Review

Molecular Cancer Therapeutics

BCL2 Inhibitors as Anticancer Drugs: A Plethora of Misleading BH3 Mimetics

Ryan S. Soderquist and Alan Eastman

Abstract

Antiapoptotic BCL2 proteins play a major role in tumor cell survival. Hence, BCL2 inhibitors have been developed as direct inducers of apoptosis. ABT-199 (venetoclax) received breakthrough therapy designation from the FDA due to its apparent efficacy in CLL and AML. However, resistance to ABT-199 is mediated by other BCL2 proteins including $BCLX_L$ and MCL1. Considerable effort has been expended seeking novel "BH3 mimetics" that inhibit all of these BCL2 proteins. While many BH3 mimetics inhibit BCL2 proteins *in vitro*, they fail to directly inhibit them in intact cells. Many BH3 mimetics induce the unfolded protein response culminating in induction of the proapoptotic protein NOXA, which in turn inhibits MCL1. We propose simple experiments to validate BH3 mimetics in cells. A true BCL2 inhibitor will rapidly induce apoptosis in chronic lymphocytic leukemia cells *ex vivo*. A $BCLX_L$ inhibitor will rapidly induce apoptosis in platelets. Finally, a BH3 mimetic targeting MCL1 will inhibit its degradation thereby inducing rapid MCL1 accumulation. Compounds that fail these tests should no longer be called BH3 mimetics. We now have a toolbox of selective inhibitors for most of the BCL2 proteins, and we hope these new tools will lead to effective treatment options for many cancers. *Mol Cancer Ther; 15(9); 2011–7. ©2016 AACR.*

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Introduction

The observation that many cancers depend on antiapoptotic BCL2 proteins, and that BCL2 proteins interact through defined BCL2-homology (BH) domains, led to the concept of BH3 mimetics as potential chemotherapeutic agents (1). A true BH3 mimetic should mimic the BH3 domain of a proapoptotic BCL2 family member, thereby inhibiting the antiapoptotic proteins by occupying their BH3-binding groove. In 2005, ABT-737 was published as a potent inhibitor of BCL2, $BCLX_L$, and BCLw (2). ABT-737 occupies the BH3-binding domain of these BCL2 proteins, and also induces apoptosis in a variety of cancer models. Subsequent medicinal chemistry led to ABT-263 (navitoclax), which had improved oral bioavailability (3). Clinical trials with navitoclax demonstrated efficacy in leukemia and some solid tumors, but it also exhibited dose-limiting toxicities including neutropenia and thrombocytopenia (4). The thrombocytopenia was attributed to the inhibition of $BCLX_L$, as platelets require $BCLX_L$ for survival (5). Subsequently, ABT-199 (venetoclax) was developed as a selective inhibitor of BCL2 and this circumvents the problem of thrombocytopenia (6).

While these early-generation BCL2 inhibitors have shown promise in the clinic, resistance is anticipated as has been observed in many *in vitro* models. This resistance can arise from the upregulation and reliance on additional antiapoptotic proteins (e.g., MCL1 and BFL1) that are not inhibited by ABT-263 or ABT-199 (7, 8). In addition, expression of $BCLX_L$ may elicit resistance to ABT-199. Many putative BH3 mimetics have been described to inhibit these other antiapoptotic proteins (i. e., MCL1 and BFL1). However, several articles have questioned the selectivity of these compounds for BCL2 proteins and highlighted the need for proper validation of putative BH3 mimetics in cell-based assays (7, 9, 10). While reviewing recent progress in the development of BH3 mimetics, Billard (11) noted that "most of these compounds did not fully meet the criteria for defining a bona fide BH3 mimetic, that is, high affinity binding to prosurvival BCL2 proteins and induction of BAX/BAK–dependent apoptosis." These were two of the criteria previously suggested by Lessene and colleagues as defining a true BH3 mimetic (12). In fact, many of these putative BH3 mimetics have been shown to disrupt interaction between BCL2 proteins *in vitro*, and induce BAX/BAK–dependent apoptosis, but unfortunately, this has too often been considered sufficient proof of their mechanism of activity in cells. In fact, Lessene and colleagues (12) proposed two additional criteria that are required to validate a BH3 mimetic: (i) that cytotoxicity should be correlated with cellular BCL2 protein family levels; and (ii) treatment of animals should trigger relevant biomarkers such as acute reduction in platelets for a $BCLX_L$ antagonist and reduced lymphocytes for a BCL2 antagonist.

The latter of these criteria can be easily established without the use of animals. A true BCL2 inhibitor such as ABT-199 will rapidly (within 6 hours) induce apoptosis in human chronic lymphocytic leukemia cells *ex vivo*. This rapid apoptosis is believed to be due to the displacement of BIM from BCL2 at the mitochondria, which then directly activates BAX/BAK (13). Similarly, human platelets can be incubated with a $BCLX_L$ inhibitor such as ABT-737 *ex vivo* resulting in rapid cleavage of gelsolin, a marker of caspase activity (5). A similar cell-based system to confirm the activity of MCL1 inhibitors is discussed in more detail below.

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Unfortunately, even these observations do not confirm that a compound directly inhibits a particular BCL2 protein in cells. The problem arises because there are a variety of ways by which BCL2 proteins can be impacted indirectly by a putative BH3 mimetic. The most common mechanism appears to be the induction of the proapoptotic protein NOXA which in turn inhibits MCL1 and BFL1, and perhaps even $BCLX_L$ and BCL2 under some circumstances (14, 15). In addition, the inhibition of one BCL2 protein can cause "partner swapping" whereby a displaced BH3-only protein can then bind to an alternate BH3 pocket, and change the apparent dependence of a cell. The BCL2 proteins are also dependent on additional posttranslational modifications that can impact their level or cellular localization (Fig. 1). These issues are elaborated further in this review.

Most BH3 Mimetics Do Not Act as BH3 Mimetics in Cells

Our initial concern for the mechanism of action of putative BH3 mimetics was raised by experiments with obatoclax that showed inhibition of phospho-ERK and MCL1 expression, events not expected for a pan-BCL2 inhibitor (10). We subsequently screened seven different BH3 mimetics, and only ABT-737 showed evidence of direct BCL2 inhibition. Our initial assay was based on the observation that BCL2 bound the proapoptotic protein BAD and, as a consequence, both proteins were detected in the pellet fraction after removal of the cytosol. Addition of ABT-737 caused rapid translocation of BAD to the cytosol. However, none of the other 6 putative mimetics dissociated BAD. From surveying expression of other BCL2 proteins, we noticed that these 6 compounds all induced NOXA. Upstream regulators of NOXA induction included phosphorylation of eIF2alpha, and the transcription factors ATF4 and ATF3 reflecting activation of the unfolded protein response (UPR), also known as the integrated stress response (10).

We were not the first to question the mechanism of action of many of these mimetics. Several years earlier, van Delft and colleagues demonstrated that 6 putative mimetics killed BAX/BAK–deficient mouse embryonic fibroblasts (MEF) implying they were not directly targeting BCL2 proteins (7). Vogler and colleagues made similar observations (9). While these observations may demonstrate that these compounds have additional activities, they do not rule out the possibility that the compounds also inhibit BCL2 proteins. van Delft and colleagues also showed that ABT-737 alone did not induce apoptosis in wild-type MEFs suggesting that MEFs are not solely dependent on BCL2 and $BCLX_L$. Indeed, when NOXA was overexpressed, ABT-737 induced BAX/BAK–dependent apoptosis. These results suggest that MEFs also rely on MCL1 and/or BFL1. Consequently, a true pan-BCL2 inhibitor would be expected to induce apoptosis in MEFs in a BAX/BAK–dependent manner. The fact that six putative mimetics killed MEFs independent of BAX/BAK appears to rule out the possibility that they act as pan-BCL2 inhibitors in these cells.

ABT-737, ABT-263, and ABT-199 are well validated as BH3 mimetics (2, 3, 6). WEHI-539 also appears to be a selective $BCLX_L$ inhibitor (16). We have tested eleven other putative BH3 mimetics and all increased NOXA expression (Table 1). We recognize this table is not a comprehensive list of all putative BH3 mimetics, but only reflects those for which we have data. However, we believe that the frequent induction of NOXA probably explains previous reports showing that these compounds can induce apoptosis in cells. For two of these compounds (S1 and gossypol), we have shown that siRNA-targeting NOXA prevented

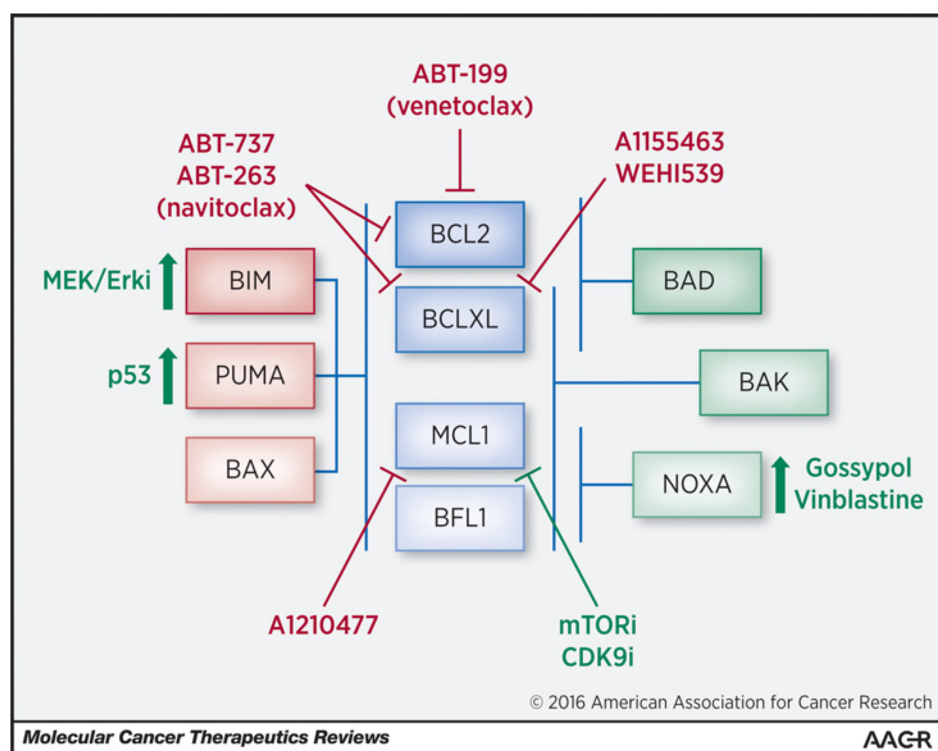

Figure 1.
The interplay between pro- and anti-apoptotic proteins, and the compounds that inhibit these interactions. The anti-apoptotic BCL2 proteins are shown in the center (blue). Proapoptotic proteins that bind to all the anti-apoptotic members are shown on the left (red), while the proapoptotic proteins with more restrictive binding capability are shown on the right (green). The sites of inhibitory action of BH3 mimetics are shown in red text. Several indirect means to modify the proapoptotic proteins are shown in green text.

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Table 1. Properties of putative BCL2 inhibitors

The good							
BH3 mimetic	**Proposed targets**	**Induces NOXA in cells**	**Activates the ISR**	**Kills CLL cells *ex vivo***	**Kills platelets *ex vivo***	**Reference**	
ABT-737/ABT263	BCL2, BCLXL	No	No	Yes	Yes	2, 3	
ABT-199	BCL2	No	No	Yes	No	6	
WEHI-539	BCLXL	No	No	No	Yes	16	
A-1155463	BCLXL	No	No	No	Yes	45	
The bad							
Putative BH3 mimetics	**Proposed targets**	**Induces NOXA in cells**	**Activates the ISR**	**Kills CLL cells *ex vivo***	**Kills platelets *ex vivo***	**Ref. to drug**	**Ref. to criticism**
Gossypol and AT-101	pan-BCL2	Yes	Yes	No	No	20	10, 18
Apogossypol	pan-BCL2	Yes	Yes	No	nd	46	10
S1	pan-BCL2	Yes	Yes	No	No	47	10, 17
HA14-1	BCL2	Yes	Yes	No	nd	48	10
2 methoxy antimycin A_3	BCL2, BCLXL	Yes	Yes	No	nd	49	10
Obatoclax (GX15-070)	pan-BCL2	Yes	Yes	No	nd	50	10
BXI-61	BCLXL	Yes	Yes	No	No	51	N/A
BXI-72	BCLXL	Yes	Yes	No	No	51	N/A
TW37	BCL2, BCLXL, MCL1	Yes	nd	nd	nd	52	53
MIM1	MCL1	Yes	Yes	No	nd	22	N/A
UMI-77	MCL1	Yes	Yes	No	nd	54	N/A

Abbreviations: ISR, integrated stress response; nd, not determined; N/A, R.S. Soderquist and A. Eastman unpublished observations.

apoptosis (as single agents or in combination with ABT-737 or ABT-199 depending on the cell system; refs. 17, 18). Given that NOXA (and other BH3-only proteins such as PUMA) can be induced by a wide array of cell-stress pathways, perhaps it is not surprising that so many putative BH3 mimetics actually function in this way. The critical point is that, when evaluating putative BH3 mimetics, one should always probe for changes in expression of BH3-only proteins which might explain how the compound functions in cells.

The targeting of these putative BH3 mimetics to one or other BCL2 protein has generally been established using *in vitro*–purified proteins, and often using truncated proteins. An assumption is often made that the concentration of drug that inhibits a target in a purified system will also be effective in cells. However, the intracellular concentrations may be much lower than the applied drug concentration because of extracellular protein binding (e.g., ABT-263 and ABT-737 bind to albumin; ref. 19), or because the cytoplasmic membrane can be an effective barrier between drug and target. The bioavailable concentration may also be limited by intracellular metabolism or protein binding. This issue may be particularly problematic with those putative BH3 mimetics that require micromolar concentrations to inhibit antiapoptotic proteins in cell-free assays. For example, ABT-737 inhibits BCL2 and $BCLX_L$ with a K_i of <1 nmol/L, but induces apoptosis in chronic lymphocytic leukemia cells at 10–100 nmol/L, suggesting that the intracellular concentration of ABT-737 is considerably lower than the applied concentration. Similarly, gossypol and S1 inhibit BCL2 binding at approximately 0.5 μmol/L (competitive displacement of a peptide in a cell-free binding assay; refs. 20, 21) but both elicit cytotoxicity at 20 μmol/L (17, 18). However, MIM1 exhibits an IC_{50} of approximately 5 μmol/L for MCL1 in a cell-free system, but the same concentration is required to reduce cell viability (22). It is possible that these compounds could act as BH3 mimetics in cells if higher intracellular concentrations could be achieved. However, this effect would likely be masked by the observed induction of NOXA. These concerns are consistent with the criteria of Lessene and colleagues suggesting that BH3 mimetics should have nanomolar potency in cell-free binding assays if they are to have on-target efficacy in cells (12).

Another confounding issue by which the selectivity of BH3 mimetics might be misassigned is that each BCL2 protein does not act in isolation. Instead, a given BH3-only protein can bind to multiple antiapoptotic proteins with varying affinities. With this in mind, it is possible for a BH3 mimetic which targets one antiapoptotic protein to indirectly inhibit an additional apoptotic protein due to displacement of a common binding partner (partner swapping). For example, we have noted that ABT-737 can effectively displace BIM which can then bind to MCL1 (10).

Partner swapping likely occurs in cells that show similar sensitivity to both BCL2- and BCLX-selective inhibitors (23). Hence, dependence on one BCL2 protein does not rule out dependence on another, particularly if both are fully loaded with BH3 proteins. The target for such a compound might be misconstrued if one were looking at only BCL2- or $BCLX_L$-binding proteins. Consequently, it is important to assay more than one interaction when evaluating putative BH3 mimetics in cells.

The opposite problem could also occur whereby a true BCL2 inhibitor might not work as predicted because BIM could swap to another BCL2 protein. For example, a BH3 mimetic targeting MCL1 could displace BIM to neutralize BCL2 or $BCLX_L$. These considerations depend on the overall binding capacity of BCL2 proteins, and in particular, the number of unoccupied binding sites. If the binding sites on BCL2 proteins were fully occupied then partner swapping would probably be unable to occur. The issue of "primed" BCL2, whereby BIM (or other BH3 protein) is constitutively bound to BCL2 proteins, has been discussed extensively by Letai (24), but we are unaware that much consideration has been given to the "reserve capacity" of the BCL2 proteins, and this could have a major impact on the response of cells to drugs. This issue of partner swapping also suggests that the level of a particular BCL2 protein may not correlate with the selectivity of a BH3 mimetic, in contradiction to the suggestion of Lessene and colleagues (12).

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A putative BH3 mimetic could also inhibit BCL2 proteins indirectly through altered expression, post-translational modifications, or changes in cellular localization of BH3-only proteins. For example, inhibition of the MEK–ERK pathway results in increased levels of BIM and thereby increased apoptosis (25). Phosphorylation of BAD by a variety of kinases can cause BAD to be displaced from BCL2 or $BCLX_L$ (26). Finally, BIM has been reported to bind BCL2 at the mitochondria and to bind dynein proteins on the microtubules (27). If a putative BH3 mimetic modifies any of these pathways, this might give the erroneous impression that the compound is a direct BCL2 inhibitor. These examples demonstrate additional mechanisms through which a putative BH3 mimetic can inhibit antiapoptotic proteins. Obatoclax has been demonstrated to inhibit the MEK–ERK pathway (10), but whether any other compounds have erroneously been characterized as BH3 mimetics due to these mechanisms is unknown.

Upregulating NOXA through the UPR Pathway

Activation of the UPR rapidly increases ATF4 and ATF3, which then form a transcriptionally active dimer that binds to the CRE site in the NOXA promoter and increases its expression (28). Importantly, many different cellular stresses can activate UPR (e.g., reactive oxygen species, increased cytosolic calcium, proteasome inhibitors; refs. 29, 30). Studies with the proteasome inhibitor bortezomib demonstrated that the induction of NOXA was not solely due to protein stabilization but rather to transcriptional upregulation, and this was proposed to contribute to the tumor-selective activity of the drug (31, 32). This apparent tumor selectivity was further supported by evidence that the transcriptional induction of NOXA occurred in a Myc-dependent manner (33). However, subsequent observations demonstrated that bortezomib increased NOXA mRNA expression through ATF4 and ATF3 transcriptional activity (28). Since this seminal paper, many different compounds have been shown to induce NOXA through this pathway, for example, thapsigargin (which increases cytosolic calcium) and fenretinide (a retinoid; ref. 34). As discussed above, we have found that 11 putative BH3 mimetics also induce NOXA (Table 1), hence this pathway may be a very common mechanism of NOXA induction. Many of the upstream components that activate the UPR differ between these compounds. For example, S1 has been shown to induce NOXA as a consequence of generating reactive oxygen species (17). Gossypol appears to be an agonist of phospholipase A2, the consequence of which is rapid increase in intracellular calcium; inhibition of PLA2 or calcium prevents NOXA induction (18).

The unanticipated induction of NOXA by all these compounds could still mean they have some therapeutic value. As the resistance to BCL2/$BCLX_L$ inhibitors is commonly due to MCL1 and/or BFL1 (8), any agent that upregulates NOXA could be a means to overcome this resistance. For example, incubation of CLL cells on stroma induces dramatic resistance to ABT-199 and ABT-737 which can be circumvented by combination with gossypol or AT-101 (18, 35). Furthermore, the fact that this pathway does not require p53 for NOXA induction increases its utility in cancers that lack functional p53. It is possible that further elucidation of these pathways may uncover novel targets for inducing NOXA, which can be exploited therapeutically.

Inhibitors of MCL1

Given the success of ABT-199 and ABT-263, and the recognition that MCL1 is a common mechanism of resistance (7, 8), many investigators have attempted to identify MCL1 inhibitors. As discussed above, most of these putative BH3 mimetics appear to inhibit MCL1 indirectly through induction of NOXA. But what would be the phenotype of a cell incubated with an inhibitor of MCL1? Two recent inhibitors provide pertinent information.

Maritoclax was recently published as a putative MCL1 inhibitor (36). Where maritoclax differs from other putative BH3 mimetics is that it does not upregulate NOXA but rather induces proteasomal degradation of MCL1. If maritoclax does block the binding of BH3-only proteins to MCL1, then the "free" MCL1 would need to be targeted by an E3 ligase for ubiquitination and degradation. There are 5 different E3 ligases capable of ubiquitinating MCL1

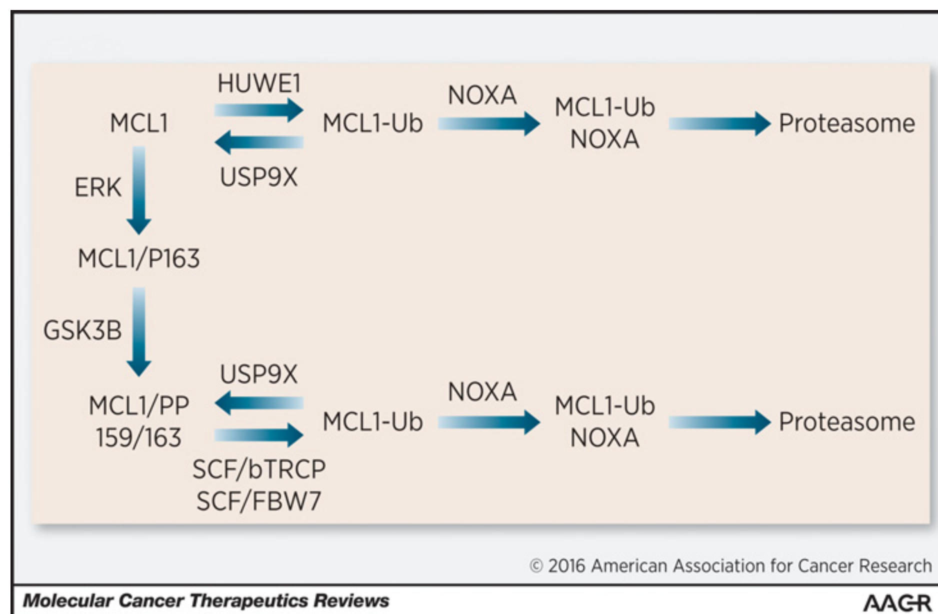

Figure 2.
Regulation of the turnover of MCL1 protein. MCL1 can be ubiquitinated by at least three E3 ligases depending upon its phosphorylation. NOXA is thought to stabilize ubiquitinated MCL1 and the MCL1:NOXA heterodimer is then degraded by the proteasome.

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(37), the most interesting of which is HUWE1 (MULE) because it targets MCL1 by directly binding to its BH3 pocket (Fig. 2). However, if maritoclax dissociates HUWE1, it should stabilize MCL1 rather than degrade it. Two other E3 ligases, SCF/β-TRCP and SCF/FBW7, ubiquitinate MCL1 after it has been phosphorylated, and perhaps this could be enhanced by maritoclax (37). However, ubiquitinated MCL1 is also a substrate for the USP9X deubiquitinase, and it has been proposed that NOXA binding to MCL1 inhibits the deubiquitination leading to degradation of the MCL1:NOXA heterodimer (38, 39). This mechanism would be inconsistent with the observed degradation of MCL1 upon incubation unless maritoclax can also inhibit MCL1 deubiquitination. A recent report has also questioned the mechanism of action of maritoclax (40). Clearly, the mechanism of action of maritoclax remains to be elucidated.

The other recently identified MCL1 inhibitor is A-1210477, which induces apoptosis in MCL1-dependent cell lines and enhances the activity of ABT-263 in other cell lines (41). This compound differs from prior BH3 mimetics in that it causes a marked accumulation of MCL1. This occurs through stabilization of MCL1 protein and not through transcriptional activation. This would be consistent with A-1210477 binding to the BH3 pocket and preventing the binding of both HUWE1 and NOXA. In retrospect, this phenotype appears consistent with what should be expected for a true MCL1-targeting BH3 mimetic. These observations with A-1210477 provide additional support for the contention that none of the previous putative BH3 mimetics are likely to act as MCL1 inhibitors in cells. However, we must note a potential concern for A-1210477 as it was recently shown to induce apoptosis in BCL2-dependent cells and in BAX/BAK knockout cells at only slightly higher concentration, suggesting it may have activities in addition to inhibition of MCL1 (42). A further caveat regarding MCL1 accumulation is that there are additional means by which this may occur; for example, it has been shown that BIM displaced from BCL2 can bind to MCL1 and increase its stability (10, 43), or alternately, MCL1 can be transcriptionally upregulated in response to ABT-737 (44).

Other candidate MCL1 inhibitors have been synthesized; those we have studied are presented in Table 1. We are unable to catalogue all of the other potential MCL1 inhibitors, but those investigators with access to them should consider comparing them to the properties discussed here.

Cellular Validation of True BH3 Mimetics

We can now conclude that a BH3 mimetic that is proposed to inhibit BCL2 should be tested against human CLL cells *ex vivo* as these cells will rapidly (<6 hours) undergo apoptosis. Similarly, a BH3 mimetic targeting $BCLX_L$ should be tested against platelets and rapid apoptosis (gelsolin cleavage) should be observed. A BH3 mimetic targeting MCL1 will induce accumulation of MCL1 protein. If these events are not observed, then the agent is unlikely to be a true BH3 mimetic.

Many cell lines have been engineered to be dependent on one or other BCL2 family member. Putative BH3 mimetics were then tested in these systems and, as discussed above, inappropriate conclusions were often drawn. A more robust system has recently been developed in which a murine leukemia cell line was engineered to express each human antiapoptotic protein singly, while the murine *MCL1* gene was deleted (albeit the cells still contain detectable murine BCLX; ref. 42). These cells were validated using BH3 profiling, and used to confirm the predicted activity of several BH3 mimetics. Suggested limitations include the residual endogenous BCL2 proteins which may provide some buffering capacity, and that the murine BH3-only proteins may differ from their human counterparts. It was proposed that these cell lines would provide an excellent model to screen and validate novel BH3 mimetics. However, they were not assayed with putative BH3 mimetics that induce NOXA. Such a BH3 mimetic might be cytotoxic to the MCL1-expressing cell line and therefore classified as having on-target action, while overlooking the fact that the activity could be due to an off-target mechanism. With these caveats, these cell lines may provide a useful alternative to CLL cells and platelets.

It must be reiterated that if a putative BH3 mimetic does induce apoptosis in any of these cell-based systems, or if it induces MCL1, this does not guarantee that the compound is a true mimetic, because there are alternate means to inhibit each BCL2 protein or increase MCL1. Keeping incubation times short (<6 hours) may avoid activation or inhibition of secondary pathways that might inhibit the BCL2 proteins. Coimmunoprecipitation assays will help to ensure that appropriate BH3 interactions are disrupted, and that apoptosis is not a consequence of partner swapping. Alternately, incubation of BH3 mimetics with mitochondria from cells known to be dependent on one BCL2 protein as discussed above (42) could be used as a secondary validation step. Potential changes in expression of other BCL2 proteins, particularly the proapoptotic BH3-only proteins, should be assessed in parallel. In summary, we strongly recommend that the term "BH3 mimetic" be reserved for those compounds that truly act as BH3 mimetics in intact cells.

We now have a toolbox of selective inhibitors for BCL2 (ABT-199), MCL1 (A-1210477), and $BCLX_L$ (A-1155463 and A-1331852, recently reported analogues of WEHI-539; refs. 23, 45). This will accelerate many studies to investigate the relative importance of each of these proteins for cell survival. Few diseases are likely to be as dependent on one BCL2 protein as CLL; the majority of cancers will probably require combinations of these agents for meaningful therapeutic impact. But will these combinations elicit unacceptable toxicity? Considering the many ways now available to inhibit MCL1 (Fig. 1), which approach will be most selective for the tumor? While there is no selective inhibitor for BCLw, it can be inhibited by ABT-737. BFL1 still awaits a selective drug. The importance of these latter two BCL2 proteins and whether they will need to be targeted remains to be determined. It is possible that they will be sufficiently inhibited as a result of swapping of BH3-binding partners displaced from other BCL2 proteins. Hopefully, targeted inhibition of these BCL2 proteins will provide significant therapeutic impact in many cancers, either as single agents or in rationally designed drug combinations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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