Adapted to Survive: Targeting Cancer Cells with BH3 Mimetics

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ABSTRACT A hallmark of cancer is cell death evasion, underlying suboptimal responses to chemotherapy, targeted agents, and immunotherapies. The approval of the anti-apoptotic BCL2 antagonist venetoclax has finally validated the potential of targeting apoptotic pathways in patients with cancer. Nevertheless, pharmacologic modulators of cell death have shown markedly varied responses in preclinical and clinical studies. Here, we review emerging concepts in the use of this class of therapies. Building on these observations, we propose that treatment-induced changes in apoptotic dependency, rather than pretreatment dependencies, will need to be recognized and targeted to realize the precise deployment of these new pharmacologic agents.

Significance: Targeting antiapoptotic family members has proven efficacious and tolerable in some cancers, but responses are infrequent, particularly for patients with solid tumors. Biomarkers to aid patient selection have been lacking. Precision functional approaches that overcome adaptive resistance to these compounds could drive durable responses to chemotherapy, targeted therapy, and immunotherapies.

INTRODUCTION

The death of cancer cells is crucial for the durability of chemotherapy, targeted therapies, and immunotherapies (1). Adaptation to these therapies, despite dramatic initial responses, suggests that fully eradicating cancer cells could reduce the emergence of acquired resistance. Thus, understanding how malignant cells die, or more precisely, why they often fail to die in response to therapy, has the potential to improve responses to a wide variety of anticancer therapeutics.

Multiple types of cell death may be involved in cancer, including apoptosis, ferroptosis, necroptosis, pyroptosis, necrosis, and others (1–5). Although the relative frequency of these cell death pathways is still incompletely characterized, most cancers have dysregulated apoptosis. For example, gain-of-function mutations in *RAS* and *RAF*, t(14;18) translocations, or loss of common tumor suppressors such as *RB* or *TP53* inhibit this type of programmed cell death to ensure survival (6–8).

Given its importance in cancer pathogenesis, significant efforts have been taken to understand how tumor cells protect themselves from apoptosis and develop therapies to reengage this process. The recent development of specific pharmacologic agents that directly target the apoptotic regulatory machinery has finally created an opportunity to target these pathways. Here, we summarize the preclinical and clinical data that illustrate the contexts in which these treatments will be applicable and propose strategies that overcome barriers to their successful clinical deployment.

BCL2 FAMILY PROTEINS REGULATE APOPTOTIC CELL DEATH IN CANCER CELLS

Apoptosis can be triggered by two different, well-characterized pathways: extrinsic and intrinsic. Extrinsic apoptosis is activated by the binding of death ligands such as Fas/APO-1, TNFα, TNF-related apoptosis-inducing ligand (TRAIL) to their respective death receptors (CD95/FasR, TNFR1, and DR4/DR5). The subsequent conformational changes lead to the formation of the dynamic multiprotein death-inducing signaling complex (DISC; refs. 9, 10). DISC enables dimerization of caspase-8, leading to its activation, which engages downstream executioner caspases and apoptosis. This process is complex and tightly regulated at several levels, as reviewed elsewhere (1).

The intrinsic pathway, also known as the mitochondrial pathway, is regulated by the BCL2 family of proteins. This

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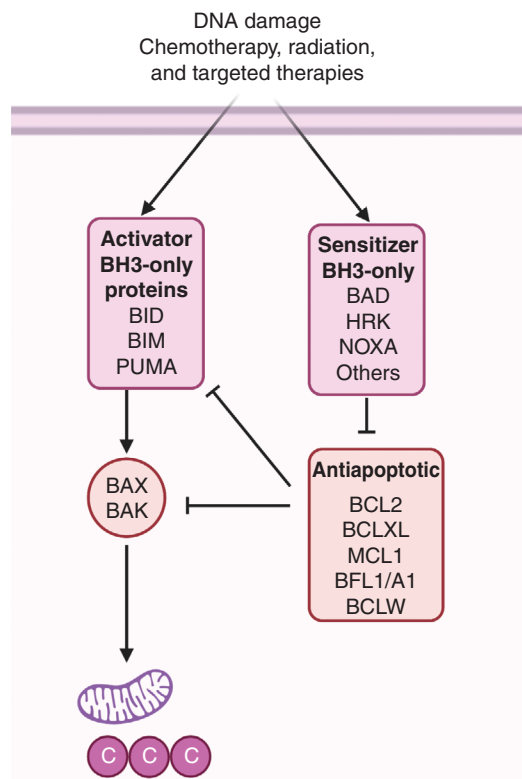The document metadata - page header indicates REVIEW, Montero and Haq.

Figure 1. The BCL2 interactome. The BCL2 family of proteins is comprised of four distinct subgroups: effectors, activators, antiapoptotics, and sensitizers. Once activated, effectors BAX and BAK induce mitochondrial outer membrane permeabilization (MOMP), leading to apoptosis. In response to therapy or oncogene activation, BH3-only activators (BID, BIM, or PUMA) engage effectors, promoting cell death. Antiapoptotic proteins (BCL2, BCLXL, MCL1, BFL1/A1, and BCLW) sequester activators or effector proteins to prevent apoptosis. BH3-only sensitizers act as selective antagonists of antiapoptotic proteins. For example, BAD has high affinity for BCL2, BCLXL, and BCLW, but not for MCL1 or BFL1. In contrast, HRK selectively binds to BCLXL, and NOXA specifically binds to MCL1. When proapoptotic members outnumber antiapoptotic, the mitochondria are permeabilized by BAX/BAK releasing cytochrome c and SMAC/DIABLO to the cytosol and engaging apoptosis.

family has different members that can be classified based on structure, function, and BCL2 homology (BH) domains (11, 12): activator members, antiapoptotic members, sensitizers, and effectors (Fig. 1). Although the execution of this pathway requires the effector members BAX and BAK (13, 14), the three other groups determine the threshold required for their activation. First, the so-called BH3-only activator proteins (e.g., BIM, BID, and PUMA), each possessing a unique BH3 domain, directly bind to effector proteins promoting conformational changes that result in effector oligomerization and mitochondrial outer membrane permeabilization (MOMP; refs. 13, 15, 16). Second, antiapoptotic proteins such as BCL2, BCLXL, MCL1, BCLW, and BFL1/A1 can directly bind and sequester both activator and activated effector proteins, preventing apoptosis (17). In fact, antiapoptotic protein neutralization is sufficient to initiate this type of programmed cell death (18). There is a fourth group in the BCL2 family of proteins, the sensitizers, each of which also possesses a unique BH3 domain but cannot directly activate

BAX and BAK. Sensitizers include BAD, HRK, BIK, NOXA, BMF, and BIK and exert a proapoptotic effect by competing for specific binding to antiapoptotic BCL2 family members and releasing the activators and effectors (19). For example, BAD has a high affinity for BCL2, BCLXL, and BCLW, but not for MCL1 or BFL1/A1. In contrast, HRK selectively binds to BCLXL, and NOXA specifically binds to MCL1 and BFL1/A1 (20). When activators outnumber and overcome inhibition by antiapoptotic proteins, they induce BAX and BAK oligomerization resulting in MOMP (21), and the release of cytochrome c, SMAC/DIABLO, and other proteins from the mitochondrial intermembrane space into the cytosol. Cytochrome c then binds to APAF1 and caspase-9 in the presence of dATP to trigger the apoptosome formation and activation of effector caspases, finally leading to apoptosis (13, 14). Inhibitor of apoptosis proteins (IAP) suppress this process by inhibiting caspases. SMAC/DIABLO in turn can block IAPs binding of caspases, allowing apoptosis to proceed (22, 23). The BCL2 family of proteins collectively represents an intricate interactome controlled at several levels: dynamic binding of its members, altered transcription and translation, and posttranslational modifications (12).

Most cancer cells have dysregulated apoptotic signaling (11, 24). Dysregulation of antiapoptotic proteins in cancer cells was initially recognized following the discovery of BCL2 as an oncogene product of the t(14:18) chromosomal translocation found in malignant lymphomas (25–28). Based on their homology to the sequence of BCL2, other proteins were later identified (29). Among them, other antiapoptotic proteins were recognized as survival factors in cancer, notably BCLXL and MCL1 (30, 31). These proteins are upregulated in different types of cancers (32–34). In addition to their higher expression of antiapoptotic BCL2 family members, most cancer cells also have higher levels of proapoptotic proteins (35). The increased binding of antiapoptotic proteins to proapoptotic BCL2 members not only prevents cell death, but also makes cancer cells more vulnerable to apoptosis compared with normal cells. Accordingly, most tumors exhibit a higher state of readiness to die, or "priming," compared to normal cells (36, 37). Cells that are more "primed" are closer to the apoptosis threshold and more sensitive to anticancer agents. Heightened apoptotic priming of cancer cells underlies the selective elimination by anticancer therapy compared with effects on most adult healthy tissues (with the notable exception of hematopoietic cells), which present low expression of apoptotic proteins and are refractory to apoptosis (38, 39).

Different anticancer therapies induce cell death through diverse effects on apoptotic signaling, including accumulation of proapoptotic BH3-only proteins, mostly activators (16, 36, 40, 41) but also sensitizers (42); decreases in antiapoptotic BCL2 family proteins (33, 43, 44); or both (45). Therefore, many anticancer agents prime cancer cells toward a proapoptotic phenotype through different mechanisms.

USING BH3 MIMETICS TO ENHANCE APOPTOTIC RESPONSES

Given the frequent dysregulation of the antiapoptotic BCL2 family members in cancer, several pharmaceutical
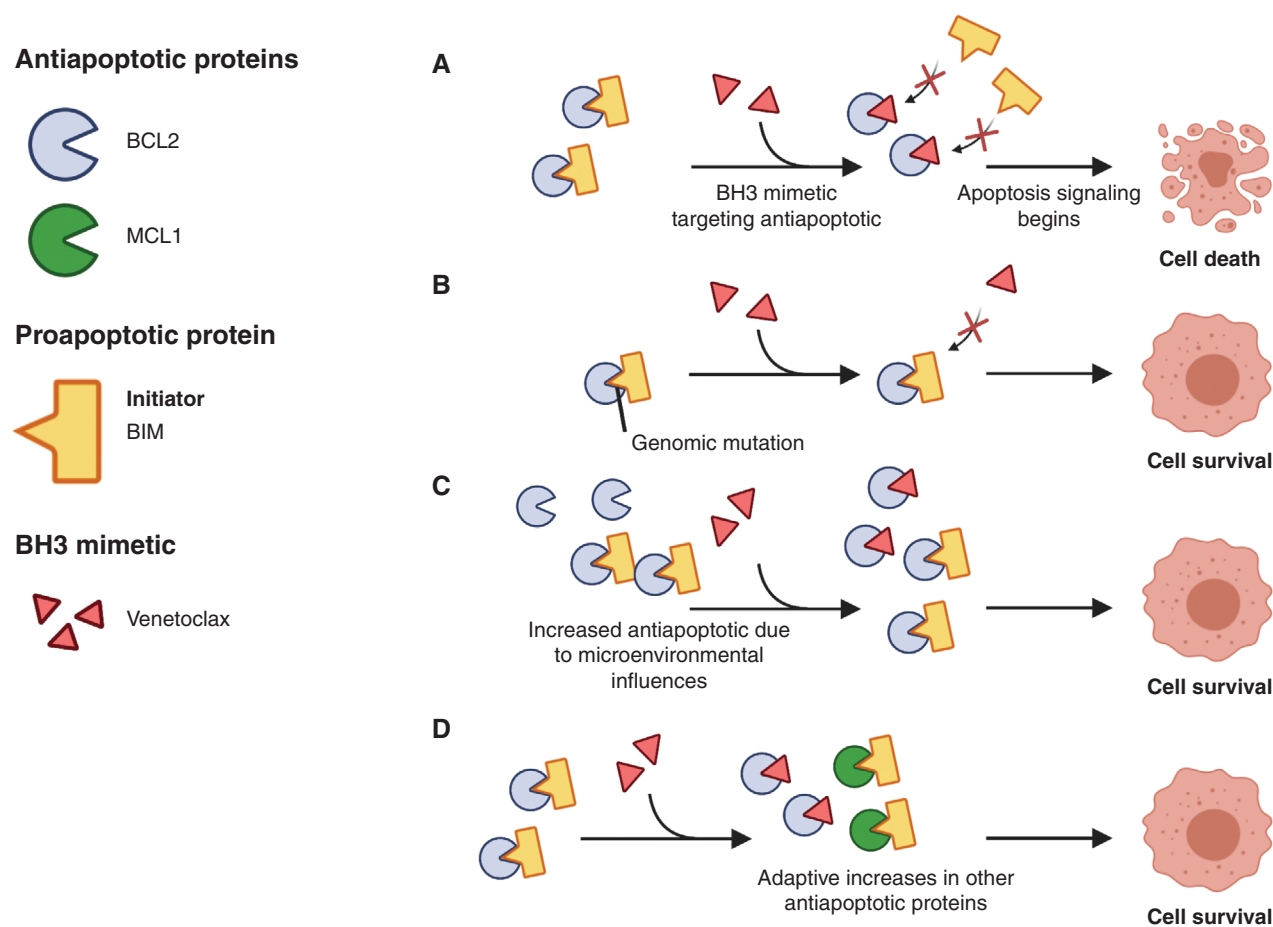
Antiapoptotic proteins

BCL2

MCL1

Proapoptotic protein

Initiator
BIM

BH3 mimetic

Venetoclax

Figure 2. Mechanisms of resistance to BH3 mimetics. For illustrative purposes, we present BCL2 as a prototypical antiapoptotic family member, BIM as a BH3-only protein, and venetoclax as the BH3 mimetic. **A,** The BH3 mimetic can disrupt binding of BCL2 to BIM, thereby enhancing BIM-dependent apoptosis. **B,** Genomic mutation that disrupts binding of the BH3 mimetic to the antiapoptotic family member. **C,** Microenvironmental influences such as IL10 or CD40 lead to enhanced expression of BCL2, limiting the effects of the BH3 mimetic. **D,** Cells adapt to conditions by upregulating alternative antiapoptotic family members, thereby reducing dependence on BCL2.

companies have developed compounds that imitate BH3-only proteins—the so-called BH3 mimetics. These small molecules bind with high affinity and specificity to the hydrophobic groove of the antiapoptotic proteins, inhibiting them and displacing bound proapoptotic proteins by imitating the action of sensitizer BCL2 proteins (Fig. 2A). The first molecule described to target BCL2 was HA14-1, which showed *in vitro* and *in vivo* activity alone or in combination with cytotoxic therapy (46). Obatoclax, which originated from a development program to therapeutically modulate BCL2 family members, followed (47) but had low affinity, had limited clinical activity, and was found to act through a BAX/BAK-independent mechanism (48). Other early attempts to target BCL2 included gossypol and oblimersen sodium (Genasense; Genta, an antisense oligonucleotide), but both were also unsuccessful, in part due to their low specificity or potency (49, 50). Abbott Laboratories (now AbbVie) developed ABT-737, the first on-target specific BH3 mimetic inhibiting BCL2, BCLXL, and BCLW (51, 52). This compound was further refined to improve its oral bioavailability, leading to the analogue ABT-263 (navitoclax; ref. 53). Navitoclax showed promising results in multiple blood cancers, particularly chronic lymphocytic leukemia (CLL), which express high levels of BCL2 (54, 55). However, this agent caused thrombocytopenia due to platelets' singular dependence on BCLXL (56). Navitoclax continues to be evaluated in multiple clinical trials but has yet to be approved by the FDA. To reduce the risk of thrombocytopenia, AbbVie developed a selective BCL2 inhibitor called ABT-199 (venetoclax; ref. 57). The clinical response of patients with CLL to venetoclax was evidence of impressive anticancer activity, leading to rapid debulking and even instances of tumor lysis syndrome (58).

Encouraged by its clinical efficacy, several pharmaceutical companies are currently developing other BH3 mimetics (Table 1). Even though inhibition of BCLXL may lead to the reported on-target thrombocytopenia (56), selective BCLXL inhibitors have also been developed for their potential anticancer activity. One of the first compounds shown to be highly selective was WEHI-539 (59), which was also effective against solid tumors. More recent derivatives of this first BCLXL inhibitor, such as A-1155463 or A-1331852 (60),

Table 1. BCL2 family member antagonists in clinical use or clinical trial development

Drug	Developer	Type	Target(s)	Development status
ABT-199 (venetoclax)	AbbVie	Small molecule	BCL2	Approved for CLL
ABT-263 (navitoclax)	AbbVie	Small molecule	BCL2, BCLXL, BCLW	Phase I/II
AMG-176	Amgen	Small molecule	MCL1	Phase I
APG-1252	Ascentage Pharma	Small molecule	BCL2, BCLXL	Phase I, II
APG-2575	Ascentage Pharma	Small molecule	BCL2	Phase I, II
AT-101	Ascenta Therapeutics	Small molecule	Pan-BCL2	Phase II; terminated
AZD-5991	AstraZeneca	Small molecule	MCL1	Phase I, II; suspended
AZD0466	AstraZeneca	Small molecule	BCL2, BCLXL	Phase I, II
BGB-11417	BeiGene	Small molecule	BCL2	Phase I/II
NU-0129	Northwestern University	siRNA	BCL2L12	Phase I; completed
Obatoclax	Teva	Small molecule	Pan-BCL2	Phase III; terminated
Oblimersen	Genta	Antisense oligonucleotide	BCL2	Phase III; halted
PRT1419	Prelude Therapeutics	Small molecule	MCL1	Phase I
S-055746	Servier; Vernalis; Novartis	Small molecule	BCL2, MCL1	Phase I; completed
S55746/BCI-201	Servier; Novartis	Small molecule	BCL2	Phase I; completed
S64315/MIK665	Novartis	Small molecule	MCL1	Phase I, II
SPC2996	Santaris Pharma	Locked nucleic acid antisense	BCL2	Phase I; completed

NOTE: Data based on ClinicalTrials.gov as of December 2021.

have also been studied, with promising preclinical results. In particular, the latter holds great promise due to its oral bioavailability (61, 62), yet none of these inhibitors have been assessed in clinical trials. In contrast, a new strategy targeting BCLXL degradation using a proteolysis-targeting chimera (PROTAC; DT2216), which has unique selectivity to target this antiapoptotic protein in tumor cells but not in platelets, is now under evaluation, with clinical potential (63). This strategy, if successful, could overcome one of the major clinical limitations of navitoclax in the treatment of patients with solid tumors.

The observation that the MCL1 antiapoptotic protein is commonly used by cancer cells to evade apoptosis (64–69) has also stimulated the development of novel targeted therapies. One of the first selective inhibitors was A-1210477, presenting excellent *in vitro* results in hematologic malignancies and solid tumors, such as breast and lung cancer cell lines (70), particularly in combination with navitoclax. Encouraged by the anticancer action of these molecules, a new generation of promising small-molecule MCL1 antagonists is now in clinical development. These include S64315/MIK665 (71), AZD-5991 (72), PRT1419 (73), and AMG-176 (74), among others, which are currently being explored in clinical trials (NCT02992483, NCT04629443, NCT03013998, NCT02675452, NCT04178902, NCT04543305, and others), mostly in hematologic malignancies. Other compounds that inhibit or degrade MCL1 have also demonstrated activity in preclinical models (75–78). Given that MCL1 is a short-lived protein (79), indirect targeting by CDK9 inhibitors, such as alvocidib, AZD4573 (NCT03263637), or voruciclib (NCT03547115), or protein selective degradation approaches

(75) have emerged as alternative strategies (80). BFL1/A1 can also be similarly targeted using CDK9 inhibitors (80) or dual inhibitors of MCL1 and BFL1 (78).

The potential for toxicities associated with MCL1 inhibition has been raised (81, 82). Toxicities associated with MCL1 inhibition by S63845 could not be fully appreciated in initial studies due to the lower affinity of this drug for murine compared with human MCL1 (81). However, MCL1 inhibition led to only modest toxicity in a humanized murine model (83). Similarly, AMG-176 was relatively well tolerated alone or when combined with venetoclax in a humanized MCL1 knock-in model (74). In some early clinical trials of MCL1 inhibitors, drugs were generally tolerated, with mostly hematologic and gastrointestinal side effects. However, a trial of AMG-397 was suspended because of cardiac toxicity. These toxicities may be related to on-target effects on cardiac myocytes, potentially limiting the drug's therapeutic window.

Other alternative approaches to restoring apoptosis are being explored. Although antiapoptotic BCL2 inhibitors lead to indirect activation of BAX and BAK, direct pharmacologic activation of BAX has recently been described in preclinical models (84–86). This approach can overcome resistance to BH3 mimetics in some cancer cells that exhibit decreased levels of BH3-only proteins. BAX is present in most cancer cells (albeit in an inactive or inhibited state) and is infrequently inactivated in cancers. A pharmacologic activator of BAX exhibited cytotoxicity in AML cells while sparing healthy cells (84). BAX activators synergize with BH3 mimetics in tumors that have decreased levels of BH3-only proteins (87).

BH3 MIMETICS HAVE LIMITED CLINICAL ACTIVITY IN MOST CANCER TYPES

BH3 mimetics have been evaluated in hundreds of clinical trials. Though a description of all these studies is beyond the scope of this review, here we summarize key observations about their use as single agents in the treatment of both hematologic and solid tumors. Except in a few specific disease types, such as hematologic malignancies like CLL or blastic plasmacytoid dendritic cell neoplasm (BPDCN; refs. 58, 88), BH3 mimetics as monotherapy have not produced high response rates.

Navitoclax was the first BH3 mimetic tested in patients with CLL, given the high expression of the BCL2 protein in CLL. Although there was clear activity, the dose-limiting thrombocytopenia associated with BCLXL inhibition led AbbVie to later investigate the BCL2-specific venetoclax in patients with CLL. Venetoclax had impressive single-agent activity, causing tumor lysis syndrome and demanding a dose-escalation strategy and clinical surveillance. It showed outstanding activity even in relapsed patients with CLL, achieving an 80% response rate with tolerable secondary effects (58). It was later approved for the treatment of patients with CLL with 17p chromosomal deletion, becoming the first BH3 mimetic permitted in the clinic for cancer treatment. Currently, venetoclax has been approved for different clinical indications in CLL, including its combination with rituximab for relapsed/refractory (R/R) patients and with obinutuzumab for first-line treatment, and further evaluated in dozens of clinical trials (89). In acute myelogenous leukemia (AML), venetoclax has been approved in combination with other chemotherapeutic agents (azacitidine, decitabine, or low-dose cytarabine), but as monotherapy the overall response rate in R/R patients was only 19% (90, 91). The European Medicines Agency (EMA) recently approved the use of venetoclax in combination treatment with a hypomethylating agent in adult patients with newly diagnosed AML who are ineligible for intensive chemotherapy.

Evidence of a role for BCL2 family dependence in solid tumors from preclinical data has similarly failed to translate into significant responses in the clinic thus far. Among the first approaches to target BCL2 family members in solid tumors was an antisense oligonucleotide specific for BCL2 mRNA (oblimersen; ref. 92). Despite encouraging initial results in human tumors, a phase III trial found that the addition of oblimersen did not improve the overall survival of patients with melanoma treated with chemotherapy. A modest increase in progression-free survival and response rate was observed (93), but the toxicity and lack of survival benefit data led the FDA to reject its application. In lung cancer, venetoclax and navitoclax had cytotoxicity in small-cell lung cancers (SCLC; refs. 94, 95). Venetoclax is now being evaluated in pediatric and young adult patients with relapsed or refractory malignancies, which includes *MYCN*-amplified neuroblastoma (NCT03236857). Nevertheless, the activity of BH3 mimetics in solid tumors has been inadequately evaluated in the clinic. BCLXL inhibition is therapeutically challenging in patients with solid tumors because of on-target thrombocytopenia. Approaches such as PROTACs and antibody–drug conjugates such as ABBV-155 could circumvent these issues, as discussed above. Data from MCL1 inhibitor trials in solid patients are also eagerly awaited (NCT04837677).

These data collectively suggest that biomarkers to enrich populations more likely to respond to treatment or approaches that combine multiple drugs may be required for maximum efficacy (91, 96).

RESISTANCE MECHANISMS TO BH3 MIMETICS

Features of the tumor cell, such as genomic mutations, or microenvironmental influences have been investigated to determine their contribution to BH3 monotherapy resistance.

Genomic Mechanisms of Resistance to BCL2 Family Inhibitors

Both preclinical and clinical data indicate that somatic mutations in BCL2 family members may disrupt binding to BH3 mimetics, leading to therapeutic resistance (Fig. 2B). *In vitro* selection with continuous exposure to ABT-199 yielded two missense mutations in the BCL2 BH3 domain (F101C and F101L) that blocked drug binding. A missense mutation in the transmembrane domain of proapoptotic BAX was also observed (97). In an analysis of 15 patients with CLL treated with venetoclax, seven patients were found to have a G101V mutation in *BCL2*, which increased with the duration of treatment (98, 99). This mutation reduces the binding of venetoclax to BCL2 180-fold (99, 100). Other mutations in *BCL2* in venetoclax-resistant patients have also been described, putatively affecting drug affinity (101, 102). In subsequent studies, patients resistant to venetoclax were found that have distinct mutations in different CLL cells, all of which were not observed before treatment (103), suggesting subclonal mechanisms of genomic resistance.

Besides mutations of BCL2 family members themselves, resistance mutations in other pathways have also been described in patients. In a study of six patients with CLL treated with venetoclax, resistance was associated with multiple genomic changes, including *SF3B1* and *TP53* mutations (104, 105). The deletion of *CDKN2A/B* has been observed in two studies of venetoclax resistance (104, 106). CRISPR-based screens have also nominated other pathways that contribute to resistance, some of which are dysregulated in cancer (104). For example, somatic mutation or the genomic loss of the SWI–SNF chromatin remodeling complex factors was found to facilitate the upregulation of BCLXL in patients, leading to resistance to ibrutinib/venetoclax combination (107). Clinical experience with other BH3 mimetics is more limited. However, preclinical data also suggest that somatic mutations may contribute to resistance to other BH3 mimetic inhibitors (107).

Translational studies have observed that *FLT3* internal tandem duplication mutations as well as *PTPN11* mutations are associated with resistance to venetoclax therapy (108), likely through their effects on MCL1 and/or BCLXL (108–111). Mutations in *KRAS* also conferred resistance to venetoclax alone or in combination, likely by downregulating BCL2 and BAX and upregulating MCL1 and BCL2A1/BFL1. *SF3B1* as well as upregulation of *BCL2A1* (BFL1) may also be potential mechanisms of resistance to venetoclax monotherapy or combinations.

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Resistance Due to Alterations in the Tumor Microenvironment

The tumor microenvironment has been proposed to regulate the sensitivity of CLL cells to BH3 mimetics (Fig. 2C). One of the first pieces of evidence was described by Vogler and colleagues, who found that after ABT-737 treatment, resistant CLL cells developed in lymph nodes (112). Using *in vitro* assays, Thijssen and colleagues demonstrated that CD40 stimulation led to resistance to ABT-199 (113). Similarly, analysis of patients with MCL and CLL treated with ibrutinib and venetoclax demonstrated heterogeneous intrinsic resistance, which was associated with microenvironmental factors such as IL10 or the CD40 ligand (114). These agonists led to enhanced expression of MCL1 and BCLXL through NF-κB signaling.

The growing appreciation of the tumor microenvironment in modulating responses to BH3 mimetics has suggested that *ex vivo* culture systems may be helpful to understand the stromal components involved in resistance (115). One approach to target these stromal components was suggested by Davids and colleagues. The authors found that CLL cells from the peripheral blood are highly primed to undergo apoptosis (116), whereas stroma sharply reduced priming. Inhibition of stromal interactions using a PI3K δ-isoform or a BTK inhibitor displaced CLL cells into the blood and increased their priming (116, 117). Similar results were observed in follicular lymphoma (118).

ADAPTIVE RESISTANCE IS COMMONLY ASSOCIATED WITH BH3 MIMETICS

Despite the aforementioned genomic and microenvironmental influences on the response of tumor cells to BH3 mimetics, these mechanisms cannot explain widespread resistance in many cancer types, including most solid tumors. In contrast, adaptive changes to individual BH3 mimetics are commonly observed, contributing to resistance in many contexts (Fig. 2D). In such instances, inhibition of a specific antiapoptotic protein leads to rapid tumor-intrinsic nongenomic adaptation through a second antiapoptotic protein. Multiple studies describe compensatory mechanisms between antiapoptotic proteins as a cross-talk to ensure the tumor's survival. The potential of BH3 mimetics simultaneously targeting several antiapoptotic proteins or in combination with other anticancer agents is a burgeoning field of investigation that will affect the clinic in the coming years.

One of the first examples of this cross-compensating mechanism was described by Yecies and colleagues when exposing lymphoma cell lines to ABT-737 (119). They observed that BIM was displaced from BCL2 to MCL1 and BFL1/A1, resulting in therapy resistance (119). In patients with CLL, Haselager and colleagues have shown that proapoptotic BIM sequentially interacts with antiapoptotic BCL2 family members, and that BCLXL is more relevant for venetoclax resistance than MCL1 (120). In B-cell lymphoma models, transcriptional remodeling has been observed following ABT-199 treatment (121). These adaptive mechanisms are not restricted to hematologic malignancies. The combination of BH3 mimetics for solid tumors is a more recent field of study, but several reports demonstrated compensation between MCL1 and BCLXL. These codependencies have been described in multiple pediatric solid tumors (122), breast cancer (123, 124), melanoma (125), non–small cell lung cancer (NSCLC; ref. 66), cervical cancer (126), and many others.

Additionally, therapy-induced senescence is becoming an expanding field of study because it may promote resistance and ultimately tumor progression, as reviewed elsewhere (127). Paradoxically, these two distinct biological processes, senescence and oncogenesis, share a common vulnerability— antiapoptotic dependence—and BH3 mimetics such as navitoclax and others are extensively explored as senolytics (128, 129). Regardless of the exact mechanism underpinning persister cancer cells' resistance to treatment, they rely on BCL2 antiapoptotic proteins for survival, making BH3 mimetics excellent candidates to enhance therapy effectiveness in combination.

Except in rare cases where the inhibition of a cell's basal apoptotic dependency is sufficient to achieve complete responses (Fig. 3A), resistance to BH3 mimetics will emerge through genetic or epigenetic changes (Fig. 3B). Recognizing the widespread adaptive changes in response to BH3 mimetics in many cancer types suggests that approaches that target adaptive changes could improve their efficacy (Fig. 3C). Here, we outline some key principles of these strategies.

Targeting Multiple Antiapoptotic BCL2 Family Members

Combining several BH3 mimetics, such as MCL1 and BCL2/BCLXL inhibitors, has had significant efficacy in preclinical *in vitro* and *in vivo* models (70, 130), leading to their current evaluation in clinical trials (e.g., NCT0321683 and NCT03672695). Even pan-BCL2 inhibition combining BH3 mimetics is now being explored for different types of cancers (70, 130, 131). Whether targeting multiple BCL2 inhibitors can safely be administered concomitantly remains to be determined. Pan-BCL2 inhibition may be tolerable because normal adult tissues are relatively refractory to apoptosis with the exception of the hematopoietic system (38, 39). However, patients will likely need to be closely monitored to control myelosuppression, cardiotoxicity, and tumor lysis syndrome in hematologic lineages (56, 57).

Combining Anticancer Therapies with BH3 Mimetics

Undoubtfully, the most studied strategy regarding BH3 mimetics is their combination with current therapies. The fundamental idea behind this approach is that tumors rapidly adapt to current treatments, both conventional chemotherapy and targeted agents, and persister cancer cells survive leading to relapse (132). The primary mechanism by which chemotherapy may synergize with BH3 mimetics is by lowering the apoptotic threshold of cells (11). Preclinical data have shown synergy between cytotoxic agents such as cytarabine and venetoclax by enhancing BH3 activity and/or suppressing MCL1 to promote apoptosis. Consistent with these observations, venetoclax was associated with deeper remissions in patients with AML when combined with chemotherapy (133, 134), and MEK inhibition synergizes with ABT-737 (135) or venetoclax (136). A recent study by Flanagan and colleagues also demonstrated the potential
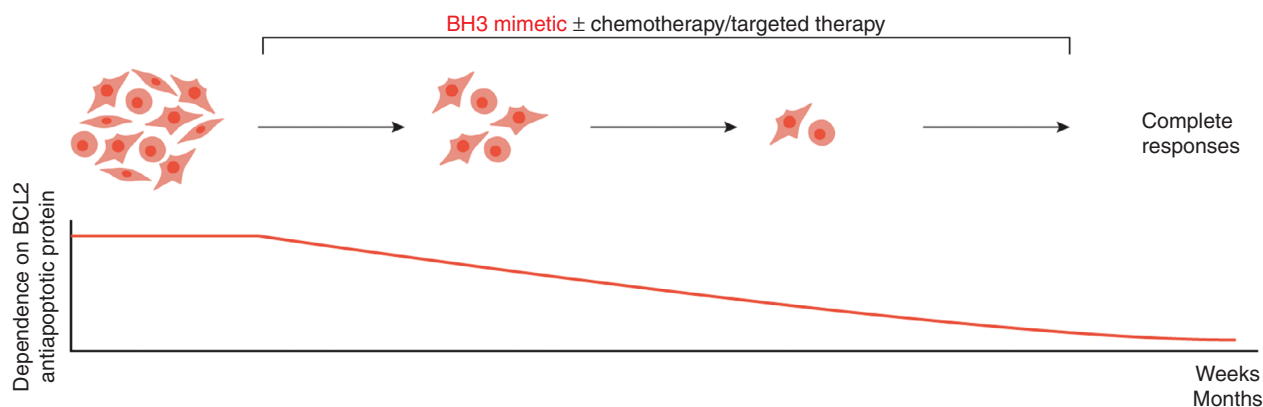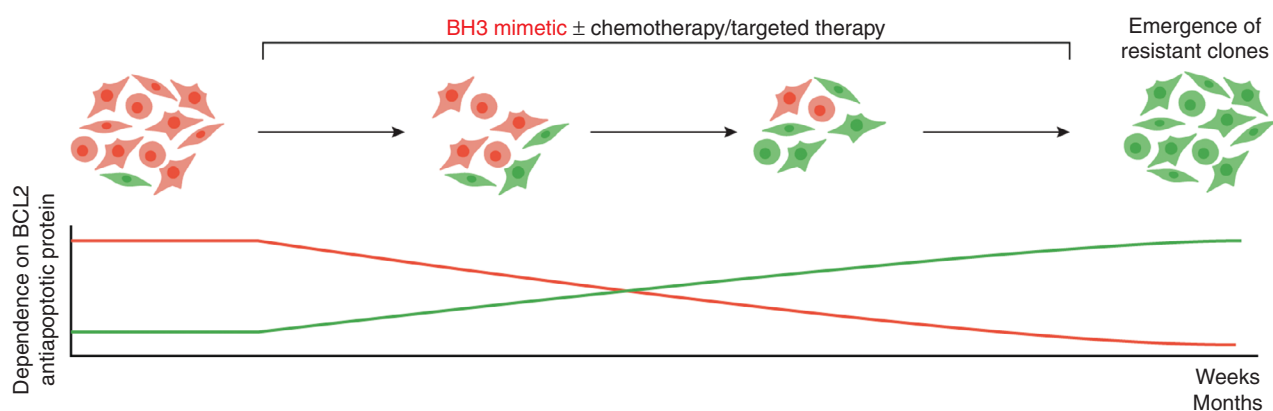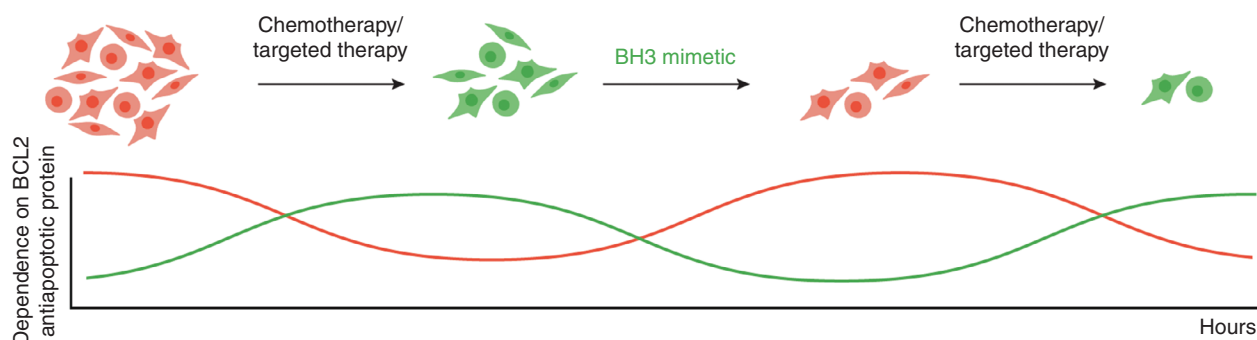
A   Basal dependence in homogeneous tumor population

B   Basal dependence in heterogeneous tumor population

C   Adaptive dependence in homogeneous population

Figure 3.   Adaptive resistance to BH3 mimetics. **A,** In the traditional model, the dependence of each cell to each antiapoptotic BCL2 member is established, thereby predicting response to a BH3 mimetic targeting this dependency. **B,** However, in a heterogeneous group of cells with different BCL2 dependencies (depicted as red or green), continuous treatment with a BH3 mimetic targeting a specific dependency (with or without targeted therapy) leads to cytotoxic responses of a subset of cells. Resistant clones with a different dependency emerge in weeks to months due to impaired binding of the drug to its target. Profiling of BCL2 dependence at the time of resistance would enable rational selection of alternative therapeutic approaches. **C,** In an adaptive model of resistance, apoptotic BCL2 member dependence is plastic. Chemotherapy or targeted therapy induces a change in the dependency of the cell to specific BH3 mimetics within hours. Profiling of the tumor will identify the specific dependency, which can then be targeted using a specific BH3 mimetic. In this model, dependence on specific BCL2 members evolves without genomic changes. Approaches that recognize and target these rapid adaptive changes may overcome resistance. In practice, both clonal evolution and adaptive resistance contribute to resistance to BH3 mimetics, although we posit that adaptive changes are more ubiquitous.

of combining epigenetic modulators with venetoclax to treat multiple myeloma (137). Other combinations of these agents with BH3 mimetics have been described for multiple hematologic malignancies (138).

Apart from the previously cited examples of BH3 mimetic combinations in hematologic malignancies, multiple reports demonstrated that BCL2/BCLXL inhibition combined with conventional chemotherapy was effective against many solid tumors, including breast cancer, ovarian cancer, neuroblastoma, NSCLC, and many others (32, 44, 130, 139–144). Also, as new compounds emerge, MCL1 inhibition has been successfully used in preclinical studies to boost standard-of-care treatments in hepatocellular carcinoma (68), breast cancer (145), ovarian cancer (130), and rhabdomyosarcoma (146), among others.

Many oncogenic kinases lead to the phosphorylation-dependent ubiquitination of BIM (147–150), suggesting that their upregulation by kinase inhibitors could enhance its levels. The stabilization of BIM through oncogenic kinase inhibitors could prime the cells toward apoptosis. This approach has the advantage of targeting cancer cells with dysregulated oncogenic signaling, potentially improving the therapeutic index over nonmalignant tissues. Many reports demonstrate that therapies that target kinase vulnerabilities in tumor cells may paradoxically induce prosurvival adaptations by antiapoptotic BCL2 family proteins (151, 152). In consequence, rational combinations with BH3 mimetics often enhance the cytotoxic effect of these targeted drugs. For instance, *PIK3CA*-mutant breast cancer is sensitive to mTOR and BCLXL inhibition (153), estrogen receptor–positive (ER+) breast cancer to tamoxifen plus venetoclax (143), and *HER2*-amplified breast cancer to lapatinib with the MCL1 inhibitor S63845 (145). For NSCLC, several combinations have been reported: gefitinib plus ABT-737 in *EGFR*-mutant tumors (36, 40), EGFR and MCL1 dual inhibition in drug-tolerant cells (154), third-generation EGFR inhibitors that target EGFR T790M with navitoclax (142), and dual MEK and MCL1 inhibition for *KRAS*-mutant tumors (155). Other examples include the aurora kinase A inhibitor MLN8237 combined with venetoclax for *MYCN*-amplified neuroblastoma (156), TORC1/2 inhibitors with navitoclax for *KRAS*- and *BRAF*-mutant colorectal cancer (43), PI3K inhibitors with BH3 mimetics against SCLC (157) and ER+ breast cancer (158), sorafenib/regorafenib plus navitoclax for hepatocellular carcinoma (159), dabrafenib/trametinib with MCL1 inhibitors for melanoma (151), and many others. Thus, the comprehensive identification of these prosurvival adaptations could enable new therapeutic strategies combining anticancer agents with BH3 mimetics to improve cancer elimination (see Fig. 3C). Although this approach seems especially promising to treat multiple solid tumors, their molecular plasticity complicates a correct prediction for treatment success (160, 161).

Targeting Mitochondrial Adaptation

The observation that mitochondrial metabolism is associated with changes in sensitivity to venetoclax/cytarabine therapy provides a rationale for using electron transport chain complex inhibitors (162, 163) and suggests that these inhibitors could overcome adaptation. Alternatively, switching combination therapies to circumvent these changes could be implemented using currently approved therapies (162).

Despite the improved understanding of the mechanisms underlying the sensitivity of some cancers to these agents, major questions remain. For example, it will be essential to evaluate if BH3 mimetics could adversely affect antitumor immunity due to some immune cells' dependency on BCL2 antiapoptotic family members. BH3 mimetics were observed to reduce selected CD4+ and CD8+ T cells, B cells, and some dendritic cells (164, 165). Nevertheless, the treatment of immunocompetent mice with MYC dysregulation with navitoclax and metformin increased the levels of tumor-infiltrating T cells and improved treatment outcomes in immunocompetent murine models (166). A recent study also demonstrated that venetoclax increases intratumoral effector T cells and cancer elimination when combined with immune-checkpoint inhibitors (167). Sharma and Allison have suggested that the killing of tumor cells could release tumor antigens, enhancing the efficacy of immunologic agents (168). Thus, there may also be a rationale for combining BCL2 inhibitors with chimeric antigen receptor (CAR) T-cell therapy, as this immune-based therapy kills tumor cells via apoptosis induction (169). Undoubtedly, the effects of BH3 mimetics on tumor immunity will add to the enormous complexity of apoptotic regulation. Clarification of the impact of apoptotic priming on tumor immunity could define specific approaches to enhance antitumor immune responses.

Overall, the clinical implementation of BH3 mimetics, as single agents but especially in combination, faces a clear problem: how to guide their use. In the next section, we cover some of the most recent approaches to answer this clear unmet need.

A NEW TYPE OF BIOMARKERS IS NEEDED FOR THE SELECTION OF BH3 MIMETICS

As described above, genomic biomarkers have not been strongly associated with clinical responses to BH3 mimetics. For example, CLL cells do not exhibit genetic alterations in BCL2, despite being strongly sensitive to venetoclax. Conversely, follicular lymphomas, which show dysregulated BCL2 expression due to the t(14;18) translocation driving the *BCL2* gene from the immunoglobulin promoter, are infrequently responsive to venetoclax as monotherapy (170). Transcripts of BCL2 family members may, to a limited extent, predict response to BH3 mimetics: navitoclax or venetoclax cytotoxicity is partially correlated with *BCL2* mRNA expression (171) but anticorrelated with *MCL1* expression (52, 172, 173). The expression of the BCL2 family of sensitizers also contributes to sensitivity. For instance, high expression of the MCL1 antagonist *NOXA* (gene name, *PMAIP1*) is associated with resistance to *BCL2L1* knockout (174). Yet these correlations are far from perfect, likely due to the multiple complex interactions of BCL2 family members as well as their numerous forms of regulation, including posttranslational modification. Measurements of these parameters, either alone or in combination, are not sufficient to predict the apoptotic response of cancer cells or the dependence on specific family BCL2 family members (175, 176). Given the relatively weak predictive capacity of individual features of the BCL2 family

members, we posit that a new generation of functional biomarkers will be needed to predict anticancer therapy induction of apoptosis.

Directly exposing patient-isolated living cancer cells to therapeutic agents *ex vivo* to determine chemosensitivity has historically been explored, but the development of novel technologies has fostered functional precision medicine. Some exciting new approaches include the direct evaluation in blood cancers (177, 178), organoids (179), pharmacoscopy (180), and cancer cell mass measurements (181). Functional assays may be particularly crucial for selecting individualized BH3 mimetics in solid tumors compared with hematologic malignancies because fewer respond to single-agent BH3 mimetics. However, a limiting factor is consistent *ex vivo* culture assays because compromised cell viability could lead to undesired phenotypic changes (182).

In this regard, the Letai laboratory developed the BH3 profiling method to rapidly identify antiapoptotic dependencies in cancer cells (176, 183–185). This functional assay uses synthetic ~20-mer BH3 peptides, mimicking BH3-only proteins, acting as a prodeath signal to induce MOMP. By using different peptides, BH3 profiling can interrogate the cancer cells and obtain precise information regarding the apoptotic status of the cell. For example, the BH3 peptides BIM and BID, which have the capacity to bind to all antiapoptotic proteins and also directly activate BAX and BAK, measure overall apoptotic priming (how close cancer cells are to the apoptotic threshold; ref. 16). Interestingly, overall pretreatment apoptotic priming by itself is a good indicator of clinical response to conventional chemotherapy (38, 39, 186). Furthermore, using peptides recapitulating the BH3 domain sequence of sensitizer proteins—such as BAD, HRK, or NOXA—BH3 profiling can identify specific antiapoptotic dependencies (21) and accurately identify BH3 mimetic cytotoxicity (187). For instance, a cell that is primed when exposed to the BAD BH3 peptide indicates BCL2/BCLXL dependence. Similarly, cytochrome c release following exposure to the HRK peptide points to BCLXL dependence. BH3 profiling analyses have evolved, and several technologies have been adapted to perform these measurements, such as fluorimetry (38, 183), flow cytometry (176, 185), microscopy (188), and even microfluidics (J. Montero; manuscript submitted). Numerous studies utilized BH3 profiling to determine the therapeutic use of BH3 mimetics for hematologic malignancies (44, 119, 184, 189–194). Therefore, the current deployment of BH3 profiling in clinical trials (NCT03593915, NCT01523977, NCT04898894, NCT03709758, and others) offers the possibility of patient stratification.

As mentioned earlier, cancer cells are overall more primed for apoptosis than normal adult tissues (38) and often rely on antiapoptotic proteins for their survival. Identifying vulnerabilities of individual tumors may be essential to guide implementation of BH3 mimetics. However, an alternative approach is to target them sequentially due to potential toxicities of simultaneous targeting of BCL2 family members. A key question with this approach is the timing and optimal sequencing of each agent and BH3 mimetic. The rationale development of such approaches will likely be aided by functionally monitoring these adaptations. Accordingly, the development of dynamic biomarkers for these combination

therapies and their deployment in selected populations will likely be required to maximize the potential of these therapeutics for patients.

Dynamic Biomarkers for BH3 Mimetics

One approach to generating novel predictive biomarkers for therapy response is termed dynamic BH3 profiling, or DBP (ref. 195; Fig. 4). DBP measures how much a given treatment primes cancer cells for apoptosis (Δ% priming) by measuring early changes in the apoptotic signaling preceding frank cell death days/weeks in advance. DBP represents an enormous technical advantage because it avoids sample deterioration due to the short *ex vivo* culture and permits directly testing therapies on patient-isolated cancer cells in less than a day. This assay allows for rapid functional analysis of many samples and treatments and has been successfully tested *in vitro*, in murine models, and on patient samples (88, 185, 195–199). DBP is superior to prior attempts to guide cancer treatment, as it is performed within a day, can be high-throughput (188), has an excellent predictive capacity, and has been validated clinically (88, 195, 198).

Furthermore, by using specific BH3 peptides mimicking sensitizer proteins (like BAD, HRK, NOXA, and others), DBP can also rapidly identify antiapoptotic adaptations upon treatment (Fig. 4). Several laboratories used this approach to determine different combinations with BH3 mimetics now explored in the clinic. For instance, DBP identified that CLL patient cells acquired resistance to BTK inhibitors, such as ibrutinib and acalabrutinib, by BCL2 adaptation, pointing to their combination with venetoclax (152). Recently, this same strategy was used to detect a rapid MCL1-mediated protection in melanoma when using MAPK inhibitors and the enhanced synergistic cytotoxicity when combined with BH3 mimetics such as S63845 or AZD-5991. Interestingly, this study showed that the observed acquired antiapoptotic dependence to the BRAF inhibitor dabrafenib was not mediated by an increase in MCL1 expression, but through a *NOXA* mRNA destabilization leading to protein decrease (151). Other novel therapeutic combinations with BH3 mimetics were recently identified using this approach in NSCLC (200), breast cancer (123, 158, 201, 202), esophageal cancer/mesothelioma (203), and pediatric cancers (146, 204), among others. Depicting the complexity of these prosurvival adaptations and antiapoptotic cross-talk is key to accurately identifying the right drug combination and the optimal sequential administration to maximize the cytotoxic effect in tumors. In this regard, DBP can be uniquely used as a predictive biomarker to track tumor adaptation to treatment and guide the combined metronomic use of anticancer agents and BH3 mimetics to increase effectiveness, avoid relapse, and reduce undesired secondary effects in patients.

Implementation of DBP into clinical practice to predict individualized responses to BH3 mimetics is feasible at present, particularly in patients with hematologic malignancies. Recently, Garcia and colleagues reported that DBP strongly predicted response of patients with AML to therapy (198). The application of similar approaches in patients with solid tumors is nonetheless more challenging because resection of metastasis with solid tumors is typically not performed prior to treatment. Adaptation of DBP for a
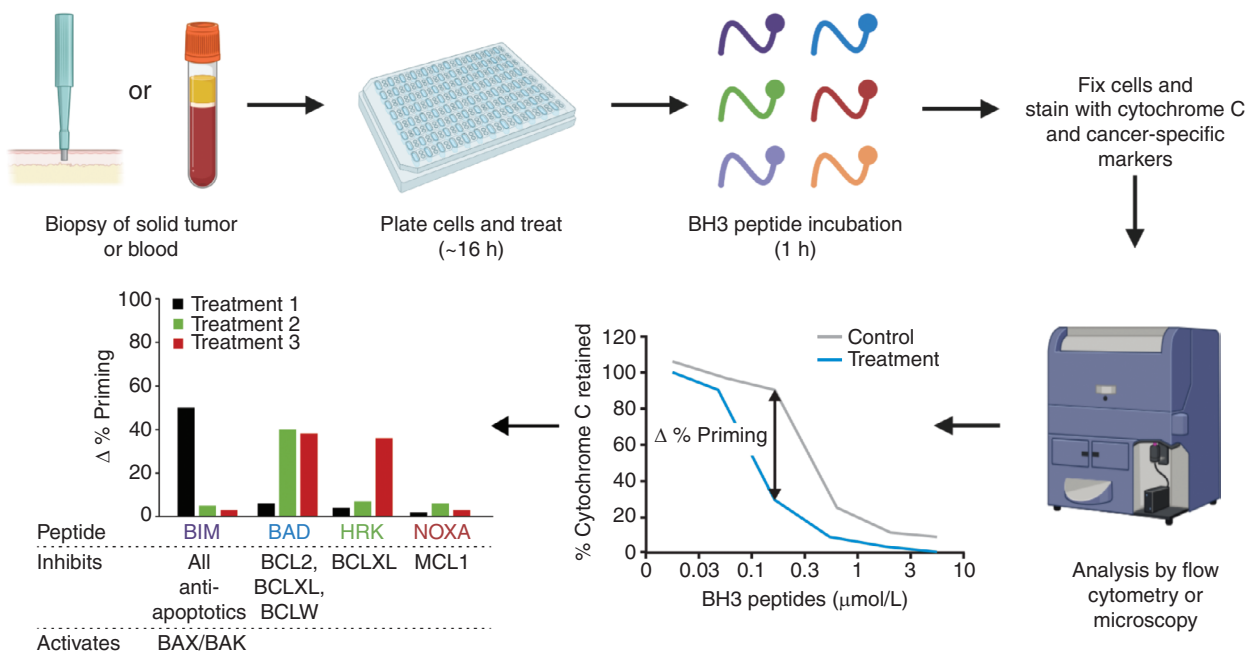
Figure 4. Scheme for the dynamic BH3 profiling of primary clinical samples. Tumor cells are isolated from either a solid tumor biopsy or blood. Cells are treated with an anticancer agent such as chemotherapy, targeted therapy, or a BH3 mimetic, followed by the addition of BH3 peptides. Mitochondrial depolarization (cytochrome c release) on tumor cells is analyzed by flow cytometry or microscopy. The percentage of change in priming is determined by comparing treated versus control cells, and this parameter predicts response to the agent. By using different BH3 peptides, BH3 profiling can predict response to treatment (using the BIM peptide) or changes in antiapoptotic dependencies (using specific BH3 peptides such as BAD, HRK, or NOXA/MCL1), which indicate potential combination therapies with BH3 mimetics. The overall time from biopsy to results is approximately 24 hours. Adapted from ref. 11 and used under a CC BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).

small number of cells (e.g., from fine needle or core biopsies) will be required. Alternatively, it may be possible to propagate cells as organoids to improve the yield of tumor cells prior to profiling. Additional challenges for DBP and other functional predictive biomarkers for precision medicine are their clinical implementation that will undoubtfully demand regulatory approval, evaluation in clinical trials, and interdisciplinary work from research laboratories, industry, and agencies.

SUMMARY AND FUTURE DIRECTIONS

Overall, the relative safety of BH3 mimetics thus far has established that targeting apoptotic signaling may be a successful approach to enhance the treatment of patients with cancer. Their combination with current anticancer therapies, conventional chemotherapy or targeted therapies, and possibly with immunotherapies or CAR T cells, could significantly improve clinical outcomes. Moreover, these therapeutic strategies could also avoid relapse and diminish undesired secondary effects by sparing nontumoral tissues protected against apoptosis.

However, the key challenge of optimizing the opportunity provided by these apoptosis-inducing drugs is their individualized deployment. Precision medicine demands new therapeutic strategies and effective biomarkers to guide their use in the clinic; however, most efforts have been guided by analyses on DNA, RNA, and cancers' molecular components

in general combined with sophisticated bioinformatic evaluations. Undoubtfully, "omics" have changed how we stratify and treat patients in the clinic, but they still present some limitations. For instance, these measurements are static, as they isolate and analyze cancer cell constituents at a given time. Therefore, by definition, omics cannot evaluate dynamic cell changes in response to a perturbation.

Accordingly, deconvolution of BCL2 family member regulation and their variable dependencies across tumor types and cells emphasize the need for rational approaches to their targeting. Biomarker-driven approaches, especially functional assays, could enable efficient strategies to overcome resistance to current generation therapies. Targeting adaptation in BCL2 family members may help realize the unprecedented opportunity to target cell death in patients with cancer. By monitoring antiapoptotic adaptations to therapy with functional assays that track how living cancer cells respond to treatment, such as DBP, we could anticipate the appearance of persister cancer cells and overcome tumor cell death evasion. This approach could identify drugs that prime cancer cells to BH3 mimetics, mostly undetectable for other technologies, making possible personalized rational combinations with these agents. Utilizing functional assays such as BH3 profiling thus may be extremely valuable to guide BH3 mimetic clinical implementation. Precision medicine is entering a new era where clinicians can rationally design sequential combinations of drugs and BH3 mimetics to maximize cancer cell killing to benefit patients.

Authors' Disclosures

Acknowledgments

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