**Cell Death &
Differentiation**

ARTICLE

BH3 profiling discriminates on-target small molecule BH3 mimetics from putative mimetics

Mariana Villalobos-Ortiz[1] · Jeremy Ryan [1] · Thelma N. Mashaka[1] · Joseph T. Opferman[2] · Anthony Letai[1,3,4,5]

Abstract

Inhibition of the anti-apoptotic machinery of cancer cells is a promising therapeutic approach that has driven the development of an important class of compounds termed "BH3 mimetics". These novel small molecules mimic BH3-only proteins by antagonizing the pro-survival function of anti-apoptotic proteins, thereby inducing apoptosis in cancer cells. To qualify as an authentic BH3 mimetic, a compound must function directly on the mitochondria of a cell of known anti-apoptotic dependence, must directly and selectively inhibit the anti-apoptotic protein with high-affinity binding, and must induce mitochondrial outer membrane permeabilization (MOMP) and apoptosis in a BAX/BAK-dependent manner. While many BH3 mimetics have entered clinical trials, the lack of a reliable validation assay to directly test the mitochondrial activity of new BH3 mimetic candidates has resulted in many misleading reports of agents touted as BH3 mimetics despite their off-target mechanisms of action. BH3 profiling probes the activity of a compound at the mitochondrial level by measuring cytochrome c release as a surrogate marker for MOMP. We propose a comprehensive biochemical toolkit consisting of BH3 profiling in parallel with high-throughput Annexin V/Hoechst viability testing to validate BH3 mimetic candidates. We tested our toolkit on eighteen different putative BH3 mimetics using a set of standardized cell lines of known anti-apoptotic dependence. Included in this set of cell lines is an apoptosis refractory BAX/BAK DKO control line to detect compounds that function independently of the BCL-2 family. Taken together, this rapid, efficient means of testing will prove advantageous as the demand for BH3 mimetics increases, particularly in the quest to identify and develop more potent MCL-1 inhibitors for use in the clinic. We strongly urge researchers utilizing BH3 mimetics in their work to use the potent and selective compounds identified with this validation toolkit instead of those lacking such potency and selectivity.

These authors contributed equally: Mariana Villalobos-Ortiz, Jeremy Ryan

Edited by G. Melino

✉ Anthony Letai
anthony_letai@dfci.harvard.edu

[1] Department of Medical Oncology, Dana-Farber Cancer Institute, 02215 Boston, MA, USA

[2] Department of Cell and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN, USA

[3] Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

[4] Harvard Medical School, Boston, MA, USA

[5] Ludwig Center at Harvard, Boston, MA, USA

Introduction

Many cancer cells must block apoptotic signaling in order to survive [1]. The mitochondrial pathway of apoptosis is regulated by more than a dozen proteins of the BCL-2 family, which is divided into three groups: the pro-apoptotic multi-domain, the anti-apoptotic multi-domain, and the BH3-only pro-apoptotic proteins. Their interactions at the mitochondrial outer membrane ultimately decide if the pore-forming multi-domain pro-apoptotic proteins BAX or BAK will undergo a conformational change, oligomerize, and form pores in the outer mitochondrial membrane [2]. Mitochondrial outer membrane permeabilization (MOMP) permits the release of intermembrane space proteins, including cytochrome c, followed by caspase activation and apoptosis. Anti-apoptotic proteins block apoptosis by binding the monomeric pro-apoptotic proteins, preventing homo- and hetero-typic interactions among pro-apoptotic proteins that are necessary for MOMP. Anti-apoptotic

proteins possess a hydrophobic groove that binds the hydrophobic face of the amphipathic, alpha-helical BH3 domain that is present in every BCL-2 family protein.

BH3 mimetics are a class of compounds that compete for this hydrophobic groove to antagonize the function of anti-apoptotic proteins. BH3 mimetics have entered the clinic and even gained regulatory approval for use in cancer. Thus, there is justifiable enthusiasm for creating novel BH3 mimetics. However, many putative BH3 mimetics are reported in the literature, and methods for validating these compounds are inconsistent so that the relative quality of different agents is sometimes uncertain. Inhibiting the anti-apoptotic members of the BCL-2 family proved to be challenging because the binding pocket is quite large, shallow, and hydrophobic. Typical small molecules cannot fully occupy this space and have struggled to be both potent and specific in their binding. The search for compounds that mimic the BH3 domain is usually guided by binding assays such as fluorescence polarization or surface plasmon resonance using tagged and often truncated versions of the multi-domain anti-apoptotic proteins. While these methods ignore other interactions, such as those of the mitochondrial membrane and its occupants, they have been useful in identifying, constructing, and validating early compounds.

Confusion regarding the method of action of many early inhibitors led to questions of how to better define a BCL-2 family inhibitor [3]. Binding assays alone could not predict or discriminate between on-target direct interactions, indirect cellular effects stemming from upstream signaling, or predict deleterious off-target effects. The ideal BH3 mimetic should operate directly on mitochondria, would selectively induce MOMP in mitochondria dependent on the specific anti-apoptotic protein target(s), and would require BAX and BAK for MOMP. Moreover, the cellular toxicity should track closely with the potency in inducing MOMP by direct mitochondrial exposure.

To perform testing of BH3 mimetics, we have assembled a toolkit of cell lines and BH3 peptides. Four cell lines with enforced expression of human BCL-2, BCL-XL, MCL-1, or BFL-1/A1 and one additional line that has homozygous deletion of both BAX and BAK have been engineered on a common murine B-ALL background driven by BCR-ABL with homozygous deletion of p16ARF and murine MCL-1 [4]. These five lines encompass the most common anti-apoptotic dependencies, including apoptosis refractory BAX/BAK DKO cells, that serve as an important control for the detection of mitochondrial or cellular effects that are independent of the BCL-2 family and therefore undesirable in BH3 mimetics. Because their mitochondrial phenotypes are stable and predictable, they are excellent standards to compare the behavior of new BH3 mimetics. In addition, we have assembled a panel of synthetic BH3 peptides [5–7] of known selectivity of interaction with the anti-apoptotic

proteins listed above, which can function as important positive and negative controls for credentialing our mito-chondrial testing of BH3 mimetics. We perform this via a BH3 profiling method we call iBH3 [8], a method by which we expose mitochondria to standardized concentrations of BH3 peptides and measure MOMP via release of cytochrome c via flow cytometry.

We have tested eighteen different putative BH3 mimetics using our toolkit. We find that several possess the properties expected of bona fide, selective, and potent BH3 mimetics, but that several demonstrate important deficiencies, calling into serious question their mechanism of action. Our simple, scalable method can improve the efficiency of testing potency and validating the method of action for future BH3 mimetics as the demand for this new class of therapeutics grows.

Materials and methods

Cell culture

Murine cells bearing human anti-apoptotic proteins were created and characterized for anti-apoptotic dependence previously [4]. They were maintained in RPMI with 10% FBS, L-glutamine, NEAA, HEPES, pen/strep, and 8 uL pure 2-mercaptoethanol per liter of media. Cells were maintained at 37 °C and 5% CO_2 at 100% humidity. Cell lines were tested for mycoplasma using a Lookout PCR mycoplasma testing kit (Sigma–Aldrich) and found to be negative.

Compounds

ABT-737, Sabutoclax, BDA-366, and UMI-77 (SellekChem, Houston, TX, USA), ABT-263 (navitoclax), WEHI-539, A-1210477, and TW-37 (ApexBio, Boston, MA USA), ABT-199 (venetoclax) and maritoclax (Medchem Express, Monmouth Junction, NJ USA), S63845 (Active Biochem, Kowloon, Hong Kong), A-1331852, A-1155463, and AMG176 (Chemietek, Indianapolis, Indiana USA), Gossypol (Sigma–Aldrich, St. Louis, MO USA), and obato-clax (LC Labs, Woburn, MA USA) were obtained as solid stocks, dissolved in DMSO, and stored at −20 °C. AZD4320 and AZD5991 were obtained from Astra Zeneca. The Annexin V HIS plasmid was a gift of Seamus Martin, and Annexin V-Alexa647 was produced as described [9].

iBH3 profiling

BH3 profiling with the iBH3 method was performed as described [8]. Cells were suspended at 2×10^6 cells/mL in MEB2P25 buffer (150 mM mannitol, 150 mM KCl, 10 mM

HEPES, 5 mM succinate, 1 μM EDTA, 1 μM EGTA, 0.1% protease free BSA, 0.25% w/v polaxamer 188, final pH 7.4) and added to plates containing peptides at 2× their final concentration in MEB2P25 buffer with 0.002% w/v digitonin. Cells were exposed at 25 °C for 60 min before fixation, neutralization, and staining. Cells were stained with Hoechst 33342 (Thermo Fisher, Waltham, MA USA) and anti-cytochrome c (BioLegend, San Diego, CA USA). Fluorescent cytometry to measure cytochrome c intensity was performed using an Intellicyt iQue Screener Plus (Intellicyt, Albuquerque, NM USA). The raw data were converted to the cytochrome c loss parameter as described below in Data Reduction. Additional protocol details and comparison of BH3 profiling techniques can be found online at https://letailab.dana-farber.org/bh3-profiling.html. Fluorescence from obatoclax and ABT-199 were avoided by using cytometer channels that did not show significant background from these drugs.

Viability testing

Cells were plated at 0.5×10^6 cells/mL in culture medium in 96-well plates with dilutions of each compound, total volume 0.1 mL, and incubated for 24 h. 10 uL of 10X Annexin V binding buffer (100 mM HEPES, 40 mM KCl, 1.4 M NaCl, 7,5 mM MgCl2, 25 mM CaCl2 pH 7.4) with Annexin V–Alexa647 [9] and 20 ug/mL Hoechst 33342 was added and incubated at RT for 15 min. Cells were fixed by adding 30 uL of 4X Annexin V Fix buffer (4% formaldehyde, 0.5% glutaraldehyde in 1X Annexin V binding buffer) for 10 min at RT, neutralized using N2 buffer (1.7 M Tris, 1.25 M glycine pH 9.1), and analyzed for Annexin V positivity on the iQue Screener Plus. Populations were scored as Live (Hoechst + Annexin V−), early apoptotic (Hoechst + Annexin V+), and late apoptotic (Hoechst low Annexin V+). Because Hoechst 33342 stains all cells, live cells show as $2N + 4N$ populations that can persist into early apoptosis, and late apoptotic cells show a weaker staining due to nuclear degradation.

Data reduction

Our calculations were based on the distributions of cytochrome c intensity in test and control samples. For each distribution, we calculated a median fluorescence intensity (MFI). For each data point, % cytochrome c loss was calculated using the equation described in ref. [7] and listed below.

$$\% \text{Cytochrome C Loss} =$$
$$1 - \big[(\text{MFI}_{\text{Sample}} - \text{MFI}_{\text{FMO}}) / (\text{MFI}_{\text{Buffer Control}} - \text{MFI}_{\text{FMO}}) \big]$$

"Sample" is the treatment well being analyzed, "FMO" is a control with no cytochrome c antibody that defines the minimum possible cytochrome c signal, and the "Buffer control" is a well with all assay components but no peptide or drug, which defines complete cytochrome c retention. Sigmoidal dose-response curves of % cytochrome c loss versus drug concentration were then fit using a non-parametric fit with variable Hill slope and lower and upper bounds set to 0 and 100, respectively. Data were thus normalized and graphed using PRISM (Graphpad Software, San Diego, CA USA).

Results

To make a useful comparison, we first limited several factors including exposure time, compound concentration, and media composition for both mitochondrial and cellular assays. An effective BH3 mimetic should cause death quickly, often within a few hours, so we chose 24 h as our cutoff for cell viability measurements by Annexin V. While we understand that serum binding may reduce the effective concentration to which the cells are exposed, serum binding will also be present in vivo, and we controlled for this by testing all compounds under identical serum concentration. Maximum drug concentrations were chosen to ensure all compounds were soluble in assay buffers and culture media. Ranges of concentrations were chosen to ensure a good dynamic range that ideally went from no response to full response. Note that 1 μM is within this range, roughly the serum concentration achieved by the one FDA-approved BH3 mimetic, venetoclax [10], when used as recommended. For each compound tested, the above conditions were applied equally to all five cell lines in iBH3 profiling to show direct mitochondrial activity, and in cell death assays using Annexin V to show activity against intact cells. Both Annexin V and iBH3 were performed using assays terminating in fixation and performed with liquid handlers to demonstrate the utility of the assay in an automated format.

We have previously described the creation of a set of murine leukemia cell lines, each with a defined dependence on a different anti-apoptotic protein [4], including BCL-2, BCL-XL, MCL-1, and BFL-1. In addition, one control cell line lacked the pro-apoptotic effector proteins BAX and BAK. We used cellular toxicity, as measured by Annexin V, and mitochondrial toxicity, as measured by cytochrome c release on direct mitochondrial exposure, as measures of the properties of putative BH3 mimetic compounds. We first validated the anti-apoptotic dependence of the cell lines with the BH3 peptides. Figure 1a shows the interaction between BH3 peptides and the anti-apoptotic proteins, as previously determined by fluorescence polarization or surface plasmon resonance [5–7]. Figure 1b shows the MOMP response to the BH3 peptide panel. In each case, the MOMP response validated the anti-apoptotic dependence of the cell line and mitochondria. Note that the cell line lacking BAX
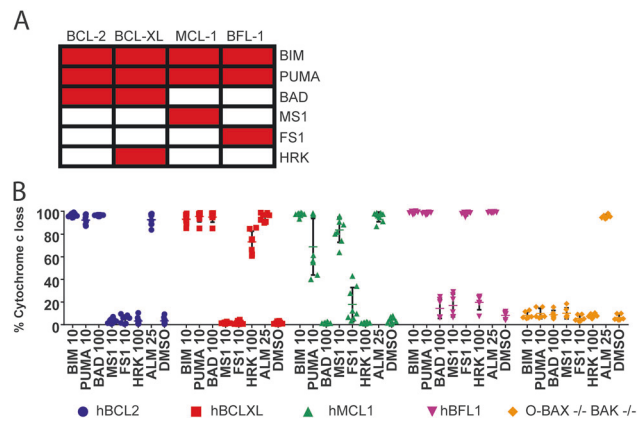
Fig. 1 a The binding pattern of anti-apoptotic proteins at the top vs. the synthetic BH3 peptides at right. A red box indicates high-affinity binding, with Kd values of <100 nM as measured by fluorescence polarization (BIM, PUMA, BAD, HRK) [5] or surface plasmon resonance (MS1, FS1) [6, 7]. b Control BH3 profiles for each cell line showing that cell line dependencies match with the interaction specificities shown in the binding assays. ALM is alamethicin, a BAX/BAK independent pore-forming peptide used as a release control. N = 6–8 and bars show mean and standard deviation

and BAK was completely resistant to BH3 peptide-induced MOMP, as expected.

We next examined six putative BH3 mimetics, including gossypol [11], obatoclax [12], subatoclax [13], maritoclax [14], TW-37 [15], and UMI-77 [16]. These compounds vary in reported target specificity from pan-inhibition to MCL-1-specific binding and have IC50 values in the upper nanomolar to micromolar range. Most of these compounds show little or no activity in iBH3 assays, indicating a lack of activity on mitochondria under conditions where there is no plasma membrane to serve as a barrier. However, all six compounds demonstrated an ability to kill primarily BFL-1- and MCL-1-dependent cells (Fig. 2). This suggests that these compounds may be able to engage the BCL-2 family indirectly by influencing protein level or modifications upstream, but not directly at the mitochondrial level. Prior studies have shown that certain putative BH3 mimetics increase NOXA protein levels, leading indirectly to inhibition of MCL-1 and cell death [3, 17–20]. Obatoclax [3], and to a lesser degree gossypol, caused death in BAX/BAK DKO cells, indicating their ability to also kill independent of intrinsic apoptosis. At the mitochondrial level, obatoclax caused cytochrome c release in mitochondria lacking BAX and BAK at similar concentrations to the other cell lines, implying it may have a direct membrane lytic function at the mitochondria at high concentrations. All six compounds failed to show strong cytochrome c release in iBH3 despite being able to selectively kill cells, and so these compounds cannot be considered potent direct BH3 mimetics. We cannot rule out participation of these compounds in signaling events upstream of the mitochondrion that less directly induce apoptosis via the mitochondrial, or intrinsic, pathway.
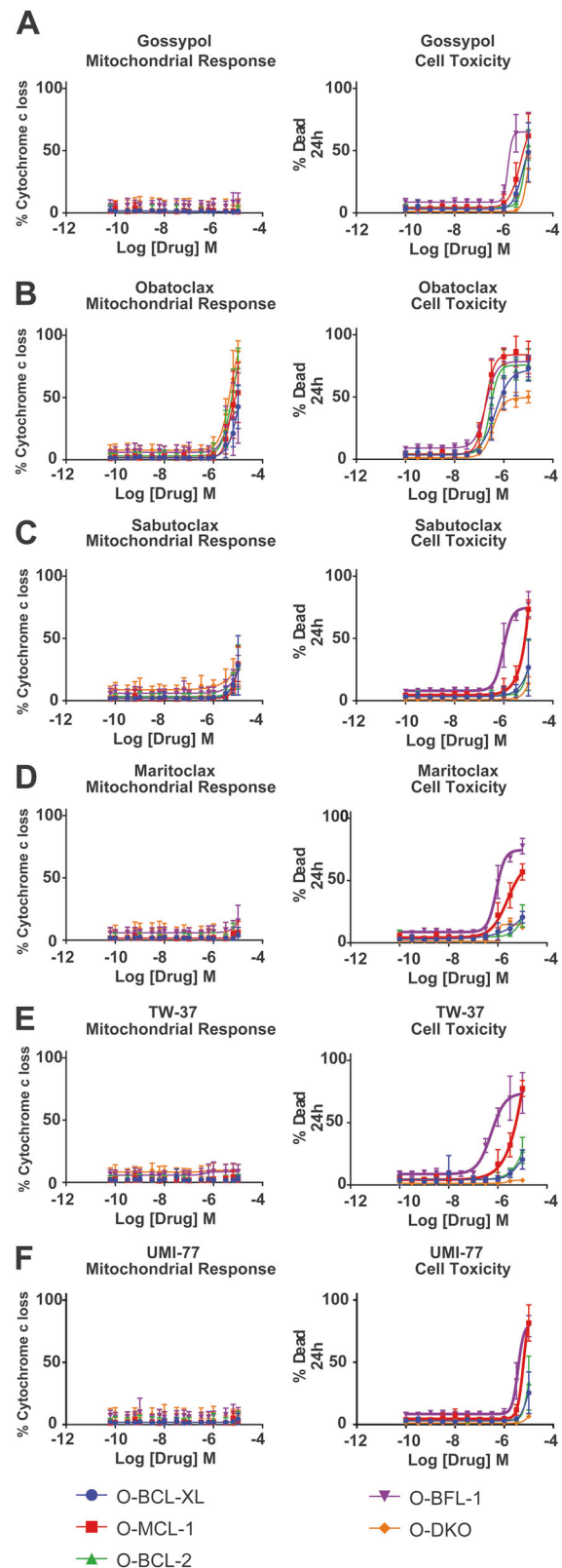
We next considered three available putative BCL-2/BCL-XL dual inhibitors: ABT-737 [21], ABT-263 [22], and AZD4320 [23]. (Fig. 3) All three compounds caused

◀ **Fig. 2** iBH3 and toxicity of putative BH3 mimetics. Direct mitochondrial response by iBH3 at 1 h (left) and cell killing as measured by Annexin V positivity at 24 h (right) using each of the five BCL-2 family model lines exposed to **a** gossypol, **b** obatoclax, **c** maritoclax, **d** sabutoclax, **e** TW37, and **f** UMI-77. For mitochondrial activity $N = 6$ or more and for viability $N = 3$ or more. Bars indicate standard deviation
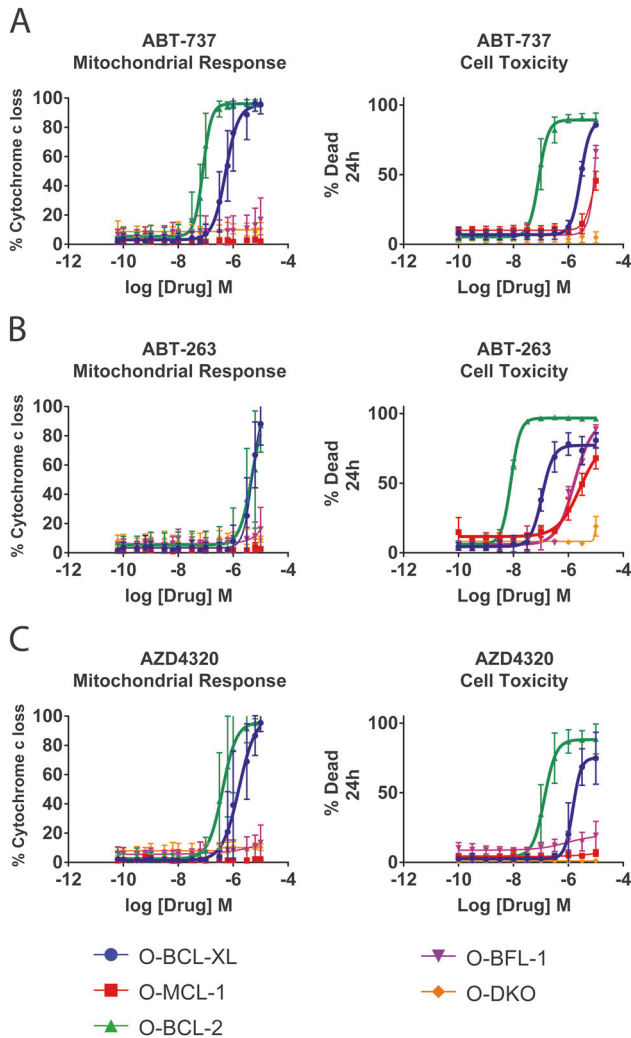

Fig. 3 Cell killing by dual BCL2/BCLXL inhibitors matches iBH3 direct mitochondrial response. Direct mitochondrial response by iBH3 at 1 h (left) and cell killing as measured by Annexin V positivity at 24 h (right). Mitochondria exposed to dual inhibitors release cytochrome c only if BCL-2- or BCL-XL-dependent. For mitochondrial activity $N = 6$ or more and for viability $N = 3$ or more. Bars indicate standard deviation

Fig. 4 a The BCL-2 specific inhibitor venetoclax (ABT-199) selectively causes cytochrome c release and cell killing if the cells are BCL-2-dependent. iBH3 response at 1 h and Annexin V positivity at 24 h is shown. **b** The putative BH4 BCL-2 antagonist lacks BCL-2 selective effect at the mitochondrial or cellular level. For mitochondrial activity $N = 6$ or more and for viability $N = 3$ or more. Bars indicate standard deviation

consistent with these compounds possessing on-target, mitochondrial-dependent killing only through competitive binding with their reported targets at the mitochondria. Similar observations of potent and selective cellular action of these compounds have also been reported [24].

The more recent BH3 mimetics focus on inhibiting a single anti-apoptotic protein. The effort is partly born out of a desire to avoid side effects. On-target thrombocytopenia in trials using ABT-263 inhibiting BCL-XL-dependent mature platelets lead to the synthesis of ABT-199 [10], a BCL-2 selective inhibitor that is now in clinical trials and approved by the FDA for CLL and AML. When tested with our toolkit, only BCL-2-dependent mitochondria responded, and only BCL-2-dependent cells were killed. In both iBH3 and Annexin V assays, ABT-199 showed activity in the low to mid-nanomolar range. Given the clinical success of this compound, future BH3 mimetics should be expected to perform to a similar degree if they are to be effective (Fig. 4a). We also studied compound BDA-366 [25, 26]. BDA-366 is reported to selectively inhibit BCL-2 protein function by binding to the BH4 domain of BCL-2. As such, it would also be expected to be selectively toxic to BCL-2-dependent cells and mitochondria. The results in Fig. 4b
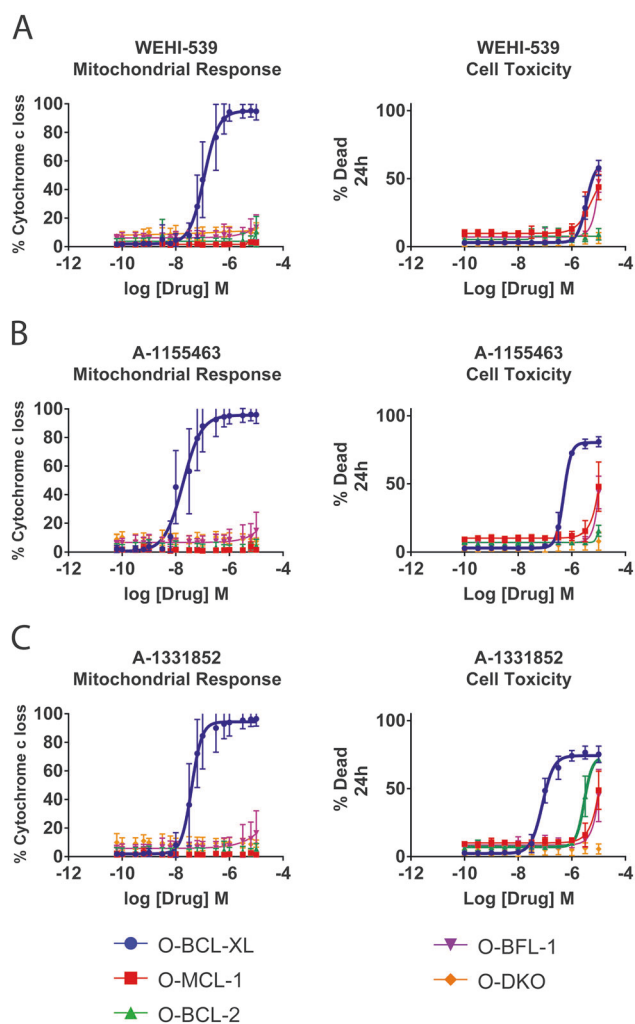
cytochrome c release from BCL-2- and BCL-XL-dependent mitochondria without a response from mitochondria of any other dependency. They also killed only those cells with the same dependencies as seen in the mitochondrial assay, although ABT-737 is relatively inefficient in killing the BCL-XL-dependent cells. We detected no response from the BAX/BAK DKO control from any compound, which is
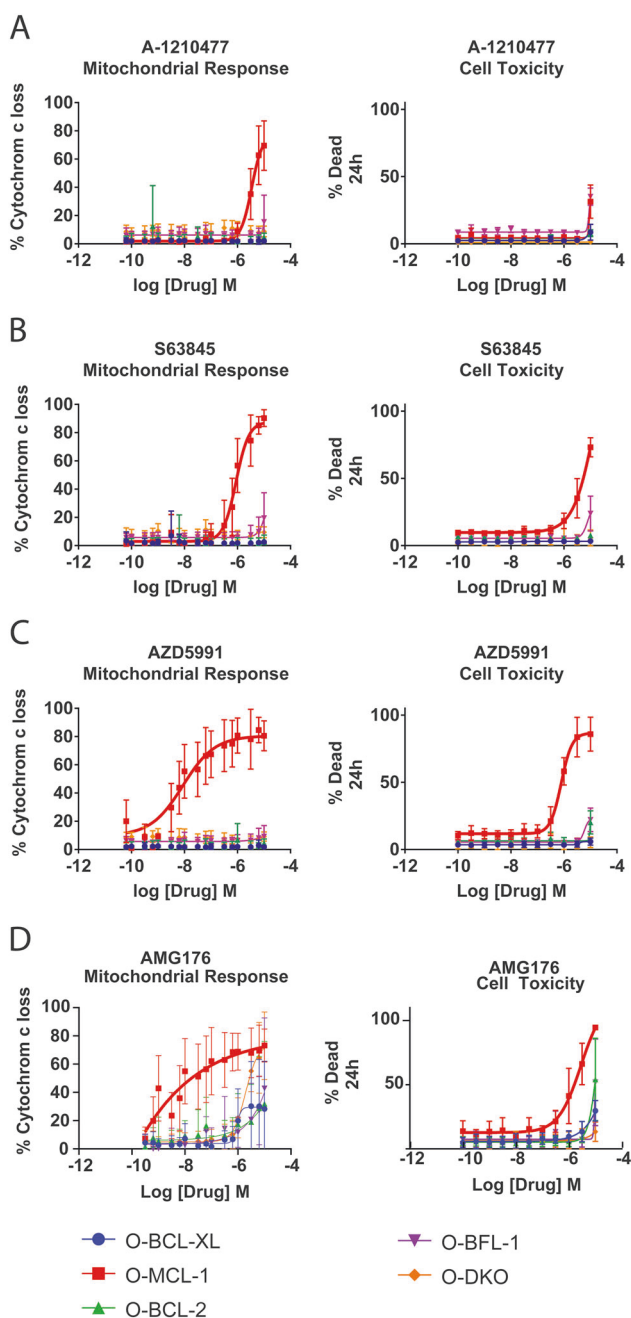
Fig. 6 The MCL-1 specific inhibitors **a** A1210477, **b** S63845, **c** AZD5991, and **d** AMG176 selectively cause cytochrome c release and cell killing if the cells are MCL-1 dependent. iBH3 response at 1 h and Annexin V positivity at 24 h is shown. For mitochondrial activity $N = 6$ or more and for viability $N = 3$ or more. Bars indicate standard deviation

and BCL-XL inhibitors, ABT-737 killed BCL-XL-dependent cells less well than the other two, while ABT-263 had less potency in mitochondrial assays. Among the selective BCL-XL inhibitors, WEHI-539 demonstrated less potent killing of the BCL-XL-dependent cell line. Among the selective MCL-1 inhibitors, A-1210477 showed inefficient killing of the MCL-1-dependent cell line. Moreover,

the extremely potent and selective induction of MOMP in MCL-1-dependent mitochondria by AZD5991 is notable, making it the most potent MCL-1 inhibitor at both the mitochondrial and cellular level of those we tested.

Seven of the test compounds did not perform as true BH3 mimetics and should be considered poor candidates for BH3 mimetic tool compounds or drugs. Gossypol, maritoclax, TW37, and UMI-77 demonstrated no mitochondrial activity at concentrations up to 10 μM. While sabutoclax, BDA-366, and especially obatoclax demonstrated mitochondrial activity at concentrations >1 μM, it is notable that they were similarly active on all mitochondria, including those lacking BAX and BAK. This strongly suggests a mechanism of action independent of the BCL-2 family.

It is worth asking how compounds lacking on-target mechanism nonetheless advanced as putative BH3 mimetics. In each case, these drugs were found to bind at least one anti-apoptotic BCL-2 family protein in a biochemical assay, although generally with modest (>100 nM) affinity. Also, in each case, these drugs demonstrated the ability to kill cells. In some cases, killing of MCL-1-dependent cells was taken as evidence that the drug selectively targeted MCL-1 [12–14]. However, we have shown previously that many drugs that do not target MCL-1 directly nonetheless selectively target MCL-1-dependent cells, perhaps because of the relative short half-life of the MCL-1 protein [2]. What was lacking in prior studies was the demonstration that the killing was taking place via direct targeting of mitochondrial BCL-2 family proteins, an absolute requirement for a true BH3 mimetic. When finally subjected to mitochondrial testing (Fig. 2), these compounds failed to demonstrate the required activity. We suggest that to avoid similar confusion in the future, all putative BH3 mimetic compounds should be subjected to a direct mitochondrial assay using mitochondria of validated dependence on anti-apoptotic BCL-2 family proteins.

It is notable that other laboratories, using different methods, have identified a lack of on-target activity for six of the drugs studied here [34–37]. It is equally notable that, notwithstanding this information, with the exception of maritoclax, even through 2018 there are publications using these compounds as if they were on-target BH3 mimetics. There are now unambiguously potent and selective BH3 mimetic inhibitors of BCL-2, BCL-XL, and MCL-1, including those we have identified above. At least one of each specificity is available from at least one commercial vendor each. We strongly urge cell death investigators interested in BH3 mimetics to restrict their experimentation to compounds with a thoroughly validated mechanism of action, and to avoid those that clearly lack such a mechanism.

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