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STATE-OF-THE-ART REVIEW

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

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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 up-regulating anti-apoptotic proteins such as BCL-2, BCL-X_L or MCL-1 to maintain their survival. However, this may come at a cost, as these cancers may also become dependent on these anti-apoptotic proteins for survival. The development and deployment of BCL-2 family inhibitors (drugs that mimic the 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.

Targeting apoptosis

The evasion of apoptosis was designated as one of six initial hallmarks of cancer by Hanahan and Weinberg [1], underscoring the important role of this pathway in survival of malignant cells. Cells undergoing neoplastic transformation experience pro-apoptotic signaling resulting from such cancer-defining cellular traits such as DNA replication stress [2], violation of cell-cycle checkpoints [3], the unfolded protein response [4] and high levels of oxidative stress [5], which must be neutralized or evaded. Several mechanisms of evasion are recurrently seen in cancers, and thus merit thorough evaluation for potential therapeutic targeting. One prominent mechanism is the up-regulation of anti-apoptotic proteins, especially B-cell lymphoma 2 (BCL-2), B-cell lymphoma X long (BCL-X_L) and myeloid cell leukemia 1 (MCL-1), that buffer pro-apoptotic signaling. These proteins, whether expressed at endogenous or increased levels, inhibit cell death by binding and sequestering either pro-apoptotic BH3-only 'activator' proteins such as BCL-2-interacting mediator of cell death (BIM), BH3-interacting domain death agonist (BID) and p53 up-regulated modulator of apoptosis (PUMA), or the pro-apoptotic effector proteins BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK) (Fig. 1). A full review of the apoptotic pathway has been provided

Abbreviations
BAK, BCL-2 homologous antagonist killer; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; BCL-X_L, B-cell lymphoma X long; BH3, BCL-2 homology 3; BID, BH3 interacting domain death agonist; BIM, BCL-2-interacting mediator of cell death; CLL, chronic lymphocytic leukemia; MCL-1, myeloid cell leukemia 1; MOMP, mitochondrial outer membrane permeabilization; PUMA, p53 up-regulated modulator of apoptosis.

elsewhere [6]. Left unchecked, activator proteins 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 inter-membrane 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 up-regulation of pro-apoptotic signals to overwhelm the anti-apoptotic reserve within a cell and trigger MOMP, or (2) directly inhibiting the activity of anti-apoptotic proteins, freeing pro-apoptotic activators to trigger MOMP. Many anti-cancer therapies (including targeted and cytotoxic chemotherapies as well as ionizing radiation) do not kill cancer cells directly, but instead stress cells by damaging critical components such as DNA [15] or microtubules [16] or block vital oncogenic signaling [17–19]. The stress response frequently includes up-regulation or activation of pro-apoptotic molecules, including activator proteins [7,20–23], and, if the up-regulation 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, i.e. they either express low levels of these proteins overall (Fig. 2A) or the anti-apoptotic proteins are actively sequestering pro-apoptotic signals (Fig. 2B,C), and are therefore unable to buffer any additional pro-death signaling. In contrast, unprimed cells contain a larger reserve of unbound anti-apoptotic proteins to bind and sequester pro-apoptotic molecules, making them more resistant to cytotoxic chemotherapies (Fig. 2D,E). Interestingly, many cancer cells are more highly primed for apoptosis than most normal cells are [24,25], and the level of apoptotic priming within tumors affects the response to conventional chemotherapy *in vivo* [24]. Differential priming is probably the most significant determinant of a therapeutic index for conventional chemotherapy in cancer [26]. Thus, conventional chemotherapy is a mechanism for selectively targeting mitochondrial apoptosis in cancer, albeit indirectly [24].

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), actively bound pro-apoptotic proteins are released, triggering MOMP (Fig. 2B,C). However, 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 exists whenever a

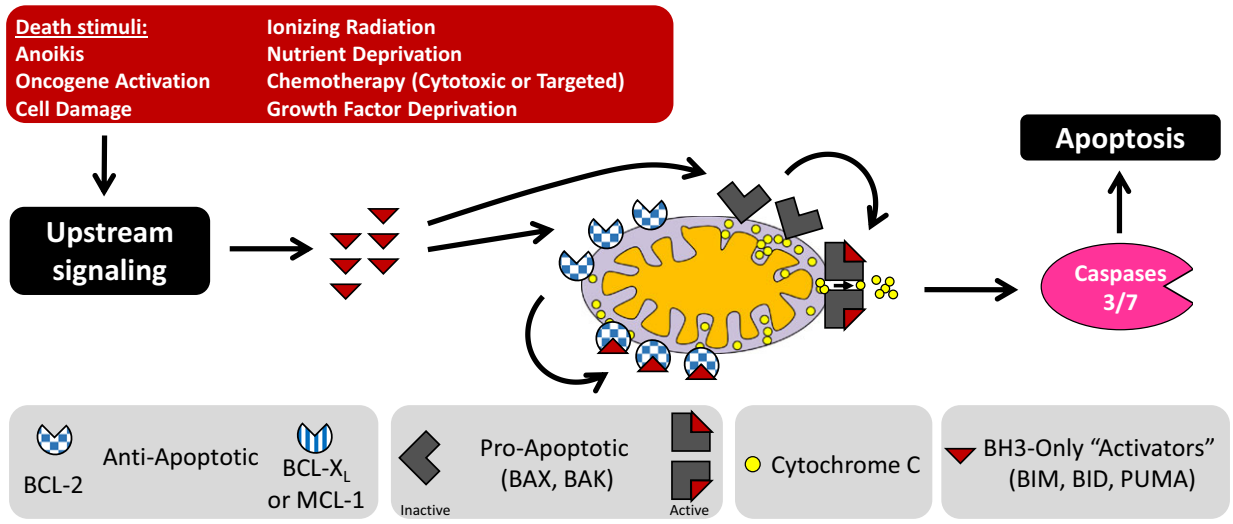

Fig. 1. The mitochondrial pathway of apoptosis. Upstream death stimuli originating from damage or stress lead to up-regulation or activation of BH3-only activator proteins. These proteins translocate to the mitochondria, where they may be bound and sequestered by anti-apoptotic proteins or activate BAX/BAK via a transient "hit and run" interaction. Once activated, BAX/BAK oligomerize and form pores in the outer mitochondrial membrane, leading to release of cytochrome *c* and other pro-apoptotic factors that activate caspases for dismantling of the cell.

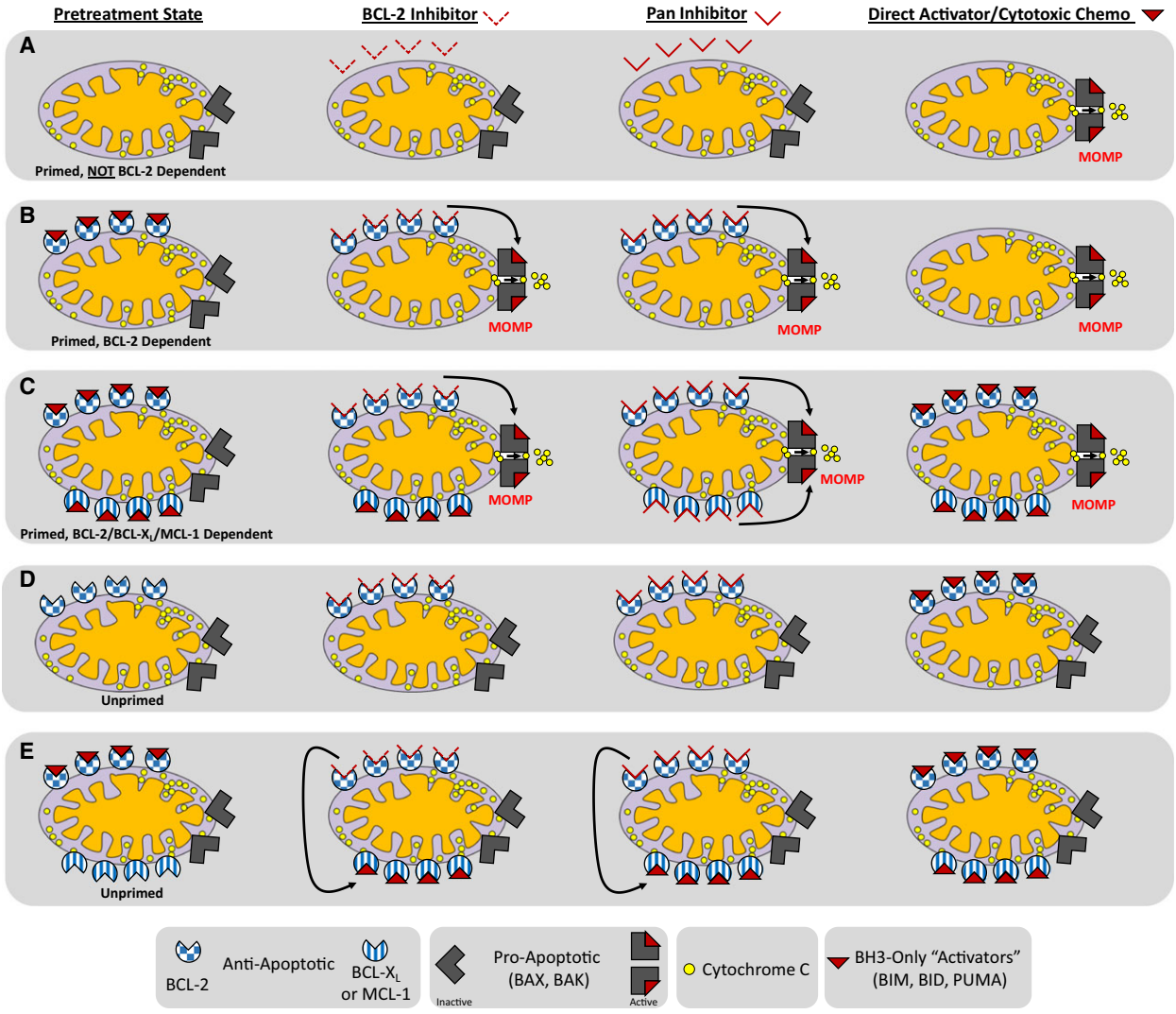

Fig. 2. Models of how mitochondrial apoptosis may be targeted directly or indirectly to induce apoptosis. (A) Cells that contain a low reserve of unbound anti-apoptotic proteins are considered to be primed for apoptosis and sensitive to cytotoxic chemotherapies but are resistant to BH3 mimetics. (B, 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) may buffer stress-induced pro-apoptotic signals and are therefore resistant to cytotoxic chemotherapies and BH3 mimetics. However, if an additional pan inhibitor or cytotoxic chemotherapy is administered, MOMP is triggered. Note that the cells in (B)–(E) show identical levels of BCL-2 expression but have varying cell fates in response to BCL-2 inhibition. In addition, cells in (B), (C) and (E) all contain BCL-2 bound to activator BH3-only protein, but have varying cell fates in response to BCL-2 inhibition.

cancer exhibits a higher dependence on specific anti-apoptotic proteins than irreplaceable vital cells do. Recent therapeutic advances have begun to exploit this therapeutic window.

Targeting BCL-2

To date, targeting BCL-2 has been advanced furthest in the clinic using a series of small molecules based on the ABT-737 compound, which inhibits BCL-2 and BCL-X_L with high specificity and selectivity. Compounds targeting anti-apoptotic BCL-2 family members and efforts to target BCL-2 directly have been reviewed previously [29,30]. 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, although single-agent biological activity

was seen in patients, particularly those with chronic lymphocytic leukemia (CLL) [33].

To avoid thrombocytopenia, attention turned to generating a small molecule that selectively antagonizes BCL-2 without affecting BCL-X_L. As several blood cancers have been shown to be dependent on BCL-2 specifically [34–39], a selective BCL-2 antagonist may be expected to have 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_L [40]. This potent selective BCL-2 inhibitor has been tested in 27 completed or ongoing clinical trials to date, mostly for hematological malignancies (https://clinicaltrials.gov/ct2/results?term = abt-199&Search = Search). Trials in patients with CLL have shown the most promise, with impressive single-agent response rates of ~ 80% in heavily pre-treated patients [41,42]. Perhaps even more impressive is that 20% were complete responders (~ 5% of whom attained 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 a non-mutated immunoglobulin heavy chain variable region [42]. The strong dependence on BCL-2 for survival in CLL is potentially due to loss or down-regulation of *miR-15a* and *miR-16-1* (in many cases as a result of deletion of 13q, a common event in CLL [43]), which leads to over-expression of BCL-2 protein [44]. However, the extremely high activity of this agent in CLL may be a double-edged sword, as several patients in early clinical trials experienced tumor lysis syndrome after initial high-dose treatment with venetoclax [40]. These reactions prompted adoption of a lead-in dosing regimen in subsequent trials to successfully reduce the incidence of tumor lysis syndrome [45]. In an attempt to build on the success of this agent, several clinical studies of venetoclax in combination with other agents that show activity in CLL are underway. The coming years will show how venetoclax, arguably the most active targeted agent yet identified for use in in CLL, may most effectively be combined with other exciting new agents for use in CLL, such as obinutuzumab, idelalisib and ibrutinib, as well as with conventional chemotherapeutic agents.

While the vast majority of CLL cases are sensitive to venetoclax, the response rates in other blood cancers, including non-Hodgkin's lymphomas, multiple myeloma, acute lymphoblastic leukemia and acute myeloid leukemia, have varied [45]. Two approaches are being pursued to potentially overcome this heterogeneity, the first being improved patient selection via use of biomarkers. A common strategy for development of a biomarker is to identify a genetic alteration in the targeted pathway that is associated with response or resistance, but this is not a promising strategy for BCL-2. As mentioned above, the disease in which the activity of ABT-199 is best, CLL, lacks any genetic alterations of the BCL-2 gene. In follicular lymphoma, in which BCL-2 is strongly over-expressed in 80–90% of patients due to a t(14;18) translocation of the BCL-2 gene and the immunoglobulin heavy-chain promoter [46], sensitivity to venetoclax is fairly modest, with a minority of patients responding [45]. This raises an important question: if BCL-2 over-expression drives development of nearly all cases of follicular lymphoma (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 diseases [47]. 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 three of 11 follicular lymphoma patients (27%) exhibited a response to ABT-199 in a recent trial, all three of the responders were among those patients treated with the highest doses of ABT-199 (≥ 600 mg) (three of six follicular lymphoma patients, 50% response rate) [45]. Another potential explanation is that follicular lymphoma cells contain other anti-apoptotic proteins (such as MCL-1) that are able to prevent apoptosis even when BCL-2 is neutralized (Fig. 2E). Further study of the role of BCL-2 in follicular lymphoma and other BCL-2-over-expressing cancers is required to address this conundrum.

Despite the absence of genetic alterations in the *BCL2* gene that predict sensitivity to venetoclax, several other genetic aberrations have been reported to correlate with the response. For instance, in multiple myeloma, there is an apparent correlation of sensitivity with the presence of the t(11;14) translocation [37], which juxtaposes IgH with *CCND1* (cyclin D1) to theoretically favor cell-cycle progression [48]. In acute myeloid leukemia, there may be a correlation with mutations in the *IDH1* or *IDH2* genes encoding isocitrate dehydrogenase [49]. However, in neither of these cases is the correlation perfect, and more clinical testing is required to determine the usefulness of these potential biomarkers. Strategies based on measurement of protein levels of BCL-2 and other anti-apoptotic proteins have been explored, but results have been inconsistent [50,51], probably 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 with known ability to inhibit BCL-2, MCL-1 or BCL-X_L [34,52,53], and detecting the extent of cytochrome *c* release. This approach may identify cancers that are dependent on BCL-2 in several hours, without the need for the protracted *ex vivo* culture required in standard cytotoxicity studies. Laboratory-based studies in conjunction with clinical trials suggest that BH3 profiling may predict the response to BCL-2 inhibition in patients [54,55]. Further testing is in progress.

The second approach to address the heterogeneity of responses to a BCL-2 inhibitor in a disease is to explore combination regimens to increase response rates. As most clinically active agents in hematological malignancies kill cells via apoptosis, there are myriad opportunities to combine them with venetoclax. In acute myeloid leukemia, combinations with hypomethylating agents (vidaza or decitabine) as well as with cytarabine are being investigated. At least in the case of the hypomethylating agents, the combination with venetoclax appears to result in a significant enhancement of clinical activity, with ~ 70% of patients showing complete clearance of their leukemia [56]. This remarkable degree of activity, combined with an acceptable toxicity profile [56], suggests that such regimens should be considered for use as outpatient induction regimens, especially in patients who are deemed poor candidates for standard induction therapy.

Although the pre-clinical and nascent clinical data indicate that BCL-2 is a valid target in several types of hematological malignancies, the data for solid tumors give a more mixed picture. When examining BCL-2 inhibitor sensitivity across cancer sub-types, it is clear that subsets of cell lines from solid malignancies such as breast [57] and lung [58] carcinomas may be sensitive to this strategy. However, patient selection becomes an even larger issue in this case, as smaller subsets of cell lines are sensitive, suggesting potential heterogeneity in responses among patients with these diseases. A mitigating factor for this issue 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%) [42].

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 cannot only predict mechanisms of resistance but also potentially act pre-emptively to avoid it. The anti-apoptotic BCL-2 family members are somewhat redundant in function, raising the possibility that inhibition of one member leads to up-regulation of another anti-apoptotic protein to maintain survival. Indeed, pre-clinical work has shown that resistance to dual BCL-2/X_L inhibition may be mediated by up-regulation of MCL-1 [59] or even increased expression of BCL-X_L [60–62], 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; aggressive lymphoma is a disease that is typically less dependent on BCL-2 for survival [45] for reasons that are unclear. A reduction in endogenous pro-apoptotic signaling may also cause resistance by eliminating the death signal to be released from BCL-2/X_L. This mechanism of resistance was reported in multiple myeloma cells in which expression of BIM and PUMA was down-regulated as they acquired resistance to ABT-737 [62]. Silencing or loss of *BIM*, *PUMA* or other pro-apoptotic genes may occur in blood cancers as well as solid tumors [63–66], and may impair sensitivity to BH3 mimetics. Finally, inactivating mutations or loss of *BAX* or *BAK* may also blunt responses to BH3 mimetics, and have been observed in clinical samples [7,67–70]. 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 that loss of BAK and BAX would confer, the rarity of this dual loss prompts 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 with non-overlapping toxicities. As single-agent venetoclax is well-tolerated, its inclusion in combinations will doubtless be explored. These combinations may include conventional chemotherapy agents, as well as more modern targeted agents with known activity against the disease. Another strategy that has yet to be explored is 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 irreversible 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 lower-level

coverage of the target, such as is typically measured as the area under the curve (AUC). So far, venetoclax has been tested mainly as a daily dose. It will be interesting to test the relative effectiveness of regimens in which higher doses are given, but perhaps only for a few days per cycle. In addition, incorporation of such pulsatile doses into a maintenance regimen may 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 may be susceptible to a BCL-2 inhibitor and also to an MCL-1 inhibitor by containing abundant BCL-2 and MCL-1 that are both completely occupied with pro-apoptotic activator or effector proteins (Fig. 2C). We have observed such conditions in cancer cells via BH3 profiling [37].

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-XL may be utilized clinically. BCL-X_L is a more attractive target in some solid malignancies, with work by several groups showing that several types of cancers up-regulate BCL-X_L to become resistant to chemotherapy [71,72]. However, profound sensitivity to single-agent treatment is limited to specific cases or cell lines, again raising the issue of patient selection [73]. Further complicating translation of BCL-X_L inhibition to clinical use is the thrombocytopenia induced by this strategy. Platelets are dependent on BCL-X_L to maintain survival [31], and are thus lost quickly after treatment with a BCL-X_L inhibitor. This observation is so consistent that any claims of an agent that operates clinically via BCL-X_L inhibition but does not induce thrombocytopenia should be viewed with skepticism. Note that megakaryocytes are unaffected, such that ABT-263 treatment stimulates the production of new platelets that are less sensitive to ABT-263 [74]. Thus, after an initial decrease, recovery of platelets occurs even during continuous dosing. This observation led to introduction of lead-in dosing to mitigate the drop in platelet count, with some effect. Indeed, serious bleeding was not observed in the patients treated [73]. Nonetheless, it should be noted that thrombocytopenia is dose-dependent, and platelets may be reduced to undetectable levels by increased dosing, such that thrombocytopenia is still a dose-limiting toxicity. Thus any effort to introduce BCL-X_L inhibition to the clinic requires the management of thrombocytopenia. However, this is a complication that 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 [75], chronic myeloid leukemia [76], B-cell acute lymphoblastic leukemia [77], hepatocellular carcinoma [78], multiple myeloma [37,79], and sub-groups of non-small cell lung cancers [80]. 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 disease [75].

Targeting MCL-1 may also show on-target toxicity, especially in cells of the myeloid lineage as well as hematopoietic stem cells [35,81,82], which may potentially be managed clinically. Perhaps more alarmingly, knockout of MCL-1 has also been shown to induce lethal cardiotoxicity in mice [83], raising the possibility that other cell types may be dependent on expression of MCL-1 for survival or other functions. However, it is not clear whether toxicity to tissues outside of the hematopoietic system is due to inhibition of the anti-apoptotic activity of MCL-1 or its newly discovered role in mitochondrial respiration [84]. Development of an agent that inhibits MCL-1's anti-apoptotic activity on the mitochondrial membrane but not its function within mitochondria would not only allow careful dissection of MCL-1 activity in these 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 utilize this strategy, including those listed above for BCL-2 inhibition. However, the most glaring issue is the lack of potent, selective MCL-1 inhibitors. Although several possible compounds have been reported [85–88], 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 [87,89]. Intense efforts to develop MCL-1 inhibitors are underway, and their progress is encouraging. However, until these are available, 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 in inducing apoptosis in cancer cells. This is due to the fact that most cells express a certain level 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 utilized [26] (Fig. 2E). Indeed, it was recently demonstrated that combining MCL-1 and BCL-2/X_L inhibitors potently induces apoptosis even in solid tumor cell lines, with significant synergy [85]. 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 priming [24]. Lack of priming may be corrected by combining these therapies with known inducers of BIM or BID, including many kinase inhibitors [22,23,90,91] or classical chemotherapeutic agents including topoisomerase inhibitors [7,92].

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 probably be tested clinically when MCL-1 inhibitors become available, especially in otherwise intractable tumors.

Concluding remarks

The excellent progress made in the past decades in 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 others that may not yet have been proposed or observed. Despite these challenges, early success in CLL is encouraging and may serve as a road map for other malignancies. What is 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 to not only identify issues as they arise, but also overcome them.

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Author contributions

Both authors wrote and edited the review.

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