Molecular Pathways: Leveraging the BCL-2 Interactome to Kill Cancer Cells—Mitochondrial Outer Membrane Permeabilization and Beyond

Hetal Brahmmbhatt1,2, Sina Oppermann2, Elizabeth J. Osterlund2,3, Brian Leber4, and David W. Andrews1,2,3

Abstract

The inhibition of apoptosis enables the survival and proliferation of tumors and contributes to resistance to conventional chemotherapy agents and is therefore a very promising avenue for the development of new agents that will enhance current cancer therapies. The BCL-2 family proteins orchestrate apoptosis at the mitochondria and endoplasmic reticulum and are involved in other processes such as autophagy and unfolded protein response (UPR) that lead to different types of cell death. Over the past decade, significant efforts have been made to restore apoptosis using small molecules that modulate the activity of BCL-2 family proteins. The small molecule ABT-199, which antagonizes the activity of BCL-2, is currently the furthest in clinical trials and shows promising activity in many lymphoid malignancies as a single agent and in combination with conventional chemotherapy agents. Here, we discuss strategies to improve the specificity of pharmacologically modulating various antiapoptotic BCL-2 family proteins, review additional BCL-2 family protein interactions that can be exploited for the improvement of conventional anticancer therapies, and highlight important points of consideration for assessing the activity of small-molecule BCL-2 family protein modulators. Clin Cancer Res; 21(12): 2671–6. ©2015 AACR.

Background

Tumorigenesis is a complex multistep process that occurs when normal cells acquire genetic or epigenetic alterations that cause dysregulated cell proliferation. Preventing apoptosis is an essential component of this process, as programmed cell death is frequently activated in response to such transformations (1). The effect of most cytotoxic chemotherapy agents that impair DNA synthesis or mitosis is to restore apoptosis. However, this strategy is often ineffective, as many tumors have acquired aberrant apoptotic signaling either during initiation or clonal progression (2). Using drugs that specifically target key components of these blocked apoptotic pathways may kill tumor cells while minimizing the severe side effects on normal cells, and therefore represents a promising strategy to improve the efficacy and specificity of anticancer therapies.

Apoptosis can be initiated via two pathways, the extrinsic and the intrinsic pathway. Whereas the extrinsic pathway is initiated through stimulation of cell surface death receptors, the intrinsic pathway is activated by many different intracellular stresses and is elicited by most chemotherapeutic agents. The BCL-2 family proteins are predominantly involved in the intrinsic pathway, in which they regulate mitochondrial outer membrane permeabilization (MOMP). MOMP results in the release of apoptosis-triggering factors, such as cytochrome c, from the mitochondrial intermembrane space into the cytoplasm in which they activate a cascade of caspases that execute widespread proteolytic events leading to cellular demise (3). MOMP marks commitment to death and is often considered to be the point of no return for a cell. Here, we review the rationale and preliminary results of recent efforts to elicit MOMP by exploiting BCL-2 family proteins as an anticancer therapy and discuss other related targets for drug development.

The BCL-2 family interactome and cancer

Over 25 BCL-2 family proteins have been identified and are classified into three groups based on function and the presence of conserved BCL-2 homology (BH) regions: (i) proapoptotic multiregion BAX and BAK oligomerize to form pores that permeabilize the mitochondrial outer membrane (MOM); (ii) proapoptotic BH3 proteins such as BID, BIM, BAD, NOXA, and PUMA are activated by various stress stimuli and directly (activator BH3) or indirectly (sensitizer BH3) activate BAX and BAK; and (iii) antiapoptotic multiregion proteins such as BCL-2, BCL-XL, and MCL-1 inhibit both classes of proapoptotic proteins. The BCL-2 family proteins are predominantly localized to the mitochondria, endoplasmic reticulum (ER) and perinuclear membrane.

The membrane is the locus of interaction and an active regulator governing binding interactions between among BCL-2 family proteins (4). Upon binding to membranes, BCL-2 family proteins undergo conformational changes that affect subsequent binding interactions. The process is initiated when activator BH3 proteins are stimulated by cellular stress signals and rapidly

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translocate to the membranes where they interact with cytoplasmic BAX or membrane-bound BAK. As a result of this binding, BAX inserts into the membrane, and both BAX and BAK undergo a series of conformational changes that allow these proteins to oligomerize, thereby forming pores which cause MOMP (5, 6). Antiapoptotic proteins inhibit activator BH3 proteins as well as activated BAX/BAK at the membrane (7, 8). Inhibition of BH3 proteins when they are sequestered by antiapoptotic proteins is called Mode 1 inhibition, and inhibition of activated BAX/BAK by antiapoptotic proteins is designated as Mode 2 (9). Sensitizer proteins function to displace activator BH3 proteins or activated BAX/BAK from antiapoptotic proteins (Fig. 1).

On the basis of the specific requirements to induce MOMP, the blocks in apoptosis that confer tumor survival can be subdivided into three classes (10). Mitochondria with a class A block have insufficient activated BH3 proteins, or all available activator BH3 proteins are sequestered by the antiapoptotic members (Mode 1 inhibition), resulting in insufficient MOMP. Mitochondria with a class B block have inactive or low levels of BAX and BAK and remain insensitive to MOMP induction even in the presence of activator BH3 proteins. Because of repeat sequences, BAX is susceptible to inactivation by microsatellite insertions (11). Mitochondria with a class C block overexpress antiapoptotic BCL-2 family members that sequester and inhibit activator BH3 proteins and activated BAX and BAK (Mode 1 or Mode 2). Overexpression of BCL-2, BCL-XL, and MCL-1 is observed in many cancers and is associated with poor survival and resistance to therapy (12, 13).

Cancer cells with a class C block can be considered “addicted” to high levels of the antiapoptotic BCL-2 proteins for survival (14). Selective antagonism of these antiapoptotic proteins using BH3 mimetics should overcome this block with reduced toxicities to normal cells. Because antiapoptotic proteins have different affinities for BH3 proteins (e.g., BCL-2 and BCL-XL bind to BAD but not NOXA whereas MCL-1 binds to NOXA but not BAD), identifying which inhibitor is involved could theoretically lead to more selective therapy (14). Using peptides from

Figure 1. Schematic of the BCL-2 family interactions at the mitochondria and ER. BCL-2 family proteins are involved in cellular processes such as apoptosis, autophagy, and the UPR, and localize at the mitochondria and ER. Various stress stimuli activate BH3 proteins. Activator BH3 proteins (BH3s) translocate to the membrane to activate BAX. BAX translocates and inserts into the MOM, where it oligomerizes to form pores releasing cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Antiapoptotic proteins function by binding and sequestering activator BH3 proteins and activated BAX at the membrane. Sensitizer BH3 proteins (BH3s) displace activator BH3 proteins or activated BAX from antiapoptotic proteins. The interactions at the ER are not well studied and the model at the ER is more speculative. At the ER, the accumulation of unfolded proteins triggers the UPR, which is mediated by IRE-1. BAX interacts with IRE-1 to sustain IRE-1 activation. In addition, pore formation by BAX oligomerization at the ER results in the release of calcium. BCL-2 interacts with IP3R to inhibit the release of calcium and with BECLIN-1 preventing autophagosome assembly to inhibit autophagy; IMM, inner mitochondrial membrane.
specific BH3 proteins to elicit MOMP in clinical cancer samples in vitro is a powerful tool to tailor patient-specific treatment approaches (10).

**Clinical–Translational Advances**

The BCL-2–BAD interaction at mitochondria: a promising clinical target

There is a strong rationale to interfere with antiapoptotic BCL-2 family members to induce selective apoptosis in cancer cells. Downregulating the expression of the prosurvival BCL-2 family proteins is one approach. Indeed, as might be expected, conventional chemotherapy drugs often modulate the expression of the BCL-2 family proteins as an indirect downstream consequence (Table 1). The first intentional approach used antisense oligonucleotide targeting of BCL-2 mRNA. This agent, oblimersen sodium, showed moderate activity in phase III clinical trials for melanoma, chronic lymphocytic leukemia (CLL), and multiple myeloma but was not approved by the FDA when it failed to reach primary survival endpoints in a phase III melanoma trial (15). Because antisense oligonucleotide therapies are limited by poor drug delivery and pharmacokinetics, modulating the interaction of BCL-2 family proteins presents an attractive alternative approach when potentially targetable binding interfaces are known.

Thus, the cytoplasmic structures of the BCL-2 family complexed with BH3 peptides from BH3 proteins have guided the discovery of small molecules that disrupt heterodimeric interactions between antiapoptotic proteins and their binding partners. The small molecules compete with the BH3 region of BH3 proteins for binding to a hydrophobic groove formed by the BH1-3 regions of the multiregion antiapoptotic proteins (16). High-throughput screening techniques and structure-based design have resulted in identification of several small-molecule antiapoptotic protein inhibitors that have relevant clinical activity when used as single agents or in combination therapy (17). Agents such as obatoclax and gossypol (AT-101) have shown some activity in phase II clinical trials as single agents and in combination with chemotherapy (Table 1; ref. 18). Unfortunately, many of these compounds have off-target cytotoxicity causing cell death even in the absence of BAX and BAK that will likely severely impair cancer cell specificity (19).

Exceptions to this observation are the AbbVie compounds. The lead compound ABT-737 was designed using nuclear magnetic resonance structure-based screening to mimic the BH3 region of BCL-2 and was dose-dependent thrombocytopenia due to on-target inhibition of BCL-XL in platelets (24). This problem was circumvented by the development of a related compound, ABT-199, that selectively targets BCL-2 not BCL-XL, and therefore does not cause thrombocytopenia (25). Although ABT-199 is active as a single agent in lymphoid malignancies such as CLL and non-Hodgkin lymphoma, tumors addicted to BCL-XL will presumably be resistant, and this remains an unmet medical need (26). Nevertheless, a strong measure of the efficacy of ABT-199 in lymphoid malignancies and the biologic importance of BCL-2 addiction was the

<table>
<thead>
<tr>
<th>Type of anticancer therapy</th>
<th>Class of anticancer drug</th>
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<td>ABT-737</td>
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<td>ABT-199</td>
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occurrence of tumor lysis syndrome in initial phase 1 trials in CLL. A gradual dose-escalation schedule has been designed and tested to prevent this and forms the basis of the current registration trials.

Targeting other BCL-2 family interactions

In live breast cancer cells, ABT-263 and ABT-737 disrupt the BCL-XL/BCL-2 complexes with BID but not with BIM at the mitochondria. This suggests that the binding properties of BIM to BCL-XL/BCL-2 differ compared with other BH3 proteins. Determining whether this is an inherent property of BIM at mitochondria or mediated by another binding site may provide useful leads for drug discovery, especially as the BIM–BCL-XL interaction does not mediate platelet survival (27).

Another important target that is not affected by ABT 263 or 199 is the antiapoptotic protein MCL-1, and tumors with higher levels of MCL-1 and lower BIM–MCL-1 ratio are associated with chemoresistance to ABT-263 treatment (12, 28, 29). MCL-1 is structurally unique from its prosurvival counterparts making targeting with small molecules challenging, as a consequence current lead compounds for MCL-1 inhibitors have weak binding affinities and lack convincing in vivo activities (30). The BH3 region recognizes four hydrophobic residue binding sites, P1–4 in the hydrophobic-binding groove. In comparison with BCL-XL, the P2 pocket of MCL-1 is larger, deeper, and expands in the presence of hydrophobic ligands; the P4 pocket is also more solvent exposed (31). ABT-737 was discovered by linking two moieties that bind the P2 and P4 pocket (20). Thus, to target MCL-1, it may be advantageous to link hydrophobic moieties that bind to the P2 while avoiding the P4 pocket to retain specificity to MCL-1. Another unique feature of MCL-1 among the antiapoptotic proteins is its short half-life due to constitutive ubiquitin-mediated proteasomal degradation (32). This is therapeutically exploitable, as many current drugs such as seliciclib and sorafenib, indirectly downregulate MCL-1 levels and may be particularly useful in combination with BCL-2 family inhibitors (33, 34).

As an alternative to targeting the antiapoptotic BCL-2 family proteins, pharmacologic activation of BAX and BAK might also serve as a promising strategy to overcome a class C block in cancer cells. In this case, the search would be for activators of these pore-forming proteins. Aside from the hydrophobic groove that binds to the BH3 region of activator proteins, an alternative binding region termed the “eat” pocket has been identified in BAX as the interaction site for BIM–BAX (35). Furthermore, after activation both BAX and BAK must oligomerize to form pores. On the basis of these multiple steps in the mediation of MOMP, several small molecules have been identified as modulators of BAX activity, each acting by a different putative mechanism that may be suited to specific combination chemotherapy with BCL-2 family inhibitors (36, 37).

The regulation of MOMP has attracted the most interest as a point of no return in cell death. However, many BCL-2 family members are also localized to the ER, where several processes relevant for cell survival are regulated by these proteins (Fig. 1). There is growing evidence that increased autophagy and activation of the unfolded protein response (UPR) drive the survival of carcinomas, leukemias, lymphomas, and gliomas in the tumor microenvironment (38). Preventing UPR may sensitize cancer cells and overcome resistance to other chemotherapy agents. BAX and BAK are inhibited by IRE-1, one of the three effector arms of UPR. Hence, either disrupting the IRE-1–BAX interaction or directly activating BAX to form pores to release intraluminal ER residing calcium may be particularly useful in cancers with constitutive UPR activation (39).

The BH3 protein BECLIN-1 is required for the initiation of autophagy but is inhibited by ER-localized BCL-2 (40). Autophagy is a survival response; however, prolonged stimulation leads to cell death (41). Disruption of the BECLIN-1–BCL-2 complex by the BH3 sensitizer protein, BAD, stimulates autophagy. Therefore, treatment with BAD mimetics, ABT-199 or ABT-737, should presumably have a similar effect. BAD mimetics alone may not be sufficient to push cells toward autophagic cell death, and consequently may confer stress tolerance and cell survival. Thus, a combination treatment with an additional autophagy-inducing agent may be more effective. In support of this strategy, ABT-737 and rapamycin showed synergistic cytotoxicity in breast cancers overexpressing BCL-2 (42). In contrast, combination treatment with an autophagy inhibitor to reduce the cytotoxic autaphagy effects of BAD mimetics may enhance apoptotic cell death induced by these agents (41). It remains to be resolved which is the better combination approach, and may be worth considering in tailoring patient treatments.

Another binding target of ER-localized BCL-2 is the 1,4,5-trisphosphate receptor (IP3R); this interaction suppresses calcium release and apoptotic signaling, and is mediated by the BH4 domain of BCL-2 binding to the regulatory coupling domain (RCD) of IP3R (43). Current BCL-2 inhibitors do not target the BH4 region. A 20–amino acid peptide of the RCD was able to disrupt the interaction between IP3R and BCL-2, and exposure to the RCD peptide significantly enhanced cell killing by ABT-737 in CLL cells (43). Because the peptide has poor cellular penetration, small peptidomimetic compounds represent a promising new approach.

Assessing apoptotic modulators in cells and tissues

Because BCL-2 family proteins adopt a different conformation in the membrane in vivo compared with isolated recombinant proteins and peptide fragments that have been used in the current screening approaches, some interactions may be missed, and the affinities of others underestimated or overestimated. For example, the detergents commonly used for immunoprecipitation to assess BCL-2 family interactions in cells can artificially create or abolish binding surfaces (4). An elegant alternative that avoids these problems is assessment of interactions by fluorescent lifetime imaging microscopy-Förster resonance energy transfer (FLIM-FRET) in live cells as we have described for BIM–BCL-XL above. This is a relatively labor-intensive process, but recent advances in fluorescent microscopy, the development of better fluorescent probes, and automated image analysis have now made high-throughput drug screening via FLIM-FRET possible (44). This technique has other desirable features, as it can be used to study interactions that have not yet been tested in vitro due to difficulty in purifying full-length proteins, and can be tailored to measure quantitatively interactions at different subcellular localizations.

In the near future, we anticipate that this technology will be able to measure protein–protein interactions in three-dimensional cultures of various cell types.

Although the circumvention of apoptosis is likely to be a widely applicable strategy to improve cancer therapy, a major clinical challenge arises from the fact that cancer is very variable genetically even within pathologic tumor subtypes, and this...
variability will contribute to differing responses to therapy. Recent attempts suggest that personalized prescriptions of anticancer drugs will be more effective than classic generic treatment protocols that are based on pathologic grade and stage. It is likely that drugs targeting the BCL-2 family proteins will have therapeutic benefit as they will synergize with conventional chemotherapies that indirectly modulate BCL-2 family protein expression (Table 1). Automated high-throughput screening of drug libraries can be used for quantitative analysis of drug synergy effects of cancer cell lines, and importantly can also assay primary cells obtained from patients with advanced forms of cancer (45, 46). In contrast with the liquid tumors, cultivation of patient-derived cancer cells from solid tumors has been challenging in the past. However, using culture techniques adapted from stem cell research, it is now feasible to investigate drug sensitivities and acquired drug resistance in cell culture models derived from biopsy samples of solid tumors (45). Using this approach, combining conventional or targeted chemotherapy and available or emerging BCL-2 family modulators will uncover exploitable private pathways to apoptosis with great clinical benefit.

References

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Development of methodology: D.W. Andrews
Writing, review, and/or revision of the manuscript: H. Brahmbhatt, S. Oppermann, E.J. Osterlund, B. Leber, D.W. Andrews

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