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Targeting Mcl-1 and other Bcl-2 family member proteins in cancer therapy

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ABSTRACT

Regulation of both the extrinsic and the mitochondria-dependent intrinsic apoptotic pathways plays a key role in the development of the hematopoietic system, for sustaining cell survival during generation of various cell types, in eliminating cells with dual identities such as CD4/CD8 double-positive cells (Hettmann, Didonato, Karin, & Leiden, 1999; Ogasawara, Suda, & Nagata, 1995), for sustaining cells during the rapid clonal expansion phase (Schirmer, Vallejo, Weyand, & Gronzy, 1998), as well as eliminating cells during the contraction phase (Yajima et al., 2006). The anti-apoptotic protein Mcl-1 is necessary for sustaining hematopoietic stem cells (HPS) (Akashi et al., 2003; Akashi, Traver, Miyamoto, & Weissman, 2000). The anti-apoptotic factors Mcl-1, Bcl-2, and Bcl-xL were also found to be over-expressed in acute myeloid leukemia (AML) (Kaufmann et al., 2016) and acute lymphocytic leukemia (ALL) (Findley, Gu, Yeager, & Zhou, 1997), suggesting that dis-regulated apoptotic processes could be a factor in the instigation of leukemia and/or its relapse. Molecules targeting these proteins were used as single agents to treat leukemia. However, by using a set of recently developed specific molecule inhibitors targeting anti-apoptotic proteins, distinct roles are being discovered for these anti-apoptotic proteins during hematopoietic and tumor development. Furthermore, using these inhibitors in proper combinations can effectively induce apoptosis in various solid tumors, even though each agent on its own cannot induce apoptosis in them. These new findings suggest that inhibiting anti-apoptotic elements can induce apoptosis without external stimuli in most cells, but it comes with a risk that some combinations could also trigger apoptosis in healthy cells. One way to address the safety issue is by limiting exposure to all the agents to only cancer cells, thus making the combination safe and effective. In this article, we review this rapidly developing idea in cancer research.

Contents

1. Introduction . . . 14
2. Mcl-1 isoforms and the non-apoptotic role of Mcl-1 . . . 14
3. Safety of S63845 . . . 14
4. Inducing apoptosis by inhibiting anti-apoptotic factors . . . 15
5. Safety of the S63845-ABT263 combination therapy . . . 15
6. Targeting subsets of Bcl-2 proteins. . . . 15
7. Combination vs. sequential administration of inhibitors . . . 17
8. Safety of the combination therapy . . . 17
9. Other means of disrupting the (Mcl-1)-(Bak/Bax) complex . . . 17
Conflict of interest . . . 18
Acknowledgments . . . 18
References . . . 18

Abbreviation: TPA, 12–0-Tetra- decanoylphorbol 13-acetate; MEF, Mouse embryonic fibroblast; HPS, Hematopoietic stem cell; LCC, Latency competent cells..

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1. Introduction

Myeloid Cell Leukemia 1 (MCL-1) was cloned from a human myeloid leukemia cell line during TPA-induced differentiation (Kozopas, Yang, Buchan, Zhou, & Craig, 1993). Based on the sequence homology, it belongs to the Bcl-2 family of anti-apoptotic genes, such as Bcl-2, Bcl-2 L10, Bcl-W and Bcl-xL (Kelekar & Thompson, 1998). In particular, Mcl-1 and Bcl-xL proteins, through their BH3 domains, bind and sequester pro-apoptotic Bak/Bax proteins on the mitochondrial outer-membrane, thus keeping them in their inactive state (Willis et al., 2005). Short of damaging mitochondria by chemical compounds (Mizuta, Shimizu, Matsuoka, Nakagawa, & Tsujimoto, 2007), activation of Bak/Bax is the only physiological means of releasing cytochrome *c* from mitochondria (Wei et al., 2001). Once cytochrome *c* is released into the cytosol through pores formed by multimerized Bak or Bax (Korsmeyer et al., 2000), it associates with caspase 9 and Apaf-1, forming a large protease complex called the apoptosome. The apoptosome degrades a great many cellular proteins, thus initiating cell death (Kim, Du, Fang, & Wang, 2005). For this reason, the moment Bak/Bax is activated is called the point of no return (Tait & Green, 2013). Therefore, Mcl-1 and Bcl-xL play crucial roles in suppressing mitochondria-dependent apoptosis.

In the last several years, there have been reports that by silencing just two genes, MCL-1 and BCL-xL using siRNA, apoptosis is induced in many cancer cells in *in vitro* experiments (Takahashi et al., 2013; Varin et al., 2010). In these reports, silencing just one of these genes was not enough to induce apoptosis. Cancer cells susceptible to the dual silencing approach included difficult-to-treat mesothelioma cells (Varin et al., 2010) and pancreatic cancer cells (Takahashi et al., 2013). These findings brought into focus three important issues in cancer and apoptosis research:

(1) Can inhibiting anti-apoptotic factors induce apoptosis without external stimuli?

(2) Can this approach induce apoptosis in any cell?

(3) How to combine anti-apoptotic inhibitors to minimize the risk of damaging healthy cells?

To address the above questions, we need to carefully interpret diverse and sometimes seemingly contradictory results from experiments involving genetic manipulations and experiments using targeted drugs. For example, (1) the Mcl-1 knockout mouse is embryonic lethal (Rinkenberger, Horning, Klocke, Roth, & Korsmeyer, 1993), (2) the cardiac specific Mcl-1 knockout mouse dies from dilated cardiomyopathy (Thomas et al., 2013), (3) the presence of Mcl-1 is necessary for the development and maintenance of lymphocytes (Opferman et al., 2003), and yet (4) a drug inhibiting Mcl-1 is relatively safe to use in mice (Kotschy et al., 2016). In this article, we review these findings, deciphering the reasons behind seemingly contradictory outcomes and through a newly gained perspective, we address the three questions posed earlier.

2. Mcl-1 isoforms and the non-apoptotic role of Mcl-1

As discussed below, Mcl-1 plays an important role in the biogenesis of mitochondria, and the loss of this function rather than the anti-apoptotic function of Mcl-1 may be the cause of embryonic lethality in the Mcl-1 knockout mouse, and cardiomyopathy in the cardio-specific knockout mouse, and possibly in the development and maintenance of HPS and lymphocyte progenitor cells (Opferman, Iwasaki, & Ong, 2005; Rasmussen et al., 2018).

The full-length 38 kDa Mcl-1 in both humans and mice localizes to the mitochondrial outer-membrane (Yang, Kozopas, & Craig, 1995). Mcl-1S, a pro-apoptotic BH3-only splice variant, was isolated from human placenta (Bae, Leo, & Yu, 2000). Furthermore, there are N-terminally truncated slightly shorter Mcl-1 isoforms in humans and mice, designated Mcl-1ΔN (De Biasio et al., 2007; Perciavalle et al., 2012), which cannot function as anti-apoptotic proteins. Using isolated mouse mitochondria, Perciavalle and co-workers showed that this processed Mcl-1ΔN translocates to the mitochondrial inner membrane facing the matrix side (Perciavalle et al., 2012), and they argued that the absence of Mcl-1ΔN may be the reason for the peri-implantation lethality of Mcl-1 knockout blastocysts (Perciavalle & Opferman, 2014; Rinkenberger et al., 1993). Even though these cells fail at peri-implantation, it is possible to recover embryos at E3.5–4.0, and generate MEFs from them (Rinkenberger et al., 1993). In Mcl-1 KO MEFs, mitochondria have poorly developed and disorganized cristae structure. In contrast, WT MEFs express both Mcl-1 and Mcl-1ΔN and their mitochondria have deeply invaginated cristae, making the mitochondria more efficient for oxidative phosphorylation and for the production and delivery of energy. Furthermore, there are indications that cells with mitochondria efficient in oxidative phosphorylation are required in peri-implantation blastocysts (Van Blerkom, Cox, & Davis, 2006). Perciavalle and co-workers suggested that Mcl-1ΔN, located in the inner mitochondrial membrane, brings respiratory chains I- IV closer together, increasing the efficiency of oxidative phosphorylation and thus facilitating the metabolic conversion necessary for survival (Perciavalle & Opferman, 2014).

The human mantel cell lymphoma-derived cell line, Z138, also makes a conversion from aerobic glycolysis to oxidative phosphorylation by increasing cristae density and cytochrome *c* content (Robinson, Dinsdale, MacFarlane, & Cain, 2012). Z318 can be converted by simply culturing cells in glucose-free medium supplemented with glutamine, alanine and sodium pyruvate. However, Mcl-1 and Mcl-1ΔN were observed apparently in the same ratio in Z318 cells regardless of the metabolic state and mitochondrial morphology, suggesting that the presence of Mcl-1ΔN is not sufficient to make the conversion (Robinson et al., 2012). Since the loss of Mcl-1 also causes failure in many mitochondrial functions, such as mitochondrial fusion (Perciavalle et al., 2012), the failure to generate Mcl-1ΔN may not be the direct cause for the failure to switch from aerobic glycolysis to oxidative phosphorylation. In any case, the presence of Mcl-1, and not necessarily Mcl-1ΔN, may be required for some cells to survive in a low glucose condition, and that is a function of Mcl-1 different from its anti-apoptotic function. Indeed, cardiac specific deletion of Mcl-1 in mice causes lethal cardiac failure in the absence of standard apoptotic markers in the dying cells (Thomas et al., 2013; Wang et al., 2013). In contrast, mitochondria from Mcl-1 depleted heart muscle cells exhibited reduced respiration and limited Ca2 + −mediated swelling (Thomas et al., 2013), suggesting that heart muscle cells died not from activated Bak/Bax, but because they were incapable of surviving in the absence of properly functioning mitochondria.

There are contradictory reports providing evidence for cleavage of Mcl-1 at different residues and by different proteases (Huang & Yang-Yen, 2010; Perciavalle et al., 2012; Warr et al., 2011). If a mutation in the MCL-1 gene is introduced, using for example CRISPR technology, making the protein resistant for Mcl-1ΔN cleavage, then whether Mcl-1ΔN is necessary for metabolic conversion can be tested.

3. Safety of S63845

S63845 is a specific molecular inhibitor of Mcl-1 that binds to the BH3 domain and inactivates the anti-apoptotic function of Mcl-1, but probably not Mcl-1's role in mitochondrial biogenesis (Perciavalle et al., 2012). Furthermore, the inhibitory effect of the anti-apoptotic function of Mcl-1 is of limited duration (Kotschy et al., 2016). It seems possible then, that hematopoietic stem cells and lymphocyte progenitor cells may withstand the temporal absence of Mcl-1 functions in mice injected with S63845. Thus, it is not surprising that S63845 is reported to have a high safety dose, well above the minimum effective dose in mice (Kotschy et al., 2016), suggesting that at least a short term loss of the anti-apoptotic function of Mcl-1 would not cause much damage to hematopoietic stem cells and T and B lymphocyte progenitor cells. It would be of great interest to know how well adult mice can tolerate

repeated injections of S63845, especially in the heart and whether S63845 affects the development and maintenance of T/B lymphocytes.

4. Inducing apoptosis by inhibiting anti-apoptotic factors

When isolated mitochondria from mouse liver, Xenopus eggs, human and mouse tissue culture cells, cancerous or otherwise, are incubated with BH3 only proteins such as truncated Bid or BimS, or BH3 peptides, they release Bak/Bax proteins residing on mitochondria from their association with Mcl-1, Bcl-xL and other anti-apoptotic proteins (Susnowa, Zhangb, Margineantub, & Hockenbery, 2009). Bak/Bax molecules become spontaneously oligomerized, releasing cytochrome *c*, Htra2/Omi and SMAC/DIABLO proteins and other intermembrane space proteins within minutes (Yamaguchi et al., 2008). There are no caspases in the solution of isolated mitochondria, and caspase inhibitors such as zVAD would not block Bak/Bax oligomerization nor release intermembrane space proteins. EM pictures taken before and after treatment with BH3-only proteins or peptides showed no breakage or rupture in the outer membranes. Under a somewhat more artificial system in which components of mitochondrial outer membranes were assembled into cytochrome *c* containing vesicles, the addition of BH3-only proteins and peptides caused rings to form on the vesicles large enough for cytochrome *c* to escape (Kuwana, Olson, Kiosses, Peters, & Newmeyer, 2016). It seems reasonable to conclude that Bak/Bax released from Mcl-1 and Bcl-xL can spontaneously form pores or rings on the mitochondrial outer membranes and release cytochrome *c* into the cytosol. Thus, cytochrome *c* release from mitochondria is a very mechanistic process involving only a few proteins. This simple mechanism is likely to be present in almost all cells, including healthy cells.

The above experimental results can be recreated using intact cells. In one such experiment, using stapled-BH3 peptides that can release both Mcl-1 and Bcl-xL from Bak/Bax in isolated mitochondria, Walensky and colleagues induced apoptosis in various human leukemic cells by adding stapled-BH3 peptides in the medium (Walensky et al., 2004). Years later, Anthony Letai's group performed a series of experiments to test the efficacy of various BH3 peptides on cells isolated from mouse tissues, comparing BH3 peptides derived from Bid, Bim, and other pro-apoptotic proteins using the loss of mitochondrial transmembrane potential as a marker for apoptotic cells (Sarosiek et al., 2017). In these experiments, BH3 peptides killed many healthy mouse tissues, young and old, and the authors suggested that tissues in the young mice are more susceptible to apoptosis than those of the old mice (Ryan & Letai, 2014). However, it must be cautioned that in some cells, the transmembrane potential is maintained for hours after the appearance of standard apoptotic markers such as caspase 3 activation and PARP cleavage (Finucane, Waterhouse, Amarante-Mendes, Cotter, & Green, 1999), and much longer than the assay's end-point used by the Letai group. Thus, the observed differences in young and old tissues may not have to come from their resistance to apoptosis. For example, the age of tissues may make a difference in the efficiency of peptides crossing the plasma membranes. These issues were not properly addressed in these articles. In any case, it seems reasonable to conclude that BH3 peptides induce apoptosis in all sorts of healthy tissues.

When a BH3 peptide is replaced with a molecular antagonist of Bcl-2 and Bcl-xL, ABT-263, Bcl-xL dissociates from Bak/Bax and cytochrome *c* is released if Mcl-1 is depleted by IL-3 withdrawal in IL-3 dependent murine polymorphic cell lines, and in small-cell lung carcinoma cell lines that express little Mcl-1 (Maurer, Charvet, Wagman, Dejardin, & Green, 2006; Tse et al., 2008). An inhibitor of both Bcl-xL and Bcl-2, ABT-737 induced apoptosis by activating caspases in platelets (Mutlu, Gyulkhandanyan, Freedman, & Leytin, 2012), even though platelets were unintended targets in these experiments because platelets do not express Mcl-1 (Debrincat et al., 2012). In another set of experiments, a molecular antagonist for Mcl-1, S63845, induced the release of cytochrome *c* in human myeloma cell line H929 (Kotschy et al., 2016), which expresses little Bcl-xL (Punnoose et al., 2016). In these experiments, S63845-induced cell death was caspase-dependent, suggesting that the death was caused by the release of cytochrome *c* and subsequent activation of apoptosomes.

Punnoose and co-workers treated human myeloma cell lines expressing various amounts of anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-xL with Bcl-2 specific ABT-199 (venetoclax), Bcl-xL selective inhibitor A-1155463, and ABT-263 (navitoclax) that inhibits both Bcl-2 and Bcl-xL, and found that Bcl-2 and Bcl-xL can competitively inhibit apoptosis (Punnoose et al., 2016), suggesting that by inhibiting all three anti-apoptotic proteins, apoptosis could be induced in most if not all myelomas.

Neither Mcl-1 nor Bcl-xL is essential in the sense that some cells can survive without them. But could there be Bak/Bax expressing cells that do not express Bak/Bax inhibitors? This theoretical question cannot be definitively answered, but it is known that to survive the forced overexpression of Bak or Bax, HEK293T cells express Mcl-1 or Bcl2L10 (Zhai, Jin, Huang, Satterthwait, & Reed, 2008), suggesting that pro-apoptotic Bak/Bax needs to be sequestered. (Expression of Bcl2L10 is found in limited tissues, and absent in the bone marrow/immune system, muscle, lung and pancreas. Among various cancer types, it is found only in renal, thyroid, liver and pancreatic cancers according to the Human Protein Atlas - www.proteinatlas.org).

In summary, it seems reasonable to assume that simultaneous inactivation of all the anti-apoptotic proteins could induce apoptosis in most cells, if not all cells. Furthermore, treating cancer cells either with S63845, or with ABT-263 would be like turning on one of the two switches that must be turned on to induce apoptosis (Fig. 1).

5. Safety of the S63845-ABT263 combination therapy

The first question to address is the safety of S63845 with ABT-263. Application of ABT-263 alone is known to transiently cause thrombocytopenia and lymphopenia (Gandhi et al., 2011; Shoemaker et al., 2008), which was to be expected from the mechanistic action of ABT-263. Application of S63845 alone, on the other hand, showed surprisingly few toxicities (Kotschy et al., 2016). As discussed earlier, based on genetic studies, at the least, we expected altered hematopoietic cell distributions. One explanation for the discrepancy is that the role of Mcl-1 as a modifier of mitochondrial biogenesis during hematopoietic development is more important, and that function is not affected by S63845. Another explanation may be that genetic modification causes the total and sustained absence of Mcl-1 in affected cells, while the effect of S63845 is transient and only affects the anti-apoptotic role of Mcl-1.

Since S63845 was found to be relatively safe to use in animals, there has been numerous studies combining S63845 with currently existing chemotherapeutic agents (Merino et al., 2017). Not surprisingly, they all showed improved outcomes. But notable in its absence was the combination of S63845 with ABT-263, possibly because of their toxicity in animals.

There is strong evidence that silencing both MCL-1 and BCL-xL could cause apoptosis in a wide variety of tissues and cell lines, including liver (Hikita et al., 2009), and megakaryocytes (Kodama et al., 2012). In megakaryocytes, when Mcl-1 and Bcl-xL were specifically deleted in Bak/Bax double deficient mice, the viability of the cell was fully restored, indicating that the loss of the anti-apoptotic functions of Mcl-1 and Bcl-xL caused apoptosis. Thus, the combination of S63845 and ABT-263 could cause not only loss of platelets but could also eliminate megakaryocytes that could generate and restore platelets in circulation. Combined with apoptosis in the liver, S63845 and ABT-263 will be a very toxic mix. But what is really dangerous is that the combination could eliminate various stem cells, making the restoration of tissues impossible.

6. Targeting subsets of Bcl-2 proteins

In the previous section, we presented the idea that by dissociating all inhibitory proteins from the Bak/Bax complex using S63845-ABT263,

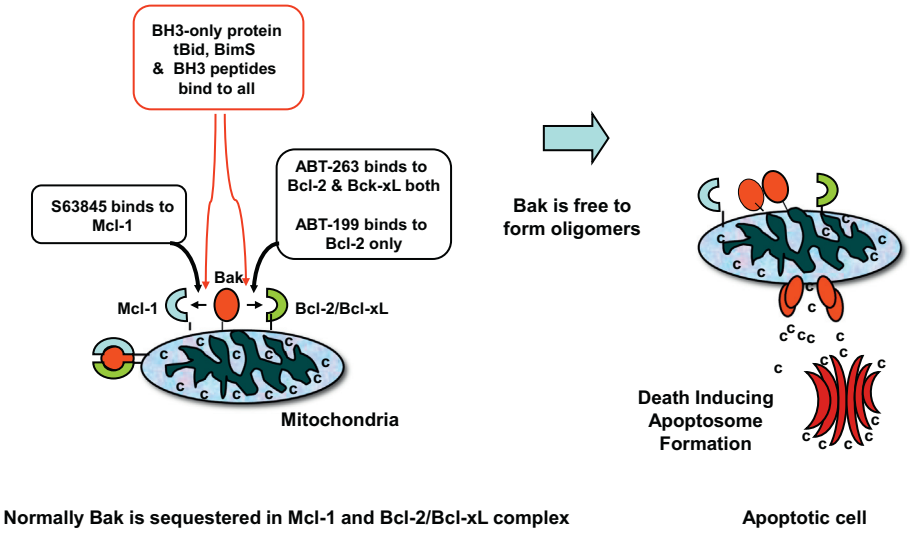

Fig. 1. Dual Switch for Mitochondria-Dependent Apoptosis.

apoptosis could be induced in nearly all cells. The question we pose here is, "If subsets of inhibitory proteins were targeted instead, would apoptosis be induced in particular cancer types?" Targeting a subset complicates the picture because it is no longer Mcl-1 vs. the rest of the Bcl-2 proteins complexed to Bak/Bax inactivated by S63845 and ABT-263. The question we pose, must address the situation where a combination of agents inhibiting Mcl-1 and Bcl-2 while leaving BcL-xL uninhibited would induce apoptosis. The question can be tested by using recently developed specific inhibitors of Mcl-1, Bcl-2 and Bcl-xL. The short answer is that it is possible to dissociate enough inhibitory molecules from the Bak/Bax complex so that a sufficient number of Bak/Bax molecules is activated by finding other activated Bak/Bax molecules in the vicinity and forming oligomers large enough to form a pore and thus be capable of releasing cytochrome *c* from mitochondria, inducing apoptosis. Choosing the right inhibitor combination to induce apoptosis for a particular cancer type, however, would be difficult. Ramsey and coworkers suggest that BH3 profiling of patient samples might be helpful to determine the right drug or combination to use (Ryan & Letai, 2014). However, it seems just as easy to employ a fluorescence-based caspase assay applied to cancer cells using a panel of Bcl-2 family targeted inhibitors either as a single agent or in combination. This way, the actual reagents instead of BH3 mimetics will be tested. These apoptosis inducers activate caspase usually within 3 h (Yamaguchi et al., 2011), which can easily be measured by mixing Ac-DEVD-AFC with lysates in a kinetic assay.

To understand what is taking place inside the cancer cell at the molecular level, however, we compare the efficacy of inhibitors as single agents and in combination with the expression pattern of Bcl-2 family member proteins. Thus, we present some concrete examples. First, platelets express only Bcl-xL to protect Bak from being activated (Vogler et al., 2015). Even in platelets, a sufficient amount of ABT-263 must be applied so that a sufficient number of Bak protein molecules are freed to form oligomers large enough to release cytochrome *c*. In the second example, Li and colleagues showed that T-cell acute lymphoblastic leukemia (T-ALL) cell lines RPMI-8402 and MOLT-3 expressed Mcl-1 as well as small amounts of Bcl-xL and Bcl-2 + Bcl-xL respectively. Treatment with S63845 was enough to induce apoptosis in both cell lines (Li, He, & Look, 2018). In the third example, when the myeloma cell line EJM expressed about the same amount of Bcl-2 and Bcl-xL, and expressed a much smaller amount of Mcl-1, 10 nM of ABT-263 was sufficient to induce apoptosis (Punnoose et al., 2016). However, the Bcl-2 specific inhibitor ABT-199 could not induce apoptosis even at 30 time higher concentration (Punnoose et al., 2016), presumably because it could release only a subset of Bak/Bax molecules inhibited by Bcl-2. In the fourth example, in the T-ALL cell line KOPT-K1, Bcl-xL, Bcl-2 and Mcl-1 are all expressed (Li et al., 2018). We could predict that single-agent applications of either S63845 or ABT-199 would not be efficient in inducing apoptosis because of the large amount of Bcl-xL expressed in these cells. However, it is not easy to predict whether the S63845-(ABT-199) combination would release enough Bak/Bax molecules to induce apoptosis efficiently, but that is what they found. However, we speculate that a prolonged treatment may select for cells with slightly elevated Bcl-xL expression, and may generate treatment-resistant colonies. These examples are summarized in Fig. 2a.

Based on the above observation, we present our model for the inhibitor-combination induced apoptosis (Fig. 2b). First, Bak/Bax must be freed from its inhibitors. Then the freed and activated Bak/Bax needs to find other freed Bak/Bax in the vicinity so that they can form a dimer. Our model is that the Bak/Bax dimer assumes a curved structure. And that process continues till the Bak/Bax oligomer becomes large enough to form a pore through which cytochrome *c* molecules can escape into the cytosol. There are other models of how Bak/Bax oligomerizes to form a pore (Westphal, Dewson, Czabotar, & Kluck, 2011; Willis, Chen, Dewson, et al., 2005). In all these models, a minimum number of freed Bak/Bax molecules is required to form a pore large enough to release cytochrome *c*. Thus, it is likely that for each cancer cell type, there is a threshold number of activated Bak/Bax molecules to form an oligomeric pore capable of releasing cytochrome *c* from mitochondria, and it does not matter what agents have freed Bak/Bax as long as the threshold amount of Bak/Bax molecules has been reached (see Fig. 2b). However, it is difficult to determine what the threshold would be, first, because determining the amount of freed Bak/Bax molecules is technically difficult, and second, because some cells have a feed-back mechanism for released cytochrome *c*; a small amount of released cytochrome *c* molecules can activate small amounts of caspase molecules, which could cleave and activate Bid or Bim proteins, converting them into BH3 only proteins (Yamaguchi et al., 2011). This feed-back mechanism exists in most cells, making apoptosis rapid and efficient, and becomes a factor when trying to determine the Bak/Bax thresholds in apoptosis, but it is not a necessary component of apoptotic cascades (Kodama et al., 2011). Thus, except for very simple cases, it is often not easy to assemble a subset of inhibitors that could induce apoptosis in a particular cancer cell line from examining its proteome. Furthermore, in a given cancer type such as T-ALL, cell lines generated from it show complex and varied Mcl-1, Bcl-2 and Bcl-xL expression profiles, making it difficult to predict what combination of inhibitory agents could be effective for each clinical case (Punnoose et al., 2016). It seems easier to isolate cancer cells and test

(a)

Cell	Mcl-1	Bcl-2	Bcl-xL	Apop inducing agent
Platelet	-	-	+++	ABT-263
RPMI-8402	++	-	+	S63845
MOLT-3	++	+	+	S63845
EMJ	+	+++	++	ABT-263
KOPT-K1	+	++	+++	S63845+ABT-199*

* Presumably ABT-263 would work as well

(b)

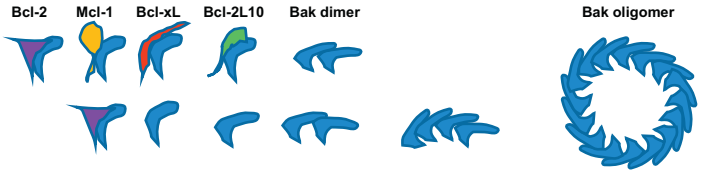

Fig. 2. Bak/Bax inhibitors and apoptosis-inducing agents. (a) Cell types with Mcl-1, Bcl-2 and Bcl-xL expression levels and likely most effective apoptosis-inducing agents. (b) Model of Bak sequestered with Bcl-2 family members and oligomerized Bak (using the asymmetric model of Bak oligomerization (Shamas-Din, Kale, Leber, & Andrews, 2013)). Upon release of the Bcl-2 family member from Bak, Bak molecules rotate and aggregate in a curving pattern that with enough molecules then closes on itself to form a pore in the mitochondrial membrane.

them in vitro with various inhibitor combinations using a fluorescence-based caspase assay.

7. Combination vs. sequential administration of inhibitors

A priori, it is difficult to determine which healthy tissues would be affected by a partial inhibition of Bcl-2 family members. But since the safety of each of these new inhibitors has been extensively studied, and if we apply them sequentially rather than apply them in combination, safety is enhanced. Li and colleagues showed that for the T-ALL cell line, KOPT-KI, it took 444 nM of ABT-199 and 111 nM S63845, used as single agents, to reduce the viability to 50% (Li et al., 2018), and the sequential application of these agents would most likely achieve 25% viability. Used in combination, however, the viability was below 1%. Furthermore, the combination of 20 nM ABT-199 and 5 nM S63845 reduced the viability to 50%. This encouraging result is due to the synergy between these two agents that happens only when two agents are applied simultaneously. In clinical settings, most cancer is a heterogeneous mix of variously mutated cancer cells. It is expected that when ABT-199 and S63845 are applied sequentially, the cells expressing almost exclusively Bcl-2 and the cells expressing almost exclusively Mcl-1 would be nearly totally eliminated. The remaining cancer cells would have only moderate amounts of Mcl-1 and Bcl-2, but they could also express any amount of Bcl-xL. Thus, there is no assurance that the remaining cells would be eliminated by the S63845-(ABT-199) combination, or by ABT-263 alone.

8. Safety of the combination therapy

When trying to predict the safety of the S63845-(ABT-199) therapy, we face exactly the same problem in trying to predict the efficacy of the combination treatment in animals, i.e., unknown toxic effects of the combination on healthy cells and lack of reliable proteomic biomarkers. Thus, systematic tests of the combination in animals are warranted. The combination of VU661013 with ABT-199, and combination of S63845 with S55746, both targeting Mcl-1 and Bcl-2, showed no obvious adverse effect in mice (Moujalled et al., 2018; Ramsey et al., 2018), suggesting that the Mcl1-Bcl2 inhibitor combination may not cause severe damage to tissues. As for other combinations, such as S63845 with ABT-263, we already expect some adverse effects, but these should be tested in animals as well.

To summarize, inhibiting subsets of Bak/Bax inhibitory proteins could induce apoptosis as long as enough Bak/Bax molecules are freed, but it is often difficult to predict the efficiency of a particular inhibitor combination based on proteomic data alone. And it is even more difficult to predict the safety of the treatment because the effects of the combination in every tissue in the body need to be predicted.

9. Other means of disrupting the (Mcl-1)-(Bak/Bax) complex

To restrict the effect of combining Mcl-1 inhibitors with Bcl-xL inhibitors, researchers focused on Mcl-1. This is because the Mcl-1 protein has a relatively short half-life in most cells (Maurer et al., 2006; Nijhawan et al., 2003). Also, the association between Bak/Bax and Mcl-1 seems to be regulated at multiple levels: at transcriptional levels of Mcl-1 (Bae et al., 2000; Wang et al., 1999), at post-transcriptional levels of Mcl-1 (Harrison et al., 2011), and post-translational levels of Mcl-1 (De Biasio et al., 2007; Maurer et al., 2006; Mojsa, Lassot, & Desagher, 2014; Zhao et al., 2007), which includes ubiquitination and protein cleavage. The molecular mechanisms underlying these processes are well studied. On the other hand, there are unusual and poorly studied phenomena such as cell-detachment that dissociates Mcl-1 from Bak and its subsequent association with Caveolin 1 (Boisvert-Adamo, Longmate, Abel, & Aplin, 2009; Chunhacha, Pongrakhananon, Rojanasakul, & Chanvorachote, 2012; Woods, Yamaguchi, Lee, Bhalla, & Wang, 2007), and Mcl-1 does not seem to associate with Bak/Bax in HL60 human promyelocytic leukemia (Ma et al., 2014). Among the elements controlling Mcl-1 and Bak/Bax association, mechanisms unique to cancer cells need to be identified so that Mcl-1 can be dissociated from Bak/Bax, priming cancer cells for ABT-263 induced apoptosis.

One of the obvious choices to disrupt Mcl-1 Bak/Bax association is IL-3. Some lymphocytes and leukemia cells are dependent on IL-3 for survival. Without this signal, Mcl-1 would be phosphorylated, ubiquinated and degraded, thus making cells sensitive to Bcl-2/Bcl-xL inhibitors (Tse et al., 2008). Extending this line of thought, some cancer cells are dependent on certain growth factors and in some cases, these growth signals drive Mcl-1 transcription. Thus, blocking the growth signals can make these cells susceptible to ABT-263 (Booy, Henson, & Gibson, 2011; Henson, Hu, & Gibson, 2006; Huang, Huang, & Yen, 2000; Schacter, Henson, & Gibson, 2014). However, in most of these cases, blocking growth factors only reduced amounts of Mcl-1 expressed, and induction of apoptosis by Bcl-xL inhibitors was not very efficient. There were attempts at inhibiting down-stream effectors of the growth factors; they usually bifurcate into two signaling cascades – MAPK pathways and PI3K-AKT pathways (Yamaguchi & Perkins, 2017a). Inhibiting PI3K, however, tends to be very toxic because PI3K is activated in healthy growing cells. In contrast, inhibiting MAPK pathways could cause cell cycle arrest (Yamaguchi & Perkins, 2017b). Since ABT-263 can induce

apoptosis in Mcl-1 depleted cells even when cells are arrested in G1 or even in quiescence (Lagadinou et al., 2013; Zeuner et al., 2014), it should cause no problem inducing apoptosis in MAPK inhibited cells. However, the PI3K pathways can also induce Mcl-1 expression (Choudhary et al., 2015), reducing the rates of ABT-263 induced apoptosis. Thus, reducing Mcl-1 protein levels by interfering with the PI3K pathways or MAPK pathways would be less efficient and less specific than inhibiting the growth signal receptor specific for the cancer.

All these approaches seek to reduce Mcl-1 protein levels in cancer cells. A totally different approach would be to convert Mcl-1 with its anti-apoptotic function into Mcl-1ΔN, which does not function as an anti-apoptotic protein. This can be done by treating cells with carbonyl cyanide chlorophenylhydrazone (CCCP), an inhibitor of oxidative phosphorylation (Warr et al., 2011), leaving the cell with only Bcl-2/Bcl-xL to sequester Bak/Bax and prevent apoptosis. However, since CCCP reduces the transmembrane potential of mitochondria, it could trigger Parkin-mediated mitophagy (Tanaka et al., 2010), which is incompatible with mitochondria-dependent apoptosis because mitochondria engulfed in phagosomes or autolysosomes cannot release cytochrome *c* into the cytosol (Zhu et al., 2013).

Yet another approach seeks to dissociate Mcl-1 from Bak/Bax by treating cells with a 2-deoxyglucose:glucose (2DG:Glu) mix plus beta-cyclodextrin (βCD). The 2DG:Glu mix is used to partially reduce hexokinase activity without inducing mitophagy, and βCD is used to sequester cholesterol at the plasma membrane so that signaling between PI3K and AKT is interrupted without causing toxicity to the cell (Yamaguchi, Perkins, & Hirota, 2015). This combination dissociates Mcl-1 from Bak/Bax very quickly, sensitizing the cell for ABT-263 induced apoptosis. This approach has an added advantage that cells exposed to all the reagents can be restricted to cancer cells outside the brain. This is because in the body, 2DG:Glu is taken up by cells with elevated glucose uptake, i.e., brain cells, muscle cells after heavy exercise, cells in inflamed tissues, and cancer cells, while βCD and ABT-263 cannot cross the blood-brain barrier. Thus, by avoiding heavy exercise and controlling for inflammation, cells exposed to all the agents can be restricted to cancer cells outside the brain. On the other hand, some cancer cells, such as about 90% of pancreatic cancer cells, express P-glycoprotein, a component of the blood-brain barrier, making ABT-263 inaccessible (Driscoll, Walsh, Larkin, Ballot, & Ooi, 2007). Furthermore, there are two more drawbacks. First, ABT-263 induces apoptosis in platelets, and the dual inhibition of Mcl-1 and Bcl-xL could induce apoptosis in GLUT1 and GLUT3 expressing maturing megakaryocytes (Fidler et al., 2017), the source of platelets. To recover from the loss of both platelets and megakaryocytes might take a week or longer, forcing the treatment to be spaced. Another drawback is that among growing malignant tumors, there may be small dormant tumors having cells with characteristics of a stem cell (Yamaguchi & Perkins, 2018) that would evade detection by natural killer cells. Massague's group called these cells, latency competent cells (LCC) (Malladi et al., 2016). Because LCC cells are not glycolytic enough to be affected by the (2DG:Glu)-(βCD)-(ABT-263) combination, they may not be induced into apoptosis. Thus, a separate therapy may be needed to induce apoptosis in these cancer stem cells.

Malignant pleural mesothelioma is an intractable cancer with no established treatment caused by long-term exposure to asbestos (Røe & Stella, 2015). There are three morphologically distinct forms of mesotheliomas: epitheloid, sarcoatoid, and biphasic, a mixture of sarcoatoid and epitheloid. Genes whose DNA has been mutated in mesotheliomas have been found in several signaling pathways, including the DNA damage response pathway, the cell cycle pathway, the MAPK-pathways, and the PI3K-AKT pathways. Mutations in multiple pathways are often found in the same patient, making it difficult to narrow down the targets in therapy to one or two (Hylebos, Van Camp, & Van Meerbeeck, 2016). It is this kind of cancer that could benefit from the combination of Mcl-1 inhibitors and Bcl-xL inhibitors. It is further noted that mesotheliomas are easily detectable with an FDG-PET scan because these tumors have elevated glucose-uptake. In conclusion, it is anticipated that the dual inhibition of Mcl-1 and Bcl-2/Bcl-xL via (2DG:Glu)-(βCD)-(ABT-263) combination therapy will become a viable option for difficult-to-treat solid tumors such as mesothelioma.

Conflict of interest

The authors declare that there are no conflicts of interest.

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