticity and allosteric regulation of the BAX structure. Indeed, structural plasticity and allosteric regulation are key properties of the BCL-2 family proteins, which seem critical in the regulation of their mitochondrial localization and protein interactions^{26-31,39-44}

In conclusion, we have elucidated a previously unrecognized pocket and an allosteric mechanism of BAX inhibition that can be utilized by small-molecule BAX inhibitors such as BAIs. BAIs can be used as tools for probing mechanisms of BAX activation and BAX-dependent cell death. Rational targeting of BAX and development of BAX inhibitors through the BAI-site offers an opportunity for therapeutic intervention in disease mediated by BAX-dependent cell death.

Online Methods

Reagents

Hydrocarbon-stapled peptides corresponding to the BH3 domain of BIM, BIM SAHB_{A2}: N-acetylated- and FITC-Ahx-EIWIAQELRS5IGDS5FNAYYA-CONH₂, where S5 represents the non-natural amino acid inserted for olefin metathesis, were synthesized, purified at >95% purity by CPC Scientific Inc. and characterized as previously described³⁰. vMIA peptide, FITC-Ahx-CEALKKALRRHRFLWQRRQRA peptide was synthesized and purified at >95% purity by Genescript²⁹. Recombinant tBID in > 95% purity by SDS-PAGE page under reducing conditions was purchased by R & D Systems. BAI1 and related compounds were purchased from Millipore, Sigma and Chembridge and their molecular identity and purity >95% was confirmed by NMR and MS analysis. BAM7 was purchased from Selleck and purity >95% was confirmed by NMR and MS analysis. Compounds were >95% pure, reconstituted in 100% DMSO and diluted in aqueous buffers or cell culture medium for assays.

Production of recombinant BAX

Human full-length (1–192) wild type BAX was cloned in pTYB1 vector (New England Biolabs) between the NdeI and SapI restriction sites. Mutations were generated using the QuickChange Lightning site directed mutagenesis kit (Agilent). Recombinant proteins were expressed in BL21 (DE3) CodonPlus (DE3)-RIPL, grown in Luria Broth media and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside. The bacterial pellet was resuspended in lysis buffer (20 mM Tris-HCl pH 7.6, 250 mM NaCl, 1 mM EDTA, and Roche complete EDTA free protease inhibitor cocktail), lysed by high pressure homogenization, and clarified by ultracentrifugation at 45,000 x g for 45 min. The supernatant was applied to 5 ml of pre-equilibrated chitin beads (New England Biolabs) in a gravity-flow column, and washed with 3 column volumes of lysis buffer. BAX was cleaved by overnight incubation using 50 mM DTT in lysis buffer. Cleaved BAX was eluted with lysis buffer, concentrated with a Centricon spin concentrator (Millipore) and purified by gel filtration using a Superdex 75 10/300 GL, column (GE Healthcare Life Sciences), pre-equilibrated with gel filtration buffer (20 mM HEPES, 150 mM KCl, pH 7.2) at 4°C. Fractions containing BAX monomer are pooled and concentrated using a 10-KDa cut-off Centricon spin concentrator (Millipore) for prompt use in biochemical and structural studies.

Microscale Thermophoresis

Recombinant BAX protein was labeled at cysteine using the Monolith Protein Labeling Kit RED-MALEIMIDE (NanoTemper Technologies), according to the instructions of the manufacturer. Briefly, proteins at concentrations of 10 μ M were incubated with 1 or 2 equivalents of dye in MST buffer (100 mM Potassium phosphate, pH 7.4, 150 mM NaCl) in the dark at room temperature for 1 hour. Unreacted dye was quenched using 5 mM DTT and removed by filtration through 10 KDa cut-off centrifugal concentrator. Degree of labeling was confirmed after purification by comparing the measured concentration of BAX and bound dye using the OD_{280 nm} and OD_{650 nm} respectively. To determine the K_d values of BAX to BAI1 and BAI2, 100 nM of labeled BAX was incubated with increasing concentrations of BAIs (150–0.5 μ M) for 10 minutes in MST buffer. Samples were sonicated in a sonicating water bath for 10 seconds, loaded into standard glass capillaries (Monolith NT.115 Capillaries), and analyzed by MST using a Monolith NT.115 Blue/Red, LED power of 30% and an IR laser power of 40% and 60%. The initial 10-second sonication was used to insure solubility of the BAI1 at the higher concentrations. Insoluble particles within the capillaries of an MST experiments can disrupt diffusion and lead to unusable data points. Binding data was analyzed using GraphPad Prism (GraphPad Software) to determine the K_d values of BAX to BAIs using a 1 to 1 binding model. For these analyses, the temperature jump (1.6 seconds) region of the MST curves was used. Labeling of one or both cysteines of BAX, or moving the site of labeling using the mutant C62S/C126S/S5C, reported essentially the same K_d.

NMR samples and spectroscopy

The uniformly ¹⁵N-labelled protein samples were prepared by growing the bacteria in minimal medium as previously described³⁹. Unlabeled and ¹⁵N-labelled protein samples were prepared in 50 mM potassium phosphate, 50 mM NaCl solution at pH 6.0 in 10 %

D₂O. All experiments were performed using an independent sample for each experimental measurement as a 55 µl sample in a 1.7 mm microtube, all samples were DMSO matched. We have tested the pH of our NMR sample with and without BAI1 up to 200 µM and found no change in pH (measured pH values of 6.023 +/-0.02 vs. 6.028 +/-0.02 n = 8). Ligand detected NMR experiments were performed using 100–150 µM BAI with and without unlabeled BAX at 10–15 µM, prepared in 50 mM potassium phosphate, 50 mM NaCl solution at pH 6.0 in 10 % D₂O. 1D ¹H- NMR spectra were recorded using standard methodologies incorporating water suppression using excitation sculpting⁴⁵. STD experiments were performed using an off resonance frequency of –2000 Hz and an on resonance frequency of 450 Hz employing a saturation time of 0.5–2 seconds using standard bruker pulse sequence⁴⁶. 1D Carr–Purcell–Meiboom–Gill (CPMG) experiments were performed using standard Bruker pulse sequence employing a relaxation delay of 200 ms⁴⁷. Correlation ¹H-¹⁵N-HSQC spectra were recorded on ¹⁵N-labelled BAX at 50 µM and titrations up to 150 µM of BAI compounds were performed. NMR spectra were acquired at 25 °C on a Bruker 600 MHz spectrometer equipped with a cryoprobe, processed using Topspin and analyzed with CCPNMR. BAX cross-peak assignments were applied as previously reported²⁶ and deposited in BMRB entry 18340³³. The weighted average chemical shift difference (CSP) was calculated as $(\delta H1)^2 + (\delta N15/5)^2$ in p.p.m. The absence of a bar indicates no chemical shift difference, the presence of a proline or a residue that is overlapped or missing and therefore not used in the analysis. The significance threshold for backbone amide chemical shift changes (0.01 p.p.m.) was calculated based on the average chemical shift across all residues plus the standard deviation, in accordance with standard methods⁴⁸. Solvent accessible surface area was probed by the addition of 10 mM hy-TEMPO (Sigma) to 50 µM ¹⁵N-BAX with and without 200 µM BAI1 measured using standard ¹H-¹⁵N-HSQC incorporating an increased recycle delay of 5 seconds³⁵. PRE were calculated as the ratio of peak intensities of BAX in the presence of hy-TEMPO to BAX without hy-TEMPO (I_{para}/I_{dia}). Mapping of chemical shifts and PRE data onto the BAX structure was performed with PYMOL (Schrödinger, LLC). Software was made available through the SBGrid collaborative network⁴⁹.

NMR-based docking calculations and molecular dynamics

Small molecule binding sites on the BAX structure (PDBID:1F16) were identified using the program SiteMap (Schrödinger, LLC, 2017)⁵⁰. NMR-guided docking of BAI1 and BAI2 into the NMR structure of BAX (PDB ID: 1F16) was performed using GLIDE (Glide, Schrödinger, LLC, 2016) with or without constraints based on residues undergoing significant chemical shifts. BAIs were converted to 3D all atom structure using LIGPREP (Schrödinger, LLC, 2016) and assigned partial charges with EPIK (Schrödinger, LLC, 2016). BAIs were docked using the Induced fit docking mode with extra precision (XP) re-scoring. To account for potential ambiguity in the NMR data, docking was repeated using a largely extended surface of BAX to include regions surrounding the proposed BAI site including the vMIA peptide but no new poses were generated. The lowest-energy docking pose is consistent with the observed NMR-chemical shift perturbation data. The lowest energy structure pose from XP docking was selected for further analysis and subjected to a 50 ns molecular dynamics (MD) simulation using DESMOND (DESMOND, version 3, Schrödinger, LLC, 2017). The lowest energy BAX structure from the NMR ensemble (PDB

ID: 1F16) was subjected to a 50 ns MD simulation using DESMOND for comparison. MD runs were performed in truncated octahedron SPC water box using OPLS_2005 force field, 300 K and constant pressure of 1.0325 bar. Clustering and analysis of the trajectory was performed with MAESTRO tools (Schrödinger, LLC, 2018). PYMOL (Schrödinger, LLC, 2018) was used for preparing the highlighted poses.

NMR-based BAX activation assay

BAX activation and oligomerization were monitored by ^1H - ^{15}N -HSQC spectra using BAX at 50 μM and BIM SAHB_{A2} at 60 μM without or with BAI1 at 100 μM in the presence of NMR buffer plus 0.25% CHAPS to stabilize the oligomeric BAX in solution and Complete EDTA free Protease inhibitor and 0.05% NaN_3 to prevent degradation. Activation was measured by the time dependent signal loss upon addition of BIM SAHB_{A2}, for this analysis a selection of well resolved residues with high starting intensity from the core domain of the protein (L25, A42, E44, L120) were monitored and normalized to a selection of residues from the flexible N-terminus of the protein (G3, S4 and G10) which show little to no signal loss during the experiment. Normalized signal loss plotted in Prism and fit to a single exponential function. Mutations were assessed as above using a single time point experiment with 50 μM BAX D84K/D86K or V83W/L120W, preincubated with 100 μM BAI1 and treated with 60 μM BIM-SAHB_{A2} for 24 hours.

BAX oligomerization by gel filtration analysis

Solution oligomerization was analyzed by size exclusion by incubating 50 μM BAX with 60 μM BIM-SAHB_{A2} with and without 100 μM BAI1 in 50 mM potassium phosphate, 150 mM NaCl solution at pH 6.0 0.25% CHAPS, for 12 hours at room temperature. oligomerization reactions were analyzed using a superdex75 gel filtration column ran in the incubation buffer.

Hydrogen-deuterium exchange mass spectrometry.—Prior to hydrogen-deuterium exchange experiments, the quench condition for best sequence coverage of BAX was optimized as previously described^{12,51}. Briefly, 3 μl of stock solution of BAX at 1.0 mg/ml was mixed with 9 μl of H_2O buffer (8.3 mM Tris, 150 mM NaCl, in H_2O , pH7.2) at 0 °C and then quenched with 18 μl of ice cold quench solutions of 0.8% formic acid, 16% Glycerol, and GdnHCl at final concentrations of 0.05 M, 0.5 M, 1.0 M and 2.0 M. The quenched samples were frozen on dry ice and then subjected to an immobilized pepsin column (1 \times 20mm, 30 mg/ ml porcine pepsin (Sigma)) for online digestion for 40 sec. The resulting peptides were collected on a C18 trap (Michrom MAGIC C18AQ 0.2 \times 2) and separated using a reversed phase C18 column (Michrom MAGIC C18AQ 0.2 \times 50 3 μm 200 Å) with a 30 min linear gradient of 0.046% (v/v) trifluoroacetic acid, 6.4% (v/v) acetonitrile to 0.03% (v/v) trifluoroacetic acid, 38.4% (v/v) acetonitrile. The effluent was directed into an Orbitrap Elite mass spectrometer (Thermo-Fisher Sci. Inc.) for MS analysis. The instrument was operated in positive ESI mode and the resolution of the instrument was set at 60,000. Proteome Discoverer software (Thermo Fisher Sci. Inc.) was used to identify the sequence of the resulting peptides. The optimal quench condition with the best coverage map of BAX (0.08 M GdnHCl in 0.8% formic acid) was used for subsequent functionally deuterated studies. Hydrogen-deuterium exchange reactions were initiated by diluting 3 μl of

pre-chilled protein stock solution (BAX monomer, 1 mg/ml, or BAX monomer with BAI1, 2 mg/ml) into 9 μ l D₂O buffer (8.3 mM Tris, 150 mM NaCl, in D₂O, pDREAD 7.2). The samples were incubated at 0 °C for 10 sec, 100 sec and 1000 sec. The exchange reaction was terminated by the addition of 18 μ l of optimized quench solution at 0 °C and samples were immediately frozen on dry ice and stored at –80°C. In addition, un-deuterated samples and equilibrium-deuterated control samples were also prepared as previously described. The deuterated samples were then loaded onto the above described instrument for HXMS analysis. The centroids of the isotopic envelopes of undeuterated, functionally deuterated, and equilibrium deuterated peptides were measured using HDExaminer, and then converted to corresponding deuteration levels with corrections for back-exchange.

Fluorescence polarization binding assays

Fluorescence polarization assays (FPA) were performed as previously described³¹. Direct binding isotherms of vMIA were measured by incubating FITC-vMIA (50 nM) with serial dilutions of full-length BAX in the presence and absence of 100 μ M BAI1. Direct binding isotherms of BIM were measured by incubating FITC-BIM-SAHB_{A2} (25 nM) with serial dilutions of full-length BAX either wild type or with mutations. Fluorescence polarization was measured for 60 min at 10 minute intervals on a F200 PRO microplate reader (TECAN). K_d values were calculated by nonlinear regression analysis of all binding curves using Graphpad Prism software fit to a 1 to 1 binding model. Competitive binding curves for vMIA with BAI1 were simulated using the program DYNAFIT (BioKin) to simulate the competition of BAI1 with vMIA for binding to BAX using a K_d of 15 μ M and 140 nM respectively⁵².

Liposomal permeabilization assay.—Lipids (Avanti Polar Lipids) at the indicated ratio: phosphatidylcholine 48%, phosphatidylinositol 10%, dioleoyl phosphatidylserine 10%, phosphatidylethanolamine, 28%, and tetraoleoyl cardiolipin 4% were mixed in total of 4 mg, dried and resuspended in 0.2 mM EDTA, 10 mM HEPES, pH 7, 200 mM KCl, and 5 mM MgCl₂ buffer with 12.5 mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) dye and 45 mM p-xylene-bis-pyridinium bromide (DPX) quencher (Molecular Probes) using a water bath sonicator. Liposomes were formed by extrusion of the suspension using polycarbonate membranes of 0.2 μ m pore size (Avanti Polar Lipids). ANTS/DPX encapsulated liposomes were purified from non-encapsulated ANT/DPX by gel filtration over a 10-ml CL2B-Sepharose (GE Healthcare Life Sciences) gravity flow column. BAX (400 nM) was pre-incubated with the indicated concentration of BAI1 in a 96-well format (Corning) at room temperature for 30 mins prior to being mixed with liposomes (10 μ L from 50 mM total lipid stock) in assay buffer (10 mM HEPES, pH 7, 200 mM KCl, 1 mM MgCl₂) to a final volume of 90 μ l. Reactions are initiated by the addition of the indicated activator at the indicated concentrations to make a final volume of 100 μ l. ANTS/DPX release was quantified based on the increase in fluorescence intensity that occurs when the ANTS fluorophore is separated from the DPX quencher upon release from the liposomes into the supernatant. Fluorescence (λ_{ex} = 355 nm and λ_{em} = 520 nm) was measured over time at 30°C using a Tecan Infinite M1000 plate reader. In the case of heat activation, reactions were set up as normal without activating peptide and the experiments recorded at 42°C for 60–90 minutes. The percentage release of ANTS/DPX at any given time point was

calculated as percentage release = $((F - F_0)/(F_{100} - F_0)) \times 100$, where F_0 and F_{100} are baseline and maximal fluorescence, respectively. 1% Triton treatment is used to determine the maximum amount of liposomal release per assay, and this value sets the 100% value.

BAX oligomerization assay

BAX oligomerization was detected using a crosslinking approach by incubating liposomes and recombinant BAX protein (400 nM) and tBID (60 nM) with and without BAI1 for 25 minutes at 30 °C followed by incubation with 10 x Bismaleimidohexane (BMH, Pierce) for 10–30 min on ice followed by quenching with 1 mM DTT for 10 minutes. Samples were denatured at 95°C with 1 x LDS loading dye (Invitrogen) and analyzed by 4–12% NuPage (Invitrogen) gel electrophoresis followed by immunoblotting with anti-BAX yth-2d2 antibody (R&D systems).

Western blotting and protein quantification

BAX samples were electrophoretically separated on 4–12% NuPage (Invitrogen) gels, transferred to mobilon-FL PVDF membranes (Millipore) and subjected to immunoblotting. For visualization of proteins with Odyssey Infrared Imaging System (LI-COR Biosciences) membranes were blocked in PBS containing 2.5% milk powder. Primary BAX yth-2d2 antibody (R&D systems) was incubated overnight at 4°C in a 1:1,000 dilution. After washing, membranes were incubated with an IRdye800-conjugated goat anti-mouse IgG secondary antibody (LI-COR Biosciences) in a 1:5,000 dilution. Protein was detected with Odyssey Infrared Imaging System. Densitometry of protein bands were acquired using a LI-COR Odyssey® scanner. Quantification and analysis was performed using the Western Analysis tool from the Image Studio 3.1 software.

BAX conformational change assay using the anti-BH3 antibody.—Exposure of the BH3 domain of BAX was assessed by immunoprecipitation with a BAX BH3-Domain specific antibody purchased from Abgent (cat. # AP1302a). Recombinant full-length BAX (200 nM) was incubated with BIM-SAHB_{A2} (800 nM) with and without BAI1 in the presence of liposomes, in assay buffer (10 mM HEPES, 200 mM KCl, 5 mM MgCl₂, pH 7.2) for 30 min. After incubation, 1 µl of BAX BH3-domain antibody was added to every sample and mixed pre-washed protein A/G beads (Santa Cruz) and incubated for 1 hr at 4°C with rotation. Beads were collected by brief spin, washed three times with 1 mL of assay buffer, and then solubilized with 25 µl LDS/DTT loading buffer. Samples were resolved by SDS-PAGE electrophoresis and western blot analysis an anti-BAX yth-2D2 antibody (Invitrogen)

Liposomal translocation assay

Liposomes of the following lipid composition (Avanti Polar Lipids): phosphatidylcholine, 47%; phosphatidylethanolamine, 27%; phosphatidylinositol, 10%; dioleoyl phosphatidylserine, 10%; tetraoleoyl cardiolipin, 4%; and biotinyl-phosphatidylethanolamine, 2% were mixed (1 mg total) and resuspended in liposome assay buffer (10 mM HEPES, 200 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, pH 7.2). The resulting slurry was vortexed for 10 min and sonicated in a sonicating water bath at 4°C for 10 min. The solution was then passed through an Avanti Mini-Extruder Set (#61000) equipped with

a 100 nm filter, followed by passage through a CL2B Sepharose column (GE Healthcare). For the liposomal translocation assay, recombinant BAX (400 nM) is pre-incubated with varying concentrations of BAI1 with the indicated activator at the indicated concentration, in liposome assay buffer in a 96-well low binding plate (Costar) for 3 mins at room temperature prior to the addition of 10 μ l of liposome preparation to a total volume of 100 μ L. Reaction mixtures were incubated for 45 min at room temperature with shaking. Streptavidin was immobilized onto 96-well EIA/RIA plates (Corning Inc.) at 5 μ g per well (incubated at 4°C over night in PBS) and blocked for 2 hr at room temperature with 3% BSA in PBS. Liposome translocation mixtures were mixed with BSA (final concentration 0.6%) immediately prior to loading on to streptavidin coated 96-well EIA/RIA plate, and incubated for 15 min at room temperature, after which 50 μ g of biotin was added to each reaction and incubated for 5 min. Non-translocated BAX was removed by 4 washes with PBS. BAX translocation was measured using either horseradish peroxidase (HRP)-N20-anti-BAX or HRP-2D2-anti-BAX antibody conjugate (Santa Cruz sc-493 HRP or sc-20067 HRP), incubated at 1/2500 dilution in 3% BSA for 1 hr at room temperature and washed 4 times with PBS, followed by incubation with 3,3',5,5'-tetramethylbenzidine HRP substrate, quenched with 0.5 M sulphuric acid and measured at 450 nm. Each well was normalized to 100% BAX translocation induced by BAX incubated with 1% triton X and liposomes.

Translocation assay by size-exclusion chromatography

Purified BAX at 1 μ M was incubated with liposomes in the presence or absence of 0.2 μ M tBID with and without 6 μ M BAI1 for 45 minutes at 30 °C. Membrane associated BAX was separated from soluble BAX by size exclusion chromatography using a 3 ml CL-2B gravity-flow gel filtration column. In total 12 fractions of 250 μ l were collected and the presence of BAX detected by western blot analysis. Fractions corresponding to liposomes were determined from control samples containing ANTS/DPX loaded liposomes and were detected by fluorescence.

Activation Temperature Measurement

Liposomal translocation assay reactions were prepared as described above with the absence of activating peptide. Reaction mixtures were heated on a PCR heat block using a temperature gradient of 35–90 °C for 60–90 minutes. Liposomes translocation mixtures were incubated with streptavidin plates and analyzed as described above. The temperature dependent activation was used to determine the activation temperature (T_m) by fitting the response using Graphpad Prism software, fit to a Boltzmann sigmoid.

Caspase-3/7 activation and cell viability assays

WT MEFs, BAK KO MEFs and BAX KO MEFs were maintained in DMEM (Life Technologies) supplemented with 10% FBS, 100 U ml⁻¹ penicillin/streptomycin, 2 mM l-glutamine, 0.1 mM MEM nonessential amino acids, and 50 μ M β -mercaptoethanol. MEFs (1×10^4 cells/well) were seeded in 96-well opaque plates for 18–24 hours and then incubated with TNF+CHX without and with BAI1 or Z-VAD-FMK in DMEM at 37°C in a final volume of 100 μ l. Caspase-3/7 activation was measured at 8 hours by addition of the Caspase-Glo 3/7 chemiluminescence reagent in accordance with the manufacturer's protocol (Promega). Luminescence was detected by a F200 PRO microplate reader (TECAN).

Caspase assays were performed in at least triplicate. Viability assays with the BAX activator, BTSA1, were performed in OCI-AML3 cells (2.5×10^3 cells/well) seeded in 384-well white plates and incubated with indicated concentrations of BTSA1 and BAI1 or DMSO in no FBS media for 2.5 hr, followed by 10% FBS replacement to a final volume of 25 μ L. Dilutions of compounds were performed using a TECAN D300e Digital Dispenser from 10 mM stocks. Cell viability was assayed at 6 hr by addition of CellTiter-Glo according to the manufacturer's protocol (Promega), and luminescence measured using a F200 PRO microplate reader (TECAN). Viability assays were performed in at least triplicate and the data normalized to vehicle-treated control wells. IC₅₀ values were determined by nonlinear regression analysis using Prism software (Graphpad).

Mitochondrial depolarization assay

BAX^{-/-}/BAK^{-/-} MEF reconstituted with human wild type BAX were plated overnight (5,000 cells/well) in a 384-well opaque plate. After 18 hrs, the media was removed and 50 μ L of fresh media was added. Cells were treated with BAI1 (5 μ M, 2.5 μ M, 1.25 μ M) or DMSO for 2 hrs; compounds were dispensed using a TECAN D300 digital dispenser. After 2 hrs, media was removed and cells were washed 3 times with 1 XPBS. Following last wash, 1X PBS was removed from every well and 50 μ L of staining solution was added directly into the plate. The staining solution contains a final concentration of 0.02 mg/mL oligomycin, 0.05 mg/mL digitonin, 2 μ M JC-1, 0.01 M 2-mercaptoethanol dissolved in MEB solution. The MEB solution contains: 150 mM mannitol, 10 mM HEPES-KOH, 50 mM KCl, 0.02 mM EGTA, 0.02 mM EDTA, 0.1% BSA, 5 mM succinate, pH 7.5. After addition of staining solution, BIM BH3 peptide, CCCP, alamethicin and DMSO were added using TECAN D300 digital dispenser. Fluorescence was measured at 545 nm excitation and 590 nm emission using the M1000 microplate reader (TECAN) at 30°. Percentage of depolarization was calculated by normalization to the solvent-only control (0% depolarization) and the positive control CCCP (100% depolarization). Mitochondrial polarization analysis was performed in triplicates.

Data Availability

Data generated or analyzed during the study and included in this published article are available from the corresponding author on a reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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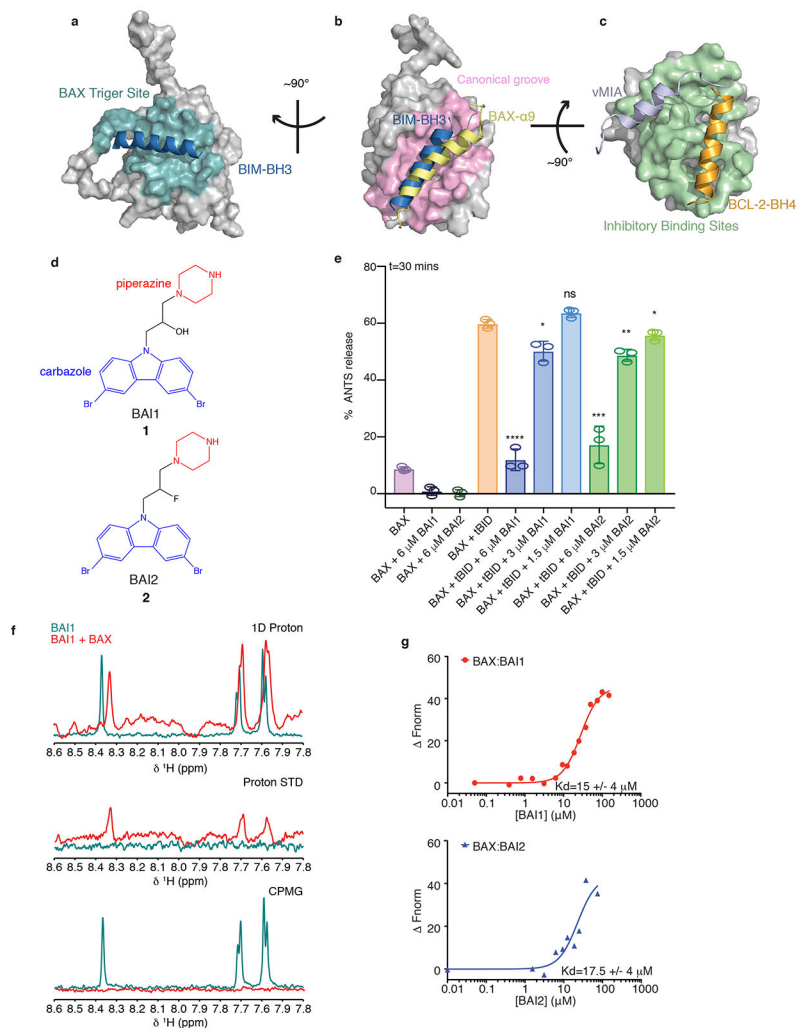


Figure 1: BIA1 and BAI2 bind directly BAX and inhibit BAX activation. Surface representation of BAX inactive monomer (grey) (PDB: 1F16) showing the location of (a) the BAX trigger site (teal) and BIM BH3 (blue) bound, (b) the canonical BH3 groove (pink) where $\alpha 9$ helix (yellow) and BIM-BH3 (blue) are bound, (c) the combined inhibitory binding site (green) where BCL-2 BH4 peptide (orange) and the vMIA peptide (violet) are bound. (d) Chemical structures of BAI1, and BAI2. The carbazole (blue) and piperazine (red) ring are labeled for clarity (e) BAX-mediated membrane permeabilization assay using liposomes with 400 nM BAX, 1.5–6 μ M BAI compounds and 30 nM tBID at t=30 minutes. Data represent mean \pm SD, n=3 replicates, data are representative of five independent experiments. Two-sided T test, **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05 ns P>0.05. (P-values were 0.000035, 0.0116, 0.0588, 0.0004, 0.00176, 0.0389, from left to right) (f) Ligand-observed 1D 1 H-NMR of the aromatic peaks of BAI1 (teal) shows chemical shift changes upon addition of inactive BAX (red). 1 H-NMR STD transfer experiment shows appearance of aromatic peaks of BAI1 in the presence of BAX (red). 1 H-NMR CPMG experiment shows peak broadening of aromatic peaks of BAI1 (teal) in the presence of BAX (red). BAX 15 μ M. BAI1 150 μ M. Data are representative of two independent experiments. (g) Microscale thermophoretic analysis of the BAX interaction with BAI1 (red) and BAI2

(blue). Single data points are recorded for each titration, data are representative of 2 technical replicates and three independent experiments.

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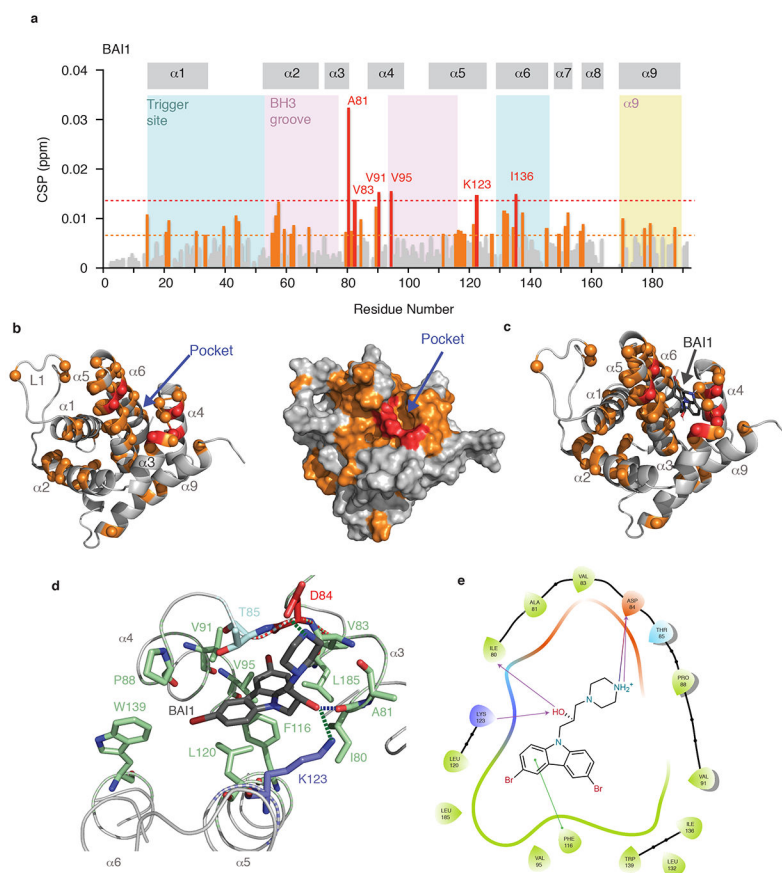


Figure 2: BAI1 binds to monomeric BAX at a novel allosteric site

(a) Measured chemical shift perturbations (CSP) of ^{15}N -BAX upon BAI1 titration up to a ratio of 1:2 BAX:BAI1, are plotted as a function of BAX residue number. Residues with chemical shift ranges over the significance threshold or 1.5 times the threshold are colored orange and red respectively. Residues associated with the canonical BH3 binding pocket and the BH3 “trigger-site” are indicated with violet and blue shading respectively, C-terminal helix $\alpha 9$ is shaded yellow. Residues showing CSP > 1.5 times the threshold are labeled for clarity. Data are representative of three independent experiments. (b) Mapping of residues undergoing significant CSP onto the ribbon and surface representation of BAX (grey) (PDB: 1F16), as in (a) in orange and red color. Residues with significant and highest CSP concentrate into a groove (blue arrow) that is formed by helices $\alpha 3$, $\alpha 4$, $\alpha 5$ and loop $\alpha 3$ – $\alpha 4$. (c) Ribbon representation of BAX and mapping of the residues undergoing significant CSP as in (b). The most energetically favorable docked structure of BAI1 calculated by the molecular docking software Glide, guided by NMR-based restraints. (d) Close-up view of the novel binding site and bound docked structure of BAI1 (grey). Favorable hydrophobic contacts, hydrogen bonds and salt-bridges between BAI1 BAX are illustrated. (e) BAX-BAI1 interaction cartoon showing key hydrophobic contacts and hydrogen bonds.

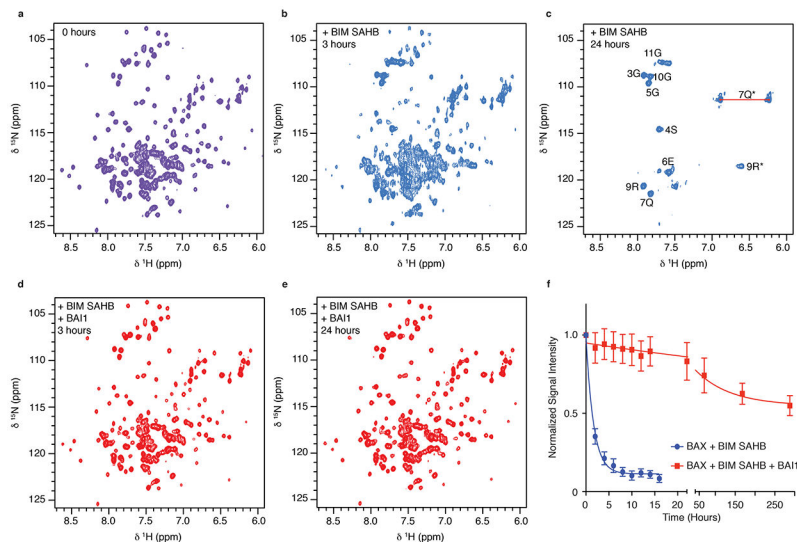


Figure 3: BAI1 allosterically inhibits BIM BH3-mediated BAX activation and oligomerization. (a-c) ^{15}N -HSQC spectra of ^{15}N -BAX, incubated with BIM SAHB at 0 hr, 3 hr, and 24 hrs. BAX activation and oligomerization was monitored by cross peaks signal loss of monomeric BAX. (d, e) ^{15}N -HSQC spectra of ^{15}N -BAX, incubated with BIM SAHB, with BAI1, at 3 hr and 24 hr. (f) NMR signal intensity of cross peaks of monomeric BAX over time for BAX incubated without (blue) and with BAI1 (red) after treatment with BIM SAHB. BAX monomer $50\ \mu\text{M}$, BAI1 $100\ \mu\text{M}$, BIM SAHB $60\ \mu\text{M}$. Data represent the mean \pm SD signal loss of 4 well resolved NMR peaks, normalized to 4 peaks with constant NMR intensity throughout the experiment. Data in (a-f) are representative of two independent experiments.

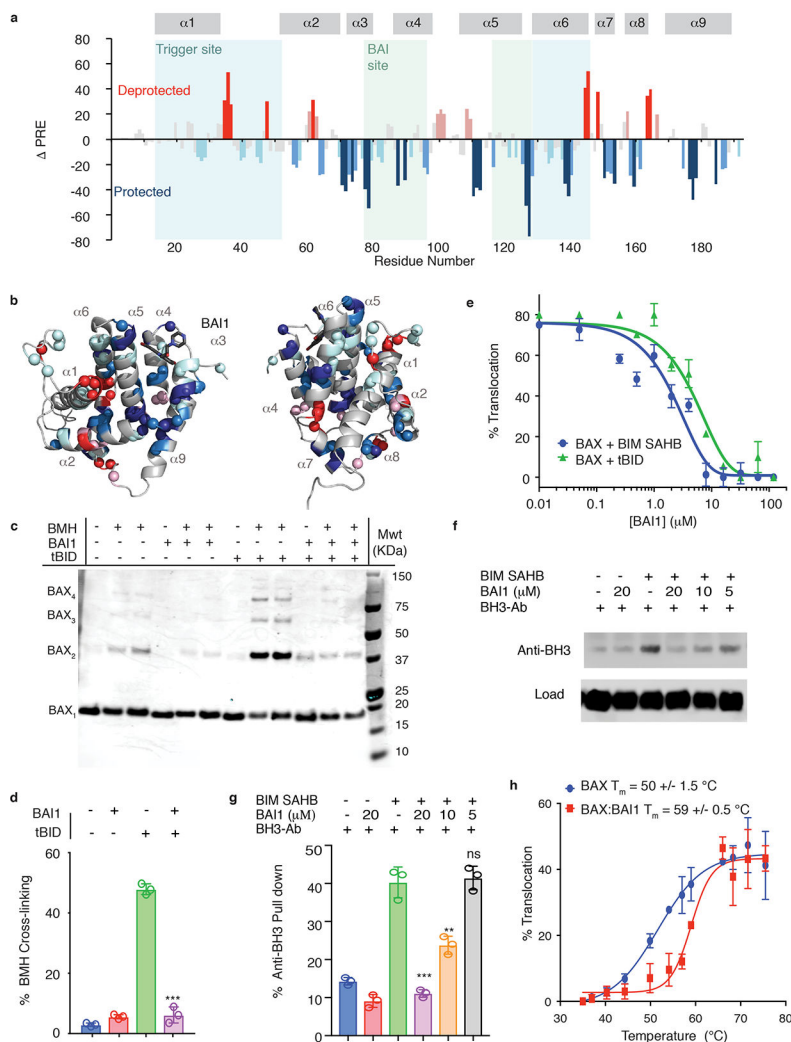


Figure 4: Structural and biochemical mechanism of BAI1-mediated BAX inhibition. (a) BAI induced differences in peak intensity ratio (PRE) of BAX cross-peaks in the presence and absence of the soluble paramagnetic agent hy-TEMPO (Iox/Ired). Residues with PRE differences over the significance threshold of 1, 2, or 3 times below the negative threshold are colored light blue, blue and dark blue respectively, and over 1 or 2 times the positive threshold are colored pink and red respectively. Residues associated with the BAI1 binding pocket and the BH3 “trigger-site” are indicated with green and blue shading respectively. (b) Ribbon representation of the lowest energy BAX:BAI1 model with mapping of backbone N atoms of the residues undergoing significant differences in PRE as in (a) in blue and red color shaded spheres. (c) BAX membrane oligomerization assay upon tBID-mediated BAX activation in the presence of liposomes with or without BAI1 measured, by BMH mediated cross-linking and analyzed by western blot analysis. (d) Quantification of western blot bands in (c) based on IR fluorescence. Data represent the mean \pm SD n=3 replicates, data are representative of 2 independent experiments (e) Quantitative BAX liposomal membrane translocation assay upon tBID-mediated (green) and BIM BH3-mediated (blue) BAX activation in the absence or presence of increasing

concentration of BAI1. Data represent mean \pm SD, n=3 replicates, data are representative of 5 independent experiments. **(f)** BIM BH3-mediated BAX activation and immunoprecipitation assay using the anti-BH3 antibody in the absence or presence of increasing concentrations of BAI1. Full blot can be found in Supplementary Figure 21 **(g)** Quantification of western blot bands in (f) based on IR fluorescence. Data represent mean \pm SD, n=3 replicates, data are representative of two independent experiments **(h)** Quantitative BAX liposomal membrane translocation assay induced by increasing temperature with and without 6 μ M BAI1, the activation temperature (T_m) and error as determined by Prism is annotated for clarity. Data represent mean \pm SD, n=3 replicates, data are representative of two independent experiments. Two-sided T test, **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05 ns P >0.05 for BAI1 treated compared to tBID or BIM alone (P-values were 0.061 for (d) and , 0.00047, 0.00469, 0.999 for (g) from left to right).

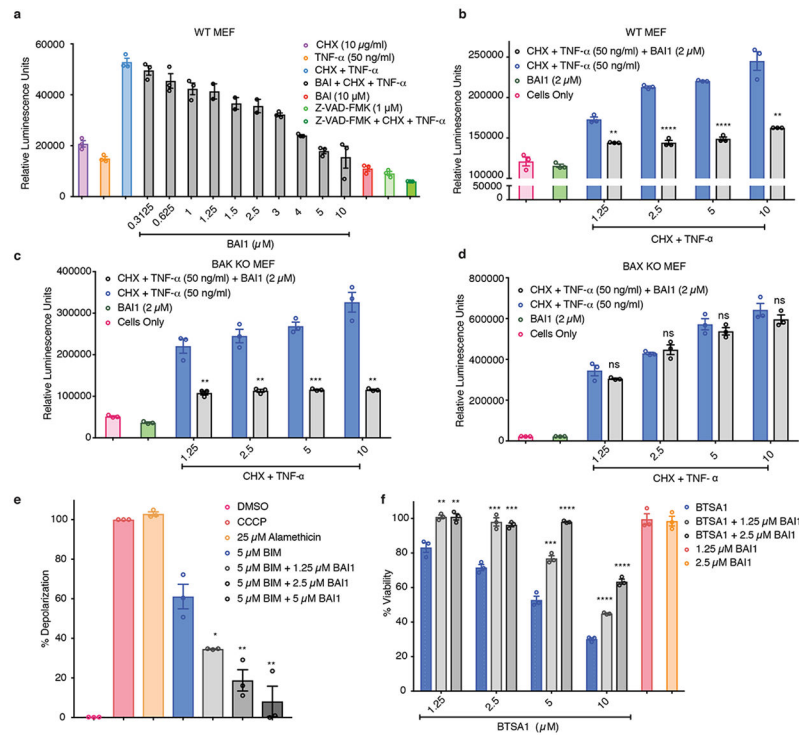


Figure 5: BAI1 inhibits cell death in BAX-dependent manner.

(a) Caspase 3/7 assay of WT MEFs upon pro-apoptotic TNF α /CHX treatment in the absence or presence of co-treatment with BAI1 at indicated doses for 8 hours. (b-d) Caspase 3/7 assay of WT MEFs, BAX KO MEFs and BAK KO MEFs upon pro-apoptotic TNF α /CHX treatment in the absence or presence of co-treatment with BAI1 at 2 μ M for 8 hours. (e) Mitochondrial depolarization measured by JC-1 staining of BAX/BAK DKO MEFs reconstituted with human BAX treated with 5 μ M BIM BH3 in the absence or presence of BAI1 at indicated doses. Data collected at 75 min after JC-1 staining. (f) Viability assay of OCI-AML3 cells upon 6 hr treatment with a BAX direct activator BTS1A1, in the absence or presence of BAI1 at indicated doses. Data represent mean \pm SD, n=3 replicates, data are representative of three independent experiments. Two-sided T test, **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05 ns P >0.05 for BAI1 treatment compared to apoptotic stimulus or BAX activator treatment. (P-values were 0.000538, 0.000023, 0.000006 and 0.0019 for (b), 0.00315, 0.00134, 0.0001, and 0.00089 for (c), 0.194, 0.495, 0.351 and 0.292 for (d), 0.01296, 0.0067 and 0.0058 for (e) and 0.0042, 0.0061, 0.00088, 0.00028, 0.00095, 0.000062 and 0.000036 for (f) from left to right).