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Directly targeting the mitochondrial pathway of apoptosis for cancer therapy with BH3 mimetics: recent successes, current challenges and future promise

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Abstract

Apoptosis within cancer cells is controlled by the BCL-2 family of proteins, making them powerful arbiters of cell fate in response to stress induced by neoplastic transformation as well as exposure to anti-cancer therapies. Many cancers evade pro-apoptotic stress signals by upregulating anti-apoptotic proteins such as BCL-2, BCL-X_L, or MCL-1 to maintain their survival. However, this putatively comes at a cost, since these cancers can also become dependent on these anti-apoptotic proteins for survival. The development and deployment of BCL-2 family inhibitors (drugs that mimic activity of pro-apoptotic BH3-only proteins or “BH3 mimetics”) is based on this paradigm and the first potent and specific molecules are now being evaluated in clinical trials. We review the recent successes in this field, the challenges currently being faced, and the promising future ahead.

Keywords

apoptosis; chemotherapy; mitochondria; BCL-2 family; therapeutics; targeted therapy

Targeting Apoptosis

The evasion of apoptosis was designated as one of six initial hallmarks of cancer by Hanahan and Weinberg¹, underscoring the important role of this pathway in malignant cell survival. Cells undergoing neoplastic transformation experience pro-apoptotic signaling from such cancer-defining cellular traits such as DNA replication stress², violation of cell cycle checkpoints³, unfolded protein response (UPR)⁴ and high levels of oxidative stress⁵, which must be neutralized or evaded. Several mechanisms of evasion are recurrently seen in cancers and thus merit not only study but also thorough evaluation for potential therapeutic targeting. One prominent mechanism is the upregulation of anti-apoptotic proteins (especially BCL-2, BCL-X_L, and MCL-1) that can buffer pro-apoptotic signaling. These proteins, whether expressed at endogenous or increased levels, inhibit cell death by binding

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and sequestering either pro-apoptotic BH3-only “activator” proteins such as BIM, BID and PUMA or pro-apoptotic effector proteins BAX and BAK (Figure 1) (for full review of apoptotic pathway see ⁶). Left unchecked, activator proteins can bind BAX/BAK^{7–10} and induce their oligomerization^{11,12}, resulting in mitochondrial outer membrane permeabilization (MOMP), release of cytochrome *c* from the mitochondrial intermembrane space, and activation of caspases for dismantling of the cell^{13,14}.

Based on our current understanding, there are two strategies that may target the evasion of apoptosis in cancer cells for therapy: 1) indirectly inducing an upregulation of pro-apoptotic signals to overwhelm the anti-apoptotic reserve within a cell and trigger MOMP or 2) directly inhibiting anti-apoptotic protein activity, freeing pro-apoptotic activators to trigger MOMP. Many anti-cancer therapies (including targeted and cytotoxic chemotherapies as well as ionizing radiation) don’t directly kill cancer cells, but instead stress cells by damaging critical components such as DNA¹⁵ or microtubules¹⁶ or block vital oncogenic signaling^{17–19}. The stress response frequently includes an upregulation or activation of pro-apoptotic molecules including activator proteins^{7,20–23} and, if the upregulation is of sufficient magnitude to overwhelm the anti-apoptotic reserve and activate BAX/BAK, the cell undergoes apoptosis. This indirect strategy is most successful in cells that are “primed” for apoptosis due to their low reserve of unbound anti-apoptotic proteins (either express low levels of these proteins overall [Figure 2A] or the anti-apoptotic proteins are actively sequestering pro-apoptotic signals [Figures 2B,C] and are therefore neutralized). In contrast, unprimed cells contain a larger reserve of unbound anti-apoptotic proteins to buffer against pro-apoptotic signaling and are therefore more resistant to cytotoxic chemotherapies (Figures 2D,E). Interestingly, many cancer cells are far more primed for apoptosis than are most normal cells^{24,25} and the level of apoptotic priming within tumors affects response to conventional chemotherapy in vivo²⁴. Differential priming is likely the most significant determinant of a therapeutic index for conventional chemotherapy in cancer²⁶. Thus, conventional chemotherapy has for many years been a mechanism for selectively targeting mitochondrial apoptosis in cancer, albeit indirectly²⁴.

Importantly, cells that contain anti-apoptotic proteins that are actively sequestering pro-apoptotic activators or even activated BAX/BAK are essentially dependent on those anti-apoptotic proteins for survival, thus making them not only primed for apoptosis, but also dependent on proteins such as BCL-2^{27,28}. Within these cells, when anti-apoptotic proteins are directly inhibited via small molecule BH3 mimetics (such as ABT-737 for BCL-2/X_L inhibition), they release any actively bound pro-apoptotic proteins, which in turn trigger MOMP (Figures 2B,C). Note that expression levels of anti- or pro-apoptotic proteins alone cannot determine sensitivity to either BCL-2 family inhibitors or cytotoxic chemotherapies, making functional tests of dependence on BCL-2 family members or overall priming necessary to predict response. A therapeutic window for directly targeting anti-apoptotic proteins would exist whenever a cancer exhibits a higher dependence on specific anti-apoptotic proteins than irreplaceable, vital cells. Recent therapeutic advances have begun to exploit this therapeutic window.

Targeting BCL-2

To date, this strategy has been advanced furthest in the clinic with the series of small molecules based on the ABT-737 compound, which inhibits BCL-2 and BCL-X_L with high specificity and selectivity (for a full review of compounds targeting anti-apoptotic BCL-2 family members see ²⁹ and for a historical review of efforts to target BCL-2 directly see ³⁰). A similar compound, ABT-263 (navitoclax), was developed to be orally bioavailable. However, early clinical trials showed that patients treated with ABT-263 suffered from thrombocytopenia due to on-target toxicity to platelets, which are dependent on BCL-X_L^{31–33}. This toxicity limited dosing, though single-agent biological activity was seen in patients, particularly in CLL³³.

To avoid thrombocytopenia, attention turned to generating a small molecule that would selectively antagonize BCL-2 without affecting BCL-X_L. Since several blood cancers have been shown to be dependent on BCL-2 specifically^{34–39}, a selective BCL-2 antagonist might be expected to maintain activity in many diseases. Another orally bioavailable compound, ABT-199 (venetoclax), was thus developed with 100-fold greater affinity for BCL-2 than BCL-X_L⁴⁰. The potent, selective BCL-2 inhibitor ABT-199 has been tested in 26 completed or ongoing clinical trials to date, mostly in hematological malignancies⁴¹. The trials in CLL have shown the most promise, with impressive single-agent response rates around 80% in heavily pre-treated patients^{42,43}. Perhaps even more impressive is that 20% were complete responders (about 5% of which attain a minimal residual disease negative state) and that the high response rate was observed across patients, including those with very poor prognostic features such as deletion 17p (loss of p53) or unmutated IGHV⁴³. The strong dependence on BCL-2 for survival in CLL is potentially due to loss or downregulation of *miR-15a* and *miR-16-1* (in many cases by deletion of 13q, a common event in CLL⁴⁴), which leads to overexpression of BCL-2 protein⁴⁵. The extremely high activity of this agent in CLL can be a double-edged sword, however, as several patients in early clinical trials experienced tumor lysis syndrome (TLS) after initial high-dose treatment with venetoclax⁴⁰. These reactions prompted the adoption of a lead-in dosing regimen in subsequent trials to successfully reduce the incidence of TLS⁴⁶. Trying to build on single-agent success, several clinical studies of venetoclax in combination with other agents with activity in CLL are underway. The coming few years will show how venetoclax, arguably the most active targeted agent yet identified in CLL, will most effectively be combined with other exciting new agents in CLL, like obinutuzumab, idelalisib, and ibrutinib, as well as with conventional chemotherapeutic agents.

While the vast majority of CLL cases are sensitive to venetoclax, the response rate in other blood cancers, including non-Hodgkin's lymphomas, multiple myeloma, acute lymphoblastic leukemia, and acute myeloid leukemia, has been more heterogeneous⁴⁶. Two approaches are being pursued to potentially overcome this heterogeneity, the first being improved patient selection via biomarkers. A common strategy for the development of a biomarker today is to seek a genetic alteration in the targeted pathway that is associated with response or resistance, yet this is not a promising strategy for BCL-2. As mentioned above, the disease where activity is best, CLL, lacks any genetic alterations of the BCL-2 gene. In follicular lymphoma, where BCL-2 is strongly overexpressed in 80–90% of patients due to a

t(14;18) translocation of the BCL-2 gene and the immunoglobulin heavy-chain promoter⁴⁷, sensitivity to venetoclax has been fairly modest, with a minority of patients responding⁴⁶. This brings up an important question: if BCL-2 overexpression drives development of nearly all FLs (ostensibly by blocking pro-apoptotic signaling during tumorigenesis), why are these lymphomas not more sensitive to BCL2 inhibition? The answer may be that although high BCL-2 expression is necessary for disease pathogenesis, it may not be necessary for disease maintenance, a phenomenon that has been observed with certain oncogenes in other diseases⁴⁸. It is also possible that the doses of ABT-199 administered in clinical trials are not sufficient to inhibit the high levels of BCL-2 present in these cells. Supporting this possibility is the observation that while only 3 out of 11 (27%) FL patients exhibited a response to ABT-199 in a recent trial, all three of the responders were among those patients treated with the highest doses (600mg) (3 of 6 FL patients, 50% response rate)⁴⁶. Another potential explanation is that FL cells contain other anti-apoptotic proteins (such as MCL-1) that are able to prevent apoptosis even when BCL-2 is neutralized (Figure 2E). Further study of the role of BCL-2 in FL and other BCL-2–overexpressing cancers is needed to answer this conundrum.

Despite the absence of genetic alterations in the *BCL2* gene to predict sensitivity to venetoclax, several other genetic aberrations have been reported to correlate with response. For instance, in multiple myeloma, there is an apparent correlation of sensitivity with the t(11;14) translocation³⁷, which juxtaposes IgH with *CCND1* (cyclin D1) to theoretically favor cell cycle progression⁴⁹. In AML, there may be a correlation with IDH1 or 2 mutations⁵⁰. In neither case is the correlation perfect, however, and more clinical testing will be needed for their evaluation. Strategies based on measurement of protein levels of BCL-2 and other anti-apoptotic proteins have been explored, but results have been inconsistent^{51,52}, likely because the property that needs to be measured is BCL-2 dependence rather than merely BCL-2 expression. Another strategy, BH3 Profiling, measures BCL-2 dependence by exposing mitochondria of cancer cells to BH3 peptides or small molecules of known ability to inhibit BCL-2, MCL-1, or BCL-X_L^{34,53,54} and detecting the extent of cytochrome *c* release. This approach can identify cancers that are dependent on BCL-2 in several hours, without the need for protracted *ex vivo* culture which is required in standard cytotoxicity studies. Lab-based studies in conjunction with clinical trials suggest that BH3 profiling can predict response to BCL-2 inhibition in patients^{55,56}. Further testing is in progress.

Another approach to address the heterogeneity of responses to a BCL-2 inhibitor in a disease is to explore combination regimens to increase response rates. Since most clinically active agents in hematologic malignancies kill cells via apoptosis, there are myriad opportunities to combine them with venetoclax. In AML, combinations with hypomethylating agents (vidaza or decitabine) as well as cytarabine are being investigated. At least in the case of the hypomethylating agents, the combination with venetoclax appears to have resulted in a significant enhancement of clinical activity, with roughly 70% of patients obtaining complete clearance of their leukemia⁵⁷. This remarkable degree of activity, with an acceptable toxicity profile⁵⁷, prompts future consideration of such regimens as outpatient induction regimens, especially in patients that are deemed poor candidates for standard induction therapy.

Although the preclinical and nascent clinical data indicates that BCL-2 is a valid target in several types of hematological malignancies, the data for solid tumors gives us a more mixed picture. When examining BCL-2 inhibitor sensitivity across all cancer subtypes, it's clear that subsets of cell lines from solid malignancies such as breast⁵⁸ and lung⁵⁹ carcinomas may be sensitive to this strategy. However, here patient selection becomes an even larger issue since smaller subsets of cell lines are sensitive, heralding potential heterogeneity in responses among patients with these diseases. A mitigating factor for this hurdle is that the toxicity profile for selective BCL-2 inhibition is manageable, with the most common adverse events recently reported in a cohort of CLL patients being mild diarrhea (52% of patients), upper respiratory tract infection (48%), nausea (47%) and neutropenia (41%)⁴³.

Finally, as with most anti-cancer therapies, the eventual development of treatment resistance is a constant concern. In the case of specific BCL-2 inhibition, we benefit from knowledge of the drug's mechanism of action and thus can not only predict mechanisms of resistance but also potentially act preemptively to avoid it. The anti-apoptotic BCL-2 family members are somewhat redundant in function, raising the possibility that the inhibition of one member will lead to upregulation of another anti-apoptotic protein to maintain survival. Indeed, preclinical work has shown that resistance to dual BCL-2/X_L inhibition can be mediated by upregulation of MCL-1⁶⁰ or even by increased expression of BCL-X_L^{61–63}, either via genetic alterations or signals from the microenvironment. In CLL patients, treatment resistance was associated with a transformation to aggressive lymphoma in 18 of 41 patients who progressed on treatment, which is a disease that is typically less dependent on BCL-2 for survival⁴⁶ for reasons that are unclear. A reduction in endogenous pro-apoptotic signaling may also cause resistance by eliminating the death signal that is to be released from BCL-2/X_L. This mechanism of resistance was reported in multiple myeloma cells that downregulated expression of BIM and PUMA as they acquired resistance to ABT-737⁶³. The silencing or loss of *BIM*, *PUMA*, or other pro-apoptotic genes can occur in blood cancers as well as solid tumors^{64–67} and could serve to impair sensitivity to BH3 mimetics. Finally, inactivating mutations or loss of *BAX* or *BAK* could also blunt responses to BH3 mimetics and have been observed in clinical samples^{7,68–71}. Although concomitant loss of BAX and BAK would confer resistance to any anti-cancer therapy that is dependent on mitochondrial apoptosis, this is not commonly observed in cancers. Given the tremendous resistance to apoptosis loss of BAK and BAX would bestow, the rarity of this dual loss provokes speculation that there may be properties of cancer cells for which BAX and BAK are beneficial.

There is a long history in cancer therapy of overcoming acquired resistance to single agents by assembling combination regimens of non-overlapping toxicities. Since single agent venetoclax is well-tolerated, its inclusion in combinations will doubtless be explored. These combinations might include conventional chemotherapy agents, as well as more modern targeted agents with known activity in the disease. Another strategy yet to be explored is more pulsatile dosing. Apoptosis is a switch-like event – once cells cross the apoptotic threshold, even for a short period of time, the cell is rapidly and irreversibly committed to programmed cell death. Therefore, it seems likely that therapeutic efficacy may be driven more by the maximum concentration achieved in cancer cells ($C_{\max}$) rather than by extended exposure to a lower level coverage of the target, such as is typically measured as area under

the curve (AUC). Thus far, venetoclax has been tested mainly as a daily dose. It will be interesting to test the relative effectiveness of regimens where higher doses are given, but perhaps only on a few days per cycle. In addition, the incorporation of such pulsatile doses into a maintenance regimen might also reduce disease progression or acquired resistance.

Targeting other anti-apoptotic proteins

While targeting of BCL-2 is most advanced clinically, cancer cells may well be dependent on other anti-apoptotic proteins. It is worth noting that dependence on one anti-apoptotic protein does not exclude dependence on others. For example, a cell could be susceptible to a BCL-2 inhibitor and also to an MCL-1 inhibitor. On a molecular level, one could envision such a cell as having abundant BCL-2 and MCL-1, but both nearly completely occupied with activator or effector pro-apoptotic proteins (Figure 1D). We have observed such conditions in cancer cells via BH3 profiling³⁷.

Targeting BCL-X_L

The earliest selective and potent compound targeting anti-apoptotic proteins that saw clinical use was the dual BCL-2/X_L inhibitor ABT-263, giving us a view into how targeting BCL-X_L may be utilized clinically. BCL-X_L is a more attractive target in some solid malignancies, with work from several groups showing that several types of cancers upregulate BCL-X_L to become resistant to chemotherapy^{72,73}. Profound sensitivity to single-agent treatment, however, is limited to specific cases or cell lines, thus again raising the issue of patient selection⁷⁴. Further complicating the translation of BCL-X_L inhibition to clinical use is the thrombocytopenia induced by this strategy. Platelets are dependent on BCL-X_L for maintaining their survival³¹ and are thus lost quickly following treatment with a BCL-X_L inhibitor. This observation is so consistent that any claims of an agent operating clinically via BCL-X_L inhibition that does not induce thrombocytopenia should be viewed with skepticism. Note that megakaryocytes are unaffected, so that ABT-263 treatment actually stimulates the production of new, young platelets which are less sensitive to ABT-263⁷⁵. Thus, after an initial fall, recovery of platelets will occur even in the face of continuous dosing. This observation led to the introduction of lead-in dosing to mitigate the nadir of platelet count, with some effect. Indeed, serious bleeding was not observed in patients treated⁷⁴. Nonetheless, it should be noted that thrombocytopenia is dose-dependent, and platelets can be driven to undetectable levels by increased dosing, so that thrombocytopenia is still a dose-limiting toxicity. Thus any effort to bring BCL-X_L inhibition to the clinic requires the management of thrombocytopenia. This, however, is a complication which most oncologists are comfortable handling.

Targeting MCL-1

Lack of effective MCL-1 inhibitors has hampered in depth study of the therapeutic potential of targeting this member of the BCL-2 family. Several malignancies have been reported to be MCL-1-dependent, most notably acute myeloid leukemia (AML)⁷⁶, chronic myeloid leukemia (CML)⁷⁷, B-cell acute lymphoblastic leukemia⁷⁸, hepatocellular carcinoma⁷⁹, multiple myeloma^{37,80}, and subgroups of non-small cell lung cancers⁸¹. One particularly

elegant study showed that conditional knockout of MCL-1 in a mouse model of acute myeloid leukemia was sufficient to induce clearance of cancer cells and cure mice. Interestingly, a survival benefit was observed from loss of just one allele of MCL-1, highlighting the potential utility of targeting MCL-1 in this disease⁷⁶.

Targeting MCL-1 may also have on-target toxicity, especially in cells of the myeloid lineage as well as hematopoietic stem cells^{35,82,83}, which may potentially be managed clinically. Perhaps more alarmingly, knockout of MCL-1 has also been shown to induce lethal cardiotoxicity in mice⁸⁴, raising the possibility that other cell types may be dependent on expression of MCL-1 for either survival or for other functions. It is not clear, however, whether toxicity to tissues outside of the hematopoietic system is due to inhibiting the anti-apoptotic activity of MCL-1 or its newly-discovered role in mitochondrial respiration⁸⁵. An inhibitor of MCL-1's anti-apoptotic activity on the mitochondrial membrane but not its function within mitochondria would not only allow for the careful dissection of MCL-1 activity in these different compartments but may eventually be the optimal strategy to pursue clinically.

Despite indications of the potential utility of targeting MCL-1 in neoplasms, several issues must be overcome to deploy this strategy, including those listed above for BCL-2 inhibition. The most glaring issue, however, is the lack of potent, selective MCL-1 inhibitors. Although several compounds have been reported^{86–89}, they either lack potency or the ability to efficiently enter cells. Although peptide-based inhibitors of MCL-1, including the BH3 domains of NOXA, BIM, BID, PUMA and even MCL-1 itself have been well characterized, their potential for in vivo use remains unknown^{88,90}. Intense efforts to develop MCL-1 inhibitors are underway, and their progress is encouraging. Until these are available, however, the determination of a therapeutic index for MCL-1 inhibitors remains speculative.

Combining Inhibitors

Concurrently inhibiting all anti-apoptotic BCL-2 family members may be particularly effective at inducing apoptosis in cancer cells. This is due to the fact that most cells express some levels of anti-apoptotic proteins and, if they remain unbound and available for sequestering pro-apoptotic molecules, they may prevent apoptosis by acting as a reserve for BH3-only proteins when selective therapies are utilized²⁶ (Figure 1C). Indeed, it was recently demonstrated that combining MCL-1 and BCL-2/X_L inhibitors can potently induce apoptosis even in solid tumor cell lines, with significant synergy⁸⁶. However, these combination therapies may not be effective against every cancer as they are still dependent on sufficient levels of activator BH3-only proteins being bound to anti-apoptotic proteins (primed for apoptosis) and many types of cancers, especially solid malignancies, do not exhibit a high level of priming²⁴. Lack of priming may be corrected by combining these therapies with known inducers of BIM or BID, including many kinase inhibitors^{22,23,91,92} or classical chemotherapeutic agents including topoisomerase inhibitors^{7,93}.

Of course, in addition to the on target toxicity of each inhibitor alone, additional toxicities may arise when these inhibitors are used in combination. Despite these concerns,

combination therapies will likely be tested clinically when MCL-1 inhibitors become available, especially in otherwise intractable tumors.

Concluding remarks

The excellent progress made in the past decades on understanding how the BCL-2 family of proteins controls survival in cancerous as well as healthy cells has provided an unprecedented opportunity to target this pathway for efficacious cancer treatment. However, there are bona fide challenges, both those discussed here and potential others that have not yet been proposed or observed. Despite these challenges, early success in CLL is encouraging and may serve as a road map in other malignancies. Perhaps most exciting is that the tools at our disposal for dissecting the activity and function of novel agents in this space are excellent, which will undoubtedly help not only identify issues as they arise, but also overcome them.

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ABBREVIATIONS

BCL-2	B-cell lymphoma 2
BCL-X_L	B-cell lymphoma X long
MCL-1	Myeloid cell leukemia 1
BAX	Bcl-2-associated X protein
BAK	Bcl-2 homologous antagonist killer
BH3	Bcl-2 homology 3
BIM	BCL-2-interacting mediator of cell death
BID	BH3 interacting domain death agonist
PUMA	p53 up-regulated modulator of apoptosis
MOMP	Mitochondrial outer membrane permeabilization
CLL	Chronic lymphocytic leukemia
IGHV	Immunoglobulin heavy chain variable region
TLS	Tumor lysis syndrome
FL	Follicular lymphoma

IDH	Isocitrate dehydrogenase
AML	Acute Myeloid Leukemia
CML	Chronic myelogenous leukemia

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Figure 1.

The mitochondrial pathway of apoptosis. Upstream death stimuli originating from damage or stress leads to the upregulation or activation of BH3-only activator proteins. These proteins translocate to the mitochondria where they can either be bound and sequestered by anti-apoptotic proteins or activate BAX/BAK. Once activated, BAX/BAK oligomerize and form pores in the outer mitochondrial membrane, leading to the release of cytochrome c and other pro-apoptotic factors which activate caspases for the dismantling of the cell.

Figure 2.

A model of how mitochondrial apoptosis can be targeted directly or indirectly to induce apoptosis. A) Cells that contain a low reserve of unbound anti-apoptotic proteins are considered to be primed for apoptosis and sensitive to cytotoxic chemotherapies yet are resistant to BH3 mimetics. B–C) Cells that contain a low reserve of unbound anti-apoptotic proteins (primed for apoptosis) and BCL-2 molecules that are actively binding and sequestering activator BH3-only proteins such as BIM, BID or PUMA, are sensitive to specific BCL-2 inhibition, pan-BCL-2 family inhibition, and cytotoxic chemotherapy. D–E) Cells that contain a high reserve of unbound anti-apoptotic proteins (unprimed) can buffer stress-induced pro-apoptotic signals and are therefore resistant to cytotoxic chemotherapies and BH3 mimetics. If additional pan inhibitor or cytotoxic chemotherapy is administered, however, MOMP would be triggered. Note that the cells in B–E contain identical levels of BCL-2 expression yet have varying cell fates in response to BCL-2 inhibition. In addition,

B, C and E all contain BCL-2 bound to activator BH3-only protein yet have varying cell fates in response to BCL-2 inhibition.

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