B Cell Lymphoma-2 (BCL-2) Homology Domain 3 (BH3) Mimetics Demonstrate Differential Activities Dependent upon the Functional Repertoire of Pro- and Anti-apoptotic BCL-2 Family Proteins*

The B cell lymphoma-2 (BCL-2) family is the key mediator of cellular sensitivity to apoptosis during pharmacological interventions for numerous human pathologies, including cancer. There is tremendous interest to understand how the proapoptotic BCL-2 effector members (e.g. BCL-2-associated X protein, BAX) cooperate with the BCL-2 homology domain only (BH3-only) subclass (e.g. BCL-2 interacting mediator of death, BIM; BCL-2 interacting-domain death agonist, BID) to induce mitochondrial outer membrane permeabilization (MOMP) and apoptosis and whether these mechanisms may be pharmacologically exploited to enhance the killing of cancer cells. Indeed, small molecule inhibitors of the anti-apoptotic BCL-2 family members have been designed rationally. However, the success of these “BH3 mimetics” in the clinic has been limited, likely due to an incomplete understanding of how these drugs function in the presence of multiple BCL-2 family members. To increase our mechanistic understanding of how BH3 mimetics cooperate with multiple BCL-2 family members in vitro, we directly compared the activity of several BH3-mimetic compounds (i.e. ABT-263, ABT-737, GX15-070, HA14.1, TW-37) in biochemically defined large unilamellar vesicle model systems that faithfully recapitulate BAX-dependent mitochondrial outer membrane permeabilization. Our investigations revealed that the presence of BAX, BID, and BIM differentially regulated the ability of BH3 mimetics to derepress proapoptotic molecules from anti-apoptotic proteins. Using mitochondria loaded with fluorescent BH3 peptides and cells treated with inducers of cell death, these differences were supported. Together, these data suggest that although the presence of anti-apoptotic BCL-2 proteins primarily dictates cellular sensitivity to BH3 mimetics, additional specificity is conferred by proapoptotic BCL-2 proteins.

Functional cooperation between multiple B cell lymphoma-2 (BCL-2) members is essential to commit a stressed cell to the mitochondrial pathway of apoptosis (1). The BCL-2 family is comprised of nearly 20 proteins that are divided into anti-apoptotic and proapoptotic members. The anti-apoptotic members (e.g. BCL-2-related protein A1, A1; B-cell lymphoma extra large, BCL-xL; and myeloid cell leukemia sequence 1, MCL-1) preserve the integrity of the outer mitochondrial membrane (OMM) by preventing proapoptotic BCL-2 proteins from forming proteolipid pores within the OMM (1–3). The process of pore formation is referred to as mitochondrial outer membrane permeabilization (MOMP), and this is a key initiating event that precipitates the release of mitochondrial factors that promote caspase activation and the hallmark features of apoptosis (3).

The proapoptotic BCL-2 proteins are further divided into two subclasses: the “effectors” and “BH3-only” members (1). The effectors (e.g. BCL-2-associated X protein, BAX) are responsible for inducing MOMP, yet they require an activation

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‡ The abbreviations used are: BCL-2, B cell lymphoma 2; A1, BCL-2-related protein A1; BAK, BCL-2 homologous antagonist killer; BAX, BCL-2-associated X protein; BCL-xL, B-cell lymphoma extra large; BH3, BCL-2 homology domain 3; BID, BH3 interacting domain death agonist; BIM, BCL-2 interacting mediator of cell death; DTT, dithiothreitol; FAM, carboxyfluorescein; LUV, large unilamellar vesicles; MCL-1, myeloid cell leukemia sequence 1; OMM, outer mitochondrial membrane; PUMA, p53-up-regulated modulator of apoptosis; TAMRA, tetramethylrhodamine; z, benzylxoycarbonyl; fmk, fluoromethyl ketone; MOMP, mitochondrial outer membrane permeabilization.
signal that is provided by transient interactions with the “direct activator” BH3-only proteins, such as BID (BCL-2-interacting domain death agonist) or BIM (BCL-2 interacting mediator of death) (4, 5). The remaining “sensitizer/derepressor” BH3-only proteins, such as BAD (BCL-2-associated death promoter) and PUMA (p53 up-regulated modulator of apoptosis) regulate the availability of direct activators by preventing BID/BIM association with the anti-apoptotic BCL-2 proteins (6–10). Together, the inhibition of the anti-apoptotic BCL-2 proteins by sensitizer/derepressor BH3-only proteins coupled with the induction of direct activators sets the pace by which MOMP and apoptosis will proceed (2).

The relatively recent development of small molecules that directly inhibit the anti-apoptotic BCL-2 members allows for the pharmacological regulation of MOMP and apoptosis (11–13). These molecules, termed BH3 mimetics, function similar to the sensitizer/derepressor BH3-only proteins by binding within the hydrophobic groove of anti-apoptotic proteins (12, 14, 15). In some cases, this association is sufficient to promote apoptosis if the treated cell harbors covert direct activator BH3-only proteins; this is exemplified by several white blood cell tumors that constitutively suppress BIM function via BCL-2/BCL-xL (16–18). However, the utility of BH3 mimetics as single agents and in combination strategies for the treatment of solid tumors remains under investigation in the laboratory and clinic.

Over the years, a panel of BH3 mimetic drugs (e.g. ABT-263, ABT-737, HA14.1, GX15-070, and TW-37) have been developed, and each demonstrate unique specificity for the anti-apoptotic BCL-2 members (13, 19–24). However, very little is known about how the presence of various pro-apoptotic BCL-2 proteins impacts upon BH3 mimetic function. Here, we directly examined the relationships between BH3 mimetics and various BCL-2 family protein functional complexes and reveal that proapoptotic BCL-2 proteins impose additional specificities for responses to these drugs.

**EXPERIMENTAL PROCEDURES**

**Reagents—**All cell culture and transfection reagents were from Invitrogen; and standard reagents were from Fisher Scientific unless indicated otherwise. Drugs were from the following companies: ABT-737 (Abbott Pharmaceuticals), ABT-263/HA14.1/GX15-070/TW-37 (Selleck), and thapsigargin/DTT (Sigma). Antibodies used for this study were as follows (clone): HA14.1/GX15-070/TW-37 (Selleck), and thapsigargin/DTT ing companies: ABT-737 (Abbott Pharmaceuticals), ABT-263/HA14.1/GX15-070/TW-37 (Selleck), and thapsigargin/DTT (Sigma). Antibodies used for this study were as follows (clone): HA14.1/GX15-070/TW-37 (Selleck), and thapsigargin/DTT

**Cell Culture and Apoptosis Assays—**HeLa cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. For cell death studies, cells were seeded for 24 h and treated as described, and floating and attached cells were harvested and stained with annexin V-FITC in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) and analyzed by flow cytometry as indicated (26).

**Heavy Membrane Isolations and Cytochrome c Release Assays—**At least 2 × 15 cm dishes at 90–95% confluency were used per treatment. Cells were harvested by trypsinization and pelleted by centrifugation at 1000 × g for 10 min. The cell pellet was washed once with mitochondrial isolation buffer (MIB; 200 mM mannitol, 68 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 0.1% BSA) and resuspended in MIB supplemented with protease inhibitors (HALT, Pierce Biotechnology). The cell suspension was incubated on ice for 20 min and homogenized using a 2-mL Potter-Elvehjem dounce. The homogenate was centrifuged for 10 min at 800 × g at 4 °C, and the supernatant was collected and centrifuged again using the same conditions to ensure that no unlysed cells or nuclei were present. The resulting supernatant was centrifuged for 10 min at 8000 × g at 4 °C. The pellet was collected as the heavy membrane (i.e. mitochondrial) fraction.

For liver mitochondrial isolations, we employed a common protocol (27). Heavy membrane fractions (referred to as mitochondria) were purified from murine liver, usually female, under 3 months, using dounce homogenization and differential centrifugation in mitochondrial isolation buffer (MIB; 200 mM mannitol, 68 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 0.1% BSA). For MOMP assays, mitochondria were incubated in MIB supplemented to 110 mM KCl (mitochondrial assay buffer), and indicated peptides and drugs were added for 30 min at 37 °C. Total cytochrome c was determined by a sample containing mitochondria solubilized in 1% CHAPS. Reactions were then fractionated into supernatant and pellet by centrifugation at 5500 × g for 5 min, and the supernatant was analyzed by SDS-PAGE and Western blot for cytochrome c. Quantification of cytochrome c release was performed by ImageJ software (NIH) and normalized to CHAPS.

**BID ΔFAM/BIM ΔFAM Release Assays—**Isolated mitochondria (50 μg) were incubated with 50 or 100 nM peptide for 30 min at 37 °C, pelleted at 8000 × g at 25 °C, resuspended in MIB supplemented with KCl (to 120 mM) and indicated BH3 mimetic or PUMA BH3 peptide (5 μM), incubated for an additional 30 min at 37 °C, pelleted at 8000 × g at 25 °C, and lysed in 100 μl of radioimmune precipitation assay buffer, and the fluorescence was detected using a Synergy H1 hybrid multi-mode microplate reader (BioTek) (excitation wavelength, 494 nm; emission wavelength, 521 nm).

**BAX Activation Assays—**For 6A7 studies, cells were treated as indicated, trypsinized, pelleted, lysed in 6A7 amino-terminal capture buffer (10 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM MgCl₂, 0.2 mM EDTA, 1% glycerol + 1% CHAPS, added fresh) incubated on ice for 10 min, and centrifuged at 20,000 × g for 10 min at 4 °C. The supernatants were combined with 2 μg of 6A7 antibody, incubated for 12 h at 4 °C with end/end mixing, an
appropriate volume of protein A/G-agarose conjugate was added, and incubated for an additional hour. The beads were washed with each of the following buffers twice, 1 ml per wash: 1) wash A consisted of 10 mM HEPES, pH 7.4, 135 mM NaCl, 2% CHAPS; pH 7.4; 2) wash B consisted of 10 mM HEPES, pH 3, 135 mM NaCl, 0.2% CHAPS) wash C consisted of 100 mM Tris HCl, pH 8.0, 100 mM NaCl. Proteins are eluted by the addition of 1× SDS-PAGE loading buffer (50 μl), denatured for 10 min at 95 °C, centrifuged for 1 min at 15,000 × g, and the supernatant was subjected to SDS-PAGE and Western blot analysis using clone N-20 for BAX detection.

Western Blot Analyses—Whole cell protein lysates were made from trypsinized cells, pelleted, resuspended in radioimmuneprecipitation assay buffer supplemented with protease inhibitors, incubated on ice for 10 min, and centrifuged for 10 min at 21,000 × g. Protein concentrations were determined by a standard BCA kit, and the lysates were then adjusted with radioimmuneprecipitation assay buffer to equal the protein concentrations. Proteins (50–100 μg/lane) were subjected to SDS-PAGE before transferring to nitrocellulose by standard Western conditions, and blocked in 5% milk/TBST and primary antibodies (1:1000 in blocking buffer) were incubated overnight at 4 °C. The secondary antibody (1:5000 in blocking buffer) was incubated at 25 °C for 1 h before standard enhanced chemiluminescence detection.

Large Unilamellar Vesicle Assays—Large unilamellar vesicle (LUV) release assays were prepared as described (5, 28). Briefly, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and cardiolipin at a ratio of 47:28:9:9:7 (4 mg total) were dried and resuspended in buffer (LUV) release assays were prepared as described (5, 28). Briefly, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and cardiolipin at a ratio of 47:28:9:9:7 (4 mg total) were dried and resuspended in buffer (0.2 mM EDTA, 10 mM HEPES, pH 7, 200 mM KCl, and 5 mM MgCl₂) containing a polyanionic dye (12.5 mM 8-aminonaphthalene-1,3,6-trisulfonic acid) and cationic quencher (45 mM p-xylene-bis-pyridinium bromide) using a water bath sonicator. Unilamellar vesicles were formed by extrusion of the suspension through indicated polycarbonate membranes (1.0 μm; Avanti). The unincorporated p-xylene-bis-pyridinium bromide and 8-aminonaphthalene-1,3,6-trisulfonic acid were removed by using a 10-ml Sepharose S-500 gravity flow column. Using a 96-well format, 100 μl of total volume per condition, LUVs, proteins, peptides, drugs, and buffers were combined as indicated, incubated for 45 min at 37 °C, and analyzed for fluorescence using a Synergy H1 hybrid multi-mode microplate reader (excitation wavelength, 355 nm; emission wavelength, 520 nm; photomultiplier tube gain, 50). For competition experiments, A1, BCL-xL, or MCL-1 was combined with BID⁰ TAMRA or BIM³ TAMRA peptides for 10 min, ABT-737 (20 μM) was added and incubated for an additional 60 min at 25 °C, and the fluorescence was measured. Higher concentrations of drugs are required in the FP assays due to the absence of LUVs. Maximal depolarization was set to the ABT-737 and BCL-xL combination, as ABT-737 was designed for BCL-xL binding (12).

RESULTS
To determine how BH3 mimetic responses are influenced by proapoptotic BCL-2 proteins, we employed a biochemically defined LUV model system that faithfully recapitulates BAX-dependent MOMP (5, 28). These LUVs are comprised of purified lipids in ratios similar to the composition of the OMM, yet in the absence of additional proteins, allow for BAX to activate, oligomerize, and permeabilize the LUV membrane (6). To examine the lowest concentrations of proapoptotic and antiapoptotic proteins required within the LUV system, we first titrated BAX (human, full-length, and untagged), in the absence and presence of BID or BIM. BAX alone demonstrated minimal, yet dose-dependent LUV permeabilization (Fig. 1A); and the presence of BID (Fig. 1B) or BIM (Fig. 1C) synergized with BAX to promote maximal dose-dependent LUV permeabilization. Based on these data, we used BAX at concentrations of 100 and 25 nm for evaluating BAX alone and in synergy with BID/ BIM, respectively, in subsequent experiments. Furthermore, 50 nM BID/BIM were determined to optimally synergize with BAX, with negligible increases in permeabilization up to 100 nM BID/BIM (Fig. 1, B–D). “BID” and “BIM” proteins are human
full-length N/C-BID and the short isoform of BIM (BIM-S), respectively (29).

Independent of direct activator proteins, high concentrations of BAX will lead to LUV permeabilization such as 100 nM BAX (Fig. 1A), due to unavoidable spontaneous activation during recombinant protein expression and/or purification. This effect can be inhibited in a dose-dependent manner by co-incubation with anti-apoptotic proteins such as A1, BCL-xL, or MCL-1 (Fig. 2A), which directly bind to active BAX. We next evaluated a panel of BH3 mimetics for their ability to derepress BAX from A1, BCL-xL, and MCL-1. These BH3 mimetics were chosen due to their common usage within the laboratory and/or clinical potential; their $K_i$ values for A1, BCL-xL, and MCL-1 are shown (Fig. 2B). Importantly, these molecules alone do not demonstrate auto-fluorescence, permeabilization activity alone, or synergy with BAX within the LUV model system (Fig. 2, C and D). When combined with BAX/A1 (Fig. 2E), BAX/BCL-xL (Fig. 2F), or BAX/MCL-1 (Fig. 2G), each BH3 mimetic produced a unique derepression profile for the BAX/anti-apoptotic BCL-2 protein combinations that is not explainable based on the $K_i$ values for anti-apoptotic BCL-2 proteins alone.

To determine whether these unexpected observations were specific to BAX/anti-apoptotic BCL-2 protein combinations, we evaluated the derepression activity of the same BH3 mimetic drug panel on the inhibitory functions of anti-apoptotic BCL-2 proteins on BID-induced BAX activation. In this scenario, low concentrations of BID (50 nM) and BAX (25 nM) synergize to permeabilize LUVs (Fig. 3A), and anti-apoptotic BCL-2 proteins inhibit this activity in a dose-dependent manner (50, 100, 175, and 250 nM) (Fig. 3A). The addition of BH3 mimetics to the BAX/BID/anti-apoptotic combinations led to differential derepression, which was dependent upon the anti-apoptotic BCL-2 within the assay. However, the derepression profiles for BAX alone (Fig. 2, D–F) differed for BID/BAX (Fig. 3, B–D), suggesting that proapoptotic BCL-2 proteins influence BH3 mimetic function.

In Fig. 2, we analyzed high concentrations of BAX, whereas in Fig. 3, a low concentration of BAX in the presence of BID was used, and these different activation scenarios could introduce unknown variation. Therefore, to compare more equivalent BAX activation scenarios, we next tested the derepression activity of the BH3 mimetic drug panel on the inhibitory func-
tions of anti-apoptotic BCL-2 proteins on BIM-induced BAX activation. In this scenario, low concentrations of BIM (50 nm) and BAX (25 nm) synergize to permeabilize LUVs (Fig. 4A), and anti-apoptotic BCL-2 proteins inhibit this activity in a dose-dependent manner (50, 100, 175, 250 nm) (Fig. 4A). Indeed, another unique derepression profile (Fig. 4, B–D) was obtained for these drugs, and interestingly, the profile differed markedly from BID-induced BAX activation (Fig. 3, B–D). For example, ABT-737 displayed marked differences for BID- and BIM-mediated derepression from A1.

The previous experiments were performed in biochemically defined conditions; we next determined whether these biochemical observations were relevant in cultured cells and isolated primary mitochondria. To generate a cellular system that harbors activated BID, we cultured HeLa cells with low levels of TNFα (0, 0.1, 0.5 ng/ml) for 7 days (8). Full-length BID is 22 kDa, cytosolic, and inactive (i.e., BID-p22) (30, 31). When proapoptotic signaling is initiated via death receptors (e.g., tumor necrosis factor receptor 1) due to the presentation of death ligands (e.g., TNFα), caspase-8 is activated, which leads to proteolytic processing of BID into an active 15-kDa fragment (i.e., BID-p15) (32). BID-p15 is rapidly sequestered by anti-apoptotic proteins on the OMM and may be pharmacologically liberated to induce apoptosis (8). We next determined whether the availability of BID-p15 cooperated with BH3 mimetics to induce apoptosis (8). We next determined whether the availability of BID-p15 cooperated with BH3 mimetics to induce apoptosis (8). We next determined whether the availability of BID-p15 cooperated with BH3 mimetics to induce apoptosis (8). We next determined whether the availability of BID-p15 cooperated with BH3 mimetics to induce apoptosis (8).

HeLa cells cultured with TNFα (0, 0.1, 0.5 ng/ml) were generated as described above, treated with indicated BH3 mimetics (5 μM) for 8 h, stained with annexin V, and analyzed by flow cytometry for apoptosis. Note that GX15-070 treatment alone promoted cell death that was independent of caspase activation at the concentration required for appropriate comparisons with the other BH3 mimetics (data not shown), so we excluded this drug from cellular apoptosis studies. All doses of TNFα and the BH3 mimetics (5 μM) failed to induce detectable apoptosis as single agents (Fig. 5, A and B), but the presence of ABT-263/ABT-737 induced massive apoptosis in all TNFα treatments. In contrast, HA14.1 and TW-37 revealed an apoptotic response that was dependent upon the higher TNFα concentration (Fig. 5A, comparing 0.1 and 0.5 ng/ml TNFα). Indeed, all cellular responses to BH3 mimetics were apoptosis, as the co-treatment with zVAD-fmk, a pan-caspase inhibitor, prevented the majority of cell death (Fig. 5B).

The observation that HA14.1 and TW-37 synergized with the higher dose of TNFα (and by extension, BID-p15) suggested that these drugs function better when BID-p15 is abundant. We isolated whole cell lysates from TNFα-cultured HeLa cells and observed that although BID-p22 expression does not change during treatments, the accumulation of BID-p15 did increase from low (0.1 ng/ml) to high (0.5 ng/ml) concentrations of TNFα (Fig. 5C). Furthermore, heavy membrane fractions (commonly referred to as “mitochondria”) were purified from the TNFα-cultured cells treated with the BH3 mimetic panel for 4 h, lysed, and assayed for associated BID-p15. Indeed, ABT-263/ABT-737 treatment completely removed BID-p15 from the heavy membrane fraction independent of TNFα concentration (Fig. 5D), which contrasted to HA14.1 and TW-37 (Fig. 5D). These drugs removed BID-p15 only in the 0.5 ng/ml TNFα treatment, which paralleled the apoptosis results in Fig. 5A.

BH3 mimetic regulated displacement of BID-p15 from the heavy membrane fraction should promote BAX activation. To determine whether BAX was activated, the TNFα-cultured cells were treated with the BH3 mimetic panel for 12 h and lysed in CHAPS buffer to preserve BAX conformations, and BAX was immunoprecipitated using an antibody (clone 6A7) that specifically recognizes the active conformation of BAX (33). Indeed,
The apoptosis and displacement results in Fig. 5, A and D, correlated with 6A7-positive BAX (Fig. 5E), whereas the expression of BAX remained consistent for all treatments (Fig. 5E).

To directly examine the ability of BH3 mimetics to release activated BID in a BID concentration-dependent manner, we loaded the anti-apoptotic BCL-2 repertoire on freshly isolated primary liver mitochondria with chemically synthesized, fluorescently labeled BID BH3 domain peptide (BIDFAM), which functions akin to BID-p15 (6). The BID BH3 domain peptide is the minimal region required to bind anti-apoptotic BCL-2 proteins (6, 7), and we quantified the extent of BIDFAM release by the BH3 mimetic panel. As with the TNFα treatments, we examined low and high levels of BIDFAM on mitochondria, 50 and 100 nM. We do not infer that these concentrations represent the physiological levels of BID-p15, but rather, they generate low and high levels of BIDFAM on mitochondria (Fig. 5F). The relative fluorescent units that bind to the OMM represent only a few percent of the total added peptide. Mitochondria activated BID in a BID concentration-dependent manner, we determined the remaining mitochondria-associated fluorescence. Whereas ABT-263 and ABT-737 released the majority of BIDFAM independent of BIDFAM concentration, HA14.1, GX15-070, and TW-37 efficiently displaced BIDFAM from the OMM only when BIDFAM was at high concentrations (Fig. 5G), which also paralleled the cell death responses in Fig. 5A. Importantly, the release of BIDFAM always paralleled cytochrome c release (Fig. 5H). The PUMA BH3 domain peptide demonstrates high affinity (−1 nM) for all members of the anti-apoptotic BCL-2 repertoire (34) and serves as a potent positive control for the release of BIDFAM (and BIMFAM, described below).

Next, we established similar model systems for BIM by examining BIMFAM loaded mitochondria, and BIM-dependent apoptosis induced by the unfolded protein response. Mitochondria with low and high BIDFAM levels (Fig. 6A) were then treated with the BH3 mimetic panel (5 μM) for 30 min at 37 °C, before determining the remaining mitochondria-associated fluorescence (Fig. 6B). In contrast to BIDFAM, ABT-263 and ABT-737 released BIMFAM in a BIMFAM concentration-dependent manner. HA14.1 and GX15-070 treatments released only a minority of BIMFAM, and TW-37 removed ~50% of BIMFAM (Fig. 6B). Importantly, the release of BIMFAM always paralleled cytochrome c release (Fig. 6C).

In cells treated with DTT (25 mM) and thapsigargin (4 μM), two inducers of the unfolded protein response that require BIM for apoptosis (35), ABT-263/ABT-737 promoted DTT- and thapsigargin-induced cell death (Fig. 6, D and E), whereas HA14.1 and TW-37 did not (Fig. 6, D and E). Similar to the BIMFAM release in Fig. 6B, increasing the DTT (5 mM) and thapsigargin (8 μM) treatments lead to more BIM expression (Fig. 6F) and dose-dependent synergy with ABT-263 and ABT-737 (Fig. 6, G and H). In contrast, HA14.1 and TW-37 remained ineffective in promoting apoptosis (Fig. 6, G and H).

Subsequently, we isolated mitochondria from cells treated with endoplasmic reticulum stress agents with or without the BH3 mimetic panel and performed Western blot analyses to determine endogenous BIM association. DTT (5 mM) and thapsigargin (8 μM) treatments led to more BIM expression (Fig. 6F) and dose-dependent synergy with ABT-263 and ABT-737 (Fig. 6, G and H). In contrast, HA14.1 and TW-37 failed to promote detectable release of BIM (Fig. 6F).

Finally, we sought to reveal a mechanism by which BH3-only proteins dictate specificity to BH3 mimetics. To determine this, we employed a fluorescence polarization approach that allowed us to monitor binding and release of TAMRA-labeled BID and BIM BH3 domain peptides (BIDTAMRA and BIMTAMRA) from

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**FIGURE 4.** BH3 mimetics display a derepression activity profile that differs for BIM-regulated BAX activation in the presence of A1, BCL-xL, and MCL-1. A, BAX (25 nM) and BIM (50 nM) were combined with anti-apoptotic proteins (A1, BCL-xL, or MCL-1; 50, 100, 175, 250 nM) for 30 min at 37 °C. B–D, BAX (25 nM), BIM (50 nM), anti-apoptotic BCL-2 proteins (A1, BCL-xL, or MCL-1; 250 nM) and BH3 mimetics (5 μM) for 30 min at 37 °C. The PUMA BH3 domain peptide is a positive control for derepression (5 μM). All data are representative of at least triplicate experiments and are reported as ± S.D. as required.
anti-apoptotic BCL-2 proteins in the same sample. BID^TAMRA and BIM^TAMRA peptides incubated with increasing concentrations of anti-apoptotic BCL-2 proteins (A1, BCL-xL, MCL-1; 0, 20, 50, 100, 200, 500, 1000, 2000, 5000 nM) undergo rapid and dose-dependent polarization (Fig. 7A).

After polarization, ABT-737 was added to promote the depolarization of BID^TAMRA and BIM^TAMRA, which is a direct measure of release from the anti-apoptotic BCL-2 protein. We narrowed our experiments to ABT-737 for four reasons: 1) this drug is widely used in the literature; 2)
served as a backbone for subsequent BH3 mimetics used in the clinic; 3) LUV, mitochondrial, and cellular responses to ABT-737 tend to be complete; and 4) including all of the drugs would require nearly 50 anti-apoptotic BCL-2/BH3 peptide/drug combinations to examine. As shown in Fig. 7B, ABT-737 differentially displaced BID<sub>TAMRA</sub> and BIM<sub>TAMRA</sub> from anti-apoptotic proteins, indicating that BH3 domains directly influence the binding of BH3 mimetics to their anti-apoptotic BCL-2 targets. The % depolarization is shown in the top panel; and the same results were normalized to release from BCL-xL as ABT-737 was originally designed to target BCL-xL (Fig. 7B). Together, these data suggest that each drug within the BH3 mimetic panel displays unique abilities to derepress BID- and BIM-dependent apoptosis and that the presence of proapoptotic BCL-2 members influences the success of BH3 mimetics to promote apoptosis.

DISCUSSION

In the current work, we identified that BH3 mimetic drugs promote differential activities that are directly influenced by the presence of proapoptotic BCL-2 proteins. Throughout most of the BH3 mimetic literature, the mechanisms of action...
for these drugs focus almost exclusively on their anti-apoptotic targets. Although this is certainly important, an overlooked additional regulator of BH3 mimetic function, as shown here, is which proapoptotic BCL-2 protein is associated with the anti-apoptotic BCL-2 protein. Although we have not examined all BH3 mimetics, our representative panel and data set suggest that defining anti-apoptotic/proapoptotic BCL-2 protein complexes is important to understanding this class of promising therapeutics.

Our data present several interesting observations. First, the literature does not provide a clear understanding of how all of the BH3 mimetics investigated within this study biophysically interact with the anti-apoptotic BCL-2 members A1, BCL-xL, or MCL-1 (Fig. 2B). However, the data presented throughout Figs. 2–7 and summarized in Fig. 7, C–F, provide evidence that functional interactions between the BH3 mimetics and anti-apoptotic targets indeed occur. For example, we cannot locate biophysical data supporting interactions for HA14.1 or TW-37 with A1, yet these drugs function to derepress proapoptotic molecules from A1 (Figs. 2E and 7D).

Second, these data provide a curious glimpse into the impact of proapoptotic BCL-2 proteins in successful derepression mediated by BH3 mimetics. For example, several cancers are dependent upon MCL-1 for survival (36, 37), and ABT-263 is described to not mediate MCL-1 inhibition. However, when MCL-1 is in complex with BAX or BIM, we detect derepression activity, but no activity is observable when BID is sequestered by MCL-1. Additionally, HA14.1 is not described to neutralize
MCL-1, yet we observed a derepressor activity for HA14.1 that is increasingly enhanced by BAX, BID, and BIM. These unpredicted activities could be mediated by the drug’s apparent $K_d$ for apo-MCL-1 or perhaps by conformational changes that occur when MCL-1 is complex with BAX, BID, or BIM that increase the affinity between MCL-1 and HA14.1. As an aside, our results are not exclusive to BAX, as BAK-dependent permeabilization demonstrated similar derepression patterns following treatment with the BH3 mimic panel (Figs. 5H and 6C).

Finally, the utility of BH3 mimetics as single agents remains to be explored and may be restricted to cells that constitutively harbor covert direct activators, promoting their release, and subsequent cell death (16–18, 38). Yet, our studies suggest that the clinical success of BH3 mimetics in combination strategies may be influenced by the following: 1) which anti-apoptotic BCL-2 proteins are expressed within the tumor cells and 2) which BH3-only protein is induced and/or activated by the other drugs within the treatment strategy. For example, a chemotherapeutic agent that promotes BIM sequestration on BCL-xL would not complement TW-37, as TW-37 preferentially disrupts BCL-xL/BID (Fig. 3C) complexes instead of BCL-xL/BIM (Fig. 4C) (38). Indeed, the complex nature of tumors that express multiple anti-apoptotic proteins surely complicates predictions, and the dynamic expression of anti-apoptotic and proapoptotic BCL-2 proteins before and after exposure to chemotherapeutics presents daunting challenges, but as clinical technologies improve to parse these events, mechanistic insights into the pathways that commit a cell to death will surely be useful.

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