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Pathways and mechanisms of venetoclax resistance

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Abstract

The approval of venetoclax, a "BH3-mimetic" antagonist of the BCL-2 anti-apoptotic protein, for chronic lymphocytic leukemia represents a major milestone in translational apoptosis research. Venetoclax has already received "breakthrough" designation for acute myeloid leukemia, and is being studied in many other tumor types. However, resistance to BCL-2 inhibitor monotherapy may rapidly ensue. Several studies have shown that the other two major anti-apoptotic BCL-2 family proteins, BCL-X_L and MCL-1, are the main determinants of resistance to venetoclax. This opens up possibilities for rationally combining venetoclax with other targeted agents to circumvent resistance. Here, we summarize the most promising combinations, and highlight those already in clinical trials. There is also increasing recognition that different tumors display different degrees of addiction to individual BCL-2 family proteins, and of the need to refine current "BH3 profiling" techniques. Finally, the successful clinical development of potent and selective antagonists of BCL-X_L and MCL-1 is eagerly awaited.

Keywords

Venetoclax; resistance; BCL-2; MCL-1; BCL-X_L; rational combinations

Introduction

The small-molecule "BH3 (BCL-2 homology domain 3)-mimetic" drug, venetoclax (Venclexta™), is currently approved by the Food and Drug Administration (FDA) for the treatment of patients with relapsed chronic lymphocytic leukemia (CLL) with a 17p deletion.[1] Venetoclax produces high overall response rates (ORRs) in heavily pre-treated, high risk subjects with CLL and, remarkably, leads to complete responses (CRs) in a substantial proportion (20%) of patients when administered as monotherapy, a feature that distinguishes it from small-molecule inhibitors of the B-cell receptor (BCR) pathway.[2] However, no patient in this phase I study had previously received BCR pathway inhibitors; additionally, in the pivotal trial in patients with relapsed or refractory CLL and deletion 17p which reported a much lower CR (or CR with incomplete count recovery, CRi) rate (8%), only five patients (5%) had previously been exposed to ibrutinib, idelalisib or other BCR pathway inhibitors.[1] An ongoing trial is investigating venetoclax monotherapy in patients

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with CLL who relapsed after or were refractory to ibrutinib and idelalisib.[3] Early results from this trial were recently presented: the ORR was 70% in the prior ibrutinib arm and 48% in the prior idelalisib arm; only 1 CRi was documented by independent review (in the prior ibrutinib arm).[3] Nevertheless, single-agent venetoclax can induce minimal residual disease (MRD)-negative CRs in some patients with relapsed/refractory CLL.[1, 2] MRD negativity has been shown to be a powerful predictor of long-term outcome in CLL, irrespective of line and type of therapy.[4]

Based on promising efficacy in patients with relapsed/refractory (R/R) acute myeloid leukemia (AML) as a single agent,[5] as well as highly encouraging results in the frontline setting in combination with hypomethylating agents (HMAs),[6] venetoclax, in combination with HMAs, has also received “breakthrough therapy designation” from the FDA for previously untreated AML in patients who cannot receive intensive chemotherapy.

Venetoclax is a *bona fide* “BH3-mimetic” (a small molecule capable of binding to and antagonizing BCL-2 family anti-apoptotic proteins by mimicking the BH3 domain of pro-apoptotic proteins). As discussed further below, many other compounds were initially considered to act as BH3-mimetics, but were found later to induce tumor cell death through other mechanisms.[7, 8] Efforts over many years to develop small-molecule inhibitors of protein-protein interactions culminated in the discovery of ABT-737, the first genuine BH3-mimetic and a specific antagonist of the anti-apoptotic proteins BCL-2, BCL-X_L, and BCL-w.[9] A clinically relevant analog, ABT-263 (navitoclax), was soon developed,[10] but marked on-target (through BCL-X_L inhibition) thrombocytopenia observed in clinical trials of this agent limited its clinical utility.[11–14] As BCL-X_L is a critical pro-survival factor for platelets,[15] venetoclax (formerly ABT-199, GDC-0199) was rationally designed by reverse engineering using the chemical scaffold of navitoclax to generate a molecule which specifically targeted BCL-2 while sparing BCL-X_L and, therefore, platelets.[16] Like ABT-737 and navitoclax, venetoclax also inhibits BCL-w. Figure 1 schematically depicts the mechanism of action of venetoclax. Venetoclax is well-absorbed orally (at least 65%), and primarily cleared by hepatic metabolism (approximately two-thirds of the administered dose), with the remainder (likely unabsorbed parent drug) excreted as the parent drug and its nitro reduction metabolite in feces.[17]

Numerous preclinical studies have investigated mechanisms of resistance to ABT-737 and explored rational combinations to circumvent resistance to this agent. Although the lack of inhibition of BCL-X_L by venetoclax is an important distinguishing feature between the two agents, many of these principles apply to venetoclax as well. Other studies have specifically evaluated resistance to venetoclax and combinations with other targeted agents. In this article, we review the major known mechanisms of resistance to venetoclax, and discuss synergistic strategies to overcome the same, some of which are already being explored in clinical trials.

The BCL-2 family, mechanism of action of BH3-mimetics and BH3 profiling

Overview of the BCL-2 family

The intrinsic (mitochondrial) pathway of apoptosis is controlled by the BCL-2 family of pro- and anti-apoptotic proteins. The apoptosis "effectors" within this family are represented by BAX and BAK, and their activation triggers mitochondrial outer membrane permeabilization (MOMP), committing the cell to apoptosis through the mitochondrial pathway.[18, 19] The anti-apoptotic proteins of the BCL-2 family are BCL-2, BCL-X_L, MCL-1, BCL-w and BFL-1/A1. The anti-apoptotic (pro-survival) proteins bind to and sequester the apoptosis effectors, as well as the apoptosis "sensitizers", BIM, BID, PUMA, NOXA, BAD, BMF, HRK and BIK.[20, 21] The five anti-apoptotic proteins and BAX/BAK all share four homologous domains referred to as BCL-2 homology 1, 2, 3 and 4 (BH1, BH2, BH3, BH4). In contrast, the eight apoptosis "sensitizers" contain only the BH3 domain and are, therefore, called "BH3-only" proteins.[18, 19] Although the major pro-apoptotic function of the BH3-only proteins is binding to the anti-apoptotic proteins, thus releasing BAX/BAK,[22] some of them (e.g., BIM, PUMA, BID) may also be able to directly activate BAX/BAK.[23–26]

Some BH3-only proteins bind to multiple anti-apoptotic proteins, e.g., BIM, PUMA, while others are more selective, e.g., BAD, NOXA.[27] Similarly, the sequestering of the apoptosis effectors by the major anti-apoptotic proteins is also somewhat selective, e.g., BAK is bound by MCL-1 and BCL-X_L, but not by BCL-2.[28] BAX, on the other hand, is bound by BCL-2.[29]

Importantly, post-translational modifications, e.g., phosphorylation, significantly impact the function of different BCL-2 family proteins; thus, BAD, which antagonizes BCL-2, BCL-X_L and BCL-w, must be dephosphorylated at two different serine residues by combined inhibition of mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling in order to perform its pro-apoptotic functions.[30] Furthermore, cellular survival signaling pathways can regulate BCL-2 family proteins, e.g., extracellular signal-regulated kinase (ERK) signaling promotes proteasomal degradation of BIM.[31]

BH3-mimetics

True BH3-mimetic compounds bind to the anti-apoptotic proteins by virtue of their structural similarity to the BH3 domains of the BH3-only proteins, displacing the latter, and inducing apoptosis through BAX/BAK activation.[32] A number of drugs (e.g., obatoclax, gossypol, AT101) that were initially considered to act as BH3-mimetics may not, in fact, do so in cells. For example, obatoclax induces autophagic cell death independent of BAX/BAK,[33] and a number of putative BH3-mimetics induce endoplasmic reticulum (ER) stress and thus up-regulate NOXA, which antagonizes MCL-1.[8] While this may certainly be a therapeutically useful attribute of these agents, it is not a "BH3-mimetic" effect.[34] ABT-737/navitoclax and venetoclax, on the other hand, represent genuine BH3-mimetics. Because of the exquisite dependence of CLL cells on BCL-2 for survival,[35] and of platelets on BCL-X_L,[15] it has been proposed that true BH3-mimetic drugs must, at a minimum, rapidly induce apoptosis in CLL cells *ex vivo* if BCL-2-targeted (e.g., venetoclax[36]) and in platelets if BCL-X_L-targeted.[37] Since NOXA binding leads to

ubiquitination and degradation of MCL-1,[38–40] a BH3-mimetic targeting MCL-1 will, in fact, lead to rapid accumulation of MCL-1 by blocking this interaction, unless the agent also happens to inhibit the USP9X deubiquitinase.[37]

BH3 profiling

BH3 profiling is a technique that exposes permeabilized tumor cells to BH3-domain-containing peptides to measure MOMP.[41] The BH3 domains in these peptides are derived from the BH3-only proteins with known specificity for anti-apoptotic BCL-2 family proteins. The assay can help separate malignant cells into three major classes: those that have functional BAX/BAK but are not significantly sequestering pro-apoptotic proteins so that they respond to activator peptides but don't respond or respond only weakly to sensitizer peptides (competent but unprimed), those that lack functional BAX/BAK (apoptotically incompetent, no response even to very high doses of activator peptides) and those that respond to both activator and sensitizer peptides (primed for apoptosis).[41] Apoptosis priming as measured by BH3 profiling correlates with chemotherapeutic success in AML,[42] and decreased apoptotic priming may underlie microenvironment-mediated resistance to chemotherapy in CLL.[43] Furthermore, BH3 profiling can help identify which anti-apoptotic BCL-2 family protein primed cells are most reliant on for survival, e.g., a response to the Bad peptide indicates dependence on BCL-2, and one to HRK peptide dependence on BCL-X_L, while a response to NOXA peptide indicates dependence on MCL-1. The use of selective small-molecule antagonists of BCL-2 (e.g., venetoclax) or BCL-X_L (discussed below) can further improve the precision of the assay in identifying the major pro-survival protein at play.

Indeed, BH3 profiling has been used as a tool to identify the cause of acquired resistance to venetoclax in multiple myeloma (MM),[44] and predict sensitivity of MM cell lines and primary plasma cells from MM patients to both venetoclax and navitoclax,[45] and is being used to select patients for a clinical trial in AML investigating flavopiridol (alvocidib), a cyclin-dependent kinase 9 (CDK9) inhibitor that down-regulates MCL-1 (NCT02520011). In T-cell acute lymphoblastic leukemia (T-ALL), a disease with a poor prognosis and no currently approved targeted therapies, BH3 profiling reveals that while most cell lines and primary patient samples display dependence on BCL-X_L, the early T-cell precursor (ETP) subtype depends on BCL-2,[46] providing rationale for targeted therapeutic intervention with venetoclax in this high-risk group of patients.[47] Indeed, the early immature T-ALL cell line, LOUCY, is exquisitely sensitive to venetoclax while more differentiated T-ALL cell lines are not, and strong synergism with cytarabine is seen.[48]

"Mitochondrial profiling" improves upon BH3 profiling by also measuring basal levels of pro-survival BCL-2 family proteins.[49] Using this technique, it has been demonstrated that the apoptogenic efficacy of venetoclax in AML cells is highly dependent on baseline levels of BCL-X_L expression.[49] Indeed, while BH3 profiling appears to accurately predict BCL-2 dependence, it may not fully identify dependence on BCL-X_L and MCL-1, since the latter appear to correlate better with protein expression of BCL-X_L and MCL-1.[49]

Major determinants of venetoclax resistance: MCL-1 and BCL-X_L

Resistance to ABT-737/navitoclax

As ABT-737 inhibits both BCL-2 and BCL-X_L, it is not surprising that the main determinant of resistance to ABT-737 is MCL-1, the other major anti-apoptotic protein of the BCL-2 family.[50] Additionally, in AML cells, phosphorylated BCL-2 conferred resistance to ABT-737, and both inhibition of BCL-2 phosphorylation and reduction of MCL-1 expression restored sensitivity to ABT-737.[51] MCL-1 phosphorylation, resulting in enhanced stability of the protein and greater sequestration of BIM, has also been shown to contribute to ABT-737 resistance in human B-cell acute lymphoblastic leukemia (B-ALL) cell lines.[52] These observations resulted in a number of rational combinations being tested in preclinical systems, adding a variety of agents that down-regulate MCL-1 to ABT-737. MCL-1 is particularly critical to the development and maintenance of AML,[53] and being a short-lived protein, is sensitive to inhibition of transcription and translation.[54] Thus, CDK9 inhibitors,[55] sorafenib,[56] MEK inhibitors,[57] dual inhibitors of PI3K and mammalian target of rapamycin (mTOR)[58] and the combination of MEK and mTOR inhibitors[59] have all been shown to synergize with ABT-737 in induction of apoptosis in AML cell lines, primary AML samples and for some combinations, murine xenograft models of AML. In lymphoma cell lines that have developed resistance to ABT-737 through long-term exposure, increased levels of BFL-1/A1 and/or MCL-1 have been demonstrated.[60] Although pro-apoptotic BIM is still displaced by ABT-737 in these resistant cells, it is sequestered by the excess BFL-1/A1 and/or MCL-1 (partner swapping).[60] The TORC1/2 inhibitor, AZD8055, reduced MCL-1 protein levels and sensitized small-cell lung cancer (SCLC) cell lines as well as xenograft models of SCLC to navitoclax.[61]

Venetoclax resistance in CLL and rational combination strategies

Mature B-cells, both normal and leukemic (CLL), are highly sensitive to BCL-2 inhibition by venetoclax, which induces BAX/BAK-mediated apoptosis triggered mainly by BIM.[62] Although circulating CLL cells are extremely sensitive to ABT-737, those cultured in artificial systems that mimic the lymph node microenvironment quickly develop a high level of resistance to ABT-737 owing to *de novo* synthesis of BCL-X_L and BCL-1/A1.[63] While BCL-X_L-mediated resistance can be counteracted by higher concentrations of ABT-737,[63] this represents a problem with venetoclax, which lacks BCL-X_L-inhibitory activity and indeed, is clinically less efficient at clearing nodal disease than blood and bone marrow.[2] The BCR signaling inhibitor, ibrutinib, on the other hand, is particularly effective at mobilizing CLL cells from lymph nodes[64, 65] and, in both *ex vivo* and *in vitro* studies in CLL, appears to synergize with venetoclax in inducing cytotoxicity, accompanied by decreases in levels of MCL-1 and BCL-X_L.[66] Both BCL-X_L and BFL-1/A1 are up-regulated in B-lymphocytes by the transcription factor, nuclear factor kappa B (NF-κB),[67] an important downstream target of ibrutinib.[68] The combination of venetoclax and ibrutinib is currently being studied in a number of ongoing clinical trials in CLL (NCT02758665, NCT02910583, NCT02756897).

Other groups have found that inhibitors of spleen tyrosine kinase (SYK), e.g., entospletinib, down-regulate MCL-1 more completely than other BCR pathway inhibitors such as ibrutinib

and idelalisib, arguing for combining these agents with venetoclax in CLL.[69] However, the clinical efficacy of SYK inhibitors as single agents in CLL has been somewhat disappointing thus far.[70] In a high-content screening system that utilized an *in vitro* microenvironment model, the multi-kinase inhibitor sunitinib was identified as the clinically available kinase inhibitor most effective at overcoming venetoclax resistance (more than ibrutinib or idelalisib).[71] The mechanism was attributed to more efficient abrogation of microenvironment-mediated up-regulation of BCL-X_L, MCL-1 and BFL-1/A1 by sunitinib.[71] Interleukin-4 (IL-4) signaling, which proceeds through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, has been identified as a resistance mechanism to BCR pathway inhibition in CLL,[72] and the dual SYK/JAK inhibitor, cerdulatinib inhibits BCR- and IL-4-induced downstream signaling in CLL cells and induces apoptosis, particularly in IGHV-unmutated samples (characterized by greater BCR-signaling capacity and response to IL-4), and also prevents anti-IgM- and nurse-like cell (NLC)-mediated production of the chemokines CCL3/CCL4.[73] Cerdulatinib was able to overcome the protection afforded to CLL cells by anti-IgM, IL-4 or NLCs by preventing up-regulation of MCL-1- and BCL-X_L, but did not affect BCL-2 levels.[73] In samples treated with IL-4, cerdulatinib synergistically induced apoptosis with venetoclax *in vitro*.[73]

Silencing of microRNA (miR) 377 may be an important mechanism underlying the BCL-X_L overexpression that drives acquired therapeutic resistance to venetoclax in CLL and B-cell lymphomas.[74] Significantly higher BCL-X_L and lower miR377 expression has also been observed in CLL cells from patients with prior exposure to chemotherapy.[74] Interestingly, it was recently demonstrated that in contrast to some other tumor types, hypoxia activates the stress kinase p38 MAPK in CLL cells, which in turn causes MCL-1 down-regulation, a high BIM:MCL-1 ratio and enhanced sensitivity to both ABT-737 and venetoclax.[75] Although none are currently in the clinic, a number of BCL-X_L and MCL-1 inhibitors are being developed (discussed below).

As noted above, inhibitors of CDK9 down-regulate MCL-1 through transcriptional repression.[76] The pan-CDK inhibitor, dinaciclib, which exhibits significant single-agent activity in relapsed/refractory CLL,[77] also lowers MCL-1 stability via inhibition of cyclin E/CDK2, promoting its ubiquitination and degradation.[78] This releases BIM from MCL-1, resulting in striking synergism against CLL cell lines and primary patient samples in combination with either ABT-737 or venetoclax.[78]

Finally, CC-115, a novel inhibitor of mTOR kinase and DNA protein kinase, induced apoptosis in CLL cell lines and patient-derived CLL cells, irrespective of *p53, ATM, NOTCH1* or *SF3B1* mutational status and inhibited BCR signaling, including in idelalisib-resistant samples.[79] CC-115, which displayed evidence of clinical activity in 8 patients with relapsed/refractory *ATM*-deleted/mutated CLL/small lymphocytic lymphoma (SLL), was able to reverse CD40-mediated resistance to both fludarabine and venetoclax.[79]

Venetoclax resistance in other lymphoid neoplasms

Follicular lymphoma (FL)—The anti-apoptotic function of BCL-2 was discovered in the context of its overexpression in FL, which is characterized by a translocation between

chromosomes 14 and 18 that juxtaposes the *BCL-2* gene to the immunoglobulin heavy chain gene.[80, 81] The sensitivity of FL cells to venetoclax has been shown to correlate with their BCL-2/BIM ratio, with cells expressing higher levels of BIM being more susceptible.[82] Venetoclax-resistant cells had increased phospho-ERK, phospho-BIM and initially, increased phospho-AKT and markers of autophagy.[82] The acquisition of these resistance phenotypes could be prevented via selective ERK/AKT inhibition or anti-CD20 antibody treatment.[82] In FL cell lines, dexamethasone and BCR signaling inhibitors synergistically induce apoptosis, accompanied by down-regulation of BCL-X_L, inhibition of ERK1/2 phosphorylation and accumulation of non-phosphorylated BIM.[83] High-BCL-2-expessing FL cell lines are less sensitive to these agents, a phenomenon that can be reversed by venetoclax.[83] However, BCL-X_L overexpression can prevent the apoptotic response to dexamethasone, BCR pathway inhibitors and to venetoclax.[83]

Diffuse large B-cell lymphoma (DLBCL)—In venetoclax-resistant DLBCL cell lines generated via chronic exposure to venetoclax, substantial AKT activation and up-regulation of MCL-1 and BCL-X_L (causing sequestration of BIM) were observed.[84] The dual PI3K/mTOR inhibitor, NVP-BEZ235, as well as the PI3Kδ inhibitor, idelalisib, down-regulated MCL-1 and re-sensitized these resistant cells to venetoclax.[84] A small interfering (si) RNA approach down-regulating AKT, MCL-1 and BCL-X_L vindicated the role of each of these cell survival factors in mediating resistance to venetoclax.[84]

Acute lymphoblastic leukemia (ALL)—In xenograft studies of childhood ALL, concurrent inhibition of both BCL-2 and BCL-X_L appears necessary for anti-leukemic efficacy and BCL-X_L expression is a key predictor of poor response to venetoclax.[85] However, mixed lineage leukemia (*MLL*)-rearranged ALL has been shown to be a notable exception, retaining substantial susceptibility to BCL-2 inhibition alone.[85] Specifically, the MLL-AF4 fusion protein product of t(4;11) leads to high levels of BCL-2 expression and sensitivity to venetoclax, as well as synergism with standard induction-type chemotherapy *in vivo*.[86] The combination of BCR-ABL tyrosine kinase inhibitors (TKIs) and venetoclax is highly synergistic *in vitro* against Philadelphia chromosome positive (Ph^+) ALL.[87] Furthermore, dasatinib and ponatinib, through their effects on the Lck/Yes novel (LYN) tyrosine kinase, may induce BIM and inhibit up-regulation of MCL-1, thus potentially circumventing the development of venetoclax resistance.[87] The dasatinib/venetoclax combination was synergistic against patient-derived Ph^+ ALL samples and in xenograft models in mice, arguing for a clinical trial of this regimen in Ph^+ ALL.[87]

Mantle cell lymphoma (MCL)—Primary MCL cells are highly sensitive to venetoclax and the BCL-2/(BCL-X_L + MCL-1) messenger RNA (mRNA) ratio is highly predictive of venetoclax sensitivity.[88] Like in CLL, nodal microenvironment-mediated NF-κB activation and consequent BCL-X_L up-regulation confer resistance to venetoclax.[88] Interestingly, the consequent loss of apoptotic priming can be overcome by the type II, glycoengineered anti-CD20 monoclonal antibody, obinutuzumab, which blocks BCL-X_L induction through NF-κB inhibition.[89] As noted above, ibrutinib can mobilize malignant lymphocytes in CLL from their nodal microenvironmental niches, as well as block their subsequent tissue homing.[64] Ibrutinib is highly active in and FDA-approved for relapsed

MCL,[90] and ibrutinib-mobilized MCL cells appear to be highly sensitive to venetoclax.[88] Accordingly, this combination is being explored in clinical trials (NCT02419560). A unique mechanism of venetoclax resistance that has been described in human MCL-like murine lymphoma cells involves the acquisition of mutations (F101C, F101L) within the BCL-2 BH3 domain that impair venetoclax binding.[91] Additionally, a mutation in BAX (G179E) that interferes with its anchoring to the mitochondrial membrane has been documented in a venetoclax-resistant human MCL cell line.[91]

Multiple myeloma (MM)—In MM, venetoclax lethality may be restricted to the subset harboring the same t(11;14) that is the hallmark of MCL and results in cyclin D1 overexpression.[92] MM is highly dependent on MCL-1 (although a subset is addicted to BCL-X_L for survival),[93] and CDK9 inhibitors exert potent cytotoxicity against MM cells through MCL-1 down-regulation.[94] Although high levels of BCL-2 expression are common in MM, *in vitro* and xenograft studies using venetoclax, A-1331852 and A-1155463, selective inhibitors of BCL-X_L, and the proteasome inhibitor, bortezomib (which leads to c-MYC-dependent induction of NOXA in malignant cells and consequent neutralization of MCL-1[95]) show that BCL-X_L and MCL-1 may be more critical for survival of MM cells than BCL-2.[96] In these studies, sensitivity to venetoclax correlated with high BCL-2 to (BCL-X_L or MCL-1) expression ratios.[92, 96] Indeed, MM cells co-expressing BCL-2 and BCL-X_L were susceptible to A-1155463 but not to venetoclax, and venetoclax resistance in MM xenograft models co-expressing BCL-2 and MCL-1 could be mitigated by bortezomib.[96] MM xenograft models expressing all three major anti-apoptotic proteins were more sensitive to the combination of bortezomib and A-1331852 than to the combination of bortezomib and venetoclax.[96] In the setting of glutamine deprivation, MM cells induce BIM but its binding to BCL-2 is enhanced, enabling continued cell survival, but also priming them for apoptosis induction by venetoclax.[97] Thus, targeting glutamine metabolism represents an attractive synthetic lethal strategy to sensitize MM cells to venetoclax.[97]

Unlike navitoclax and venetoclax, which bind to the BH3 domain of BCL-2, BCL-2 IP3 Receptor Disruptor-2 (BIRD-2) is a decoy peptide that binds to the BH4 domain of BCL-2 and disrupts its interaction with inositol-1, 4, 5-triphosphate (IP3) receptors.[98] The BCL-2/IP3 interaction normally inhibits pro-apoptotic Ca^{++} signals, and BIRD-2 has been demonstrated to induce Ca++-mediated apoptosis in CLL, MM and FL cells, including those resistant to navitoclax, venetoclax or ibrutinib.[98] Furthermore, combining BIRD-2 with either navitoclax or venetoclax potentiates apoptosis induction by the BH3-mimetics alone.[98]

Venetoclax resistance in AML

Contrary to initial expectations, AML cell lines, primary patient samples and murine primary xenografts were found to be very sensitive to venetoclax.[99] In primary patient-derived cells, the median IC_{50} was approximately 10 nmol/L, and mitochondrial apoptosis occurred within 2 hours.[99] The *ex vivo* sensitivity of AML compared favorably with those observed in CLL models.[99] In a phase II study of single-agent venetoclax (800 mg daily) in patients with relapsed/refractory AML or previously untreated patients deemed unfit for

intensive chemotherapy, the overall response rate (ORR) was 19%, and anti-leukemic activity not meeting International Working Group (IWG) criteria for response[100] was observed in another 19%.[5] Consistent with preclinical findings demonstrating particular susceptibility of isocitrate dehydrogenase 1/2 (*IDH1/2*)-mutated AML to BCL-2 inhibition,[101] 4 of 12 (33%) patients harboring *IDH1/2* mutations achieved complete response (CR) or complete response with incomplete blood count recovery (CRi).[5] BH3 profiling demonstrated an on-target, mitochondrial mechanism of action of venetoclax and expectedly, identified BCL-X_L or MCL-1 dependence as potential resistance mechanisms.[5] In a recent report, investigators characterized a panel of venetoclax-resistant myeloid leukemia cell lines derived through chronic exposure to venetoclax and found that acquired drug resistance was indeed driven by the up-regulation of MCL-1 and BCL-X_L.[102] Interestingly, not only could the resistant AML cell lines be re-sensitized to venetoclax by targeting MCL-1 and BCL-X_L, pre-emptively targeting one or both of the latter could actually delay or forestall the acquisition of venetoclax resistance.[102] These findings have obvious implications for selection of initial therapy for AML.

Based on prior work showing that MEK inhibitors efficiently down-regulate MCL-1 in AML cell lines and primary AML cells (including $CD34^+38^-123^+$ leukemia-initiating cells) and synergize *in vivo* with ABT-737 (which induces MCL-1 through ERK activation),[57] a phase I/II combination clinical trial of the MEK inhibitor, cobimetinib (Cotellic®), plus venetoclax has been initiated in patients with relapsed or refractory AML (NCT02670044).

In another arm of this trial, venetoclax is combined with idasanutlin, an inhibitor of murine double minute homolog 2 (MDM2), an important negative regulator of p53. Because of the mechanism of action of MDM2 inhibitors, i.e., activation of p53-dependent apoptosis,[103] patients must have functional, i.e., wild type *TP53*. Preclinical studies with Nutlin-3a, a first-generation MDM2 inhibitor, and ABT-737 in AML published a decade ago showed strikingly synergistic induction of mitochondrial apoptosis by the combination.[104] A key concept underlying these phenomena was p53-mediated apoptosis induction by Nutlin-3a predominantly in S-phase and G2/M cells, while ABT-737 induced apoptosis predominantly in G1, the cell cycle phase characterized by the lowest BCL-2 protein levels and BCL-2/BAX ratios, and absent BCL-2 phosphorylation.[104] Nutlin-3a also potentiates ABT-737-induced apoptosis in both proliferating and quiescent $CD34^+$ chronic myeloid leukemia (CML) progenitor cells from patients in blast crisis (BC).[105] Most recently, the combination of idasanutlin and venetoclax has been studied in *TP53*-wild type AML cell lines (including in OCI-AML3 cells, which are relatively resistant to both venetoclax and idasanutlin, and are characterized by high levels of basal MCL-1 expression) and xenograft (subcutaneous and orthotopic) mouse models.[106, 107] The combination was synergistic both *in vitro* and *in vivo*, and inhibition of MCL-1 by the combination treatment was confirmed to be contributing to the superior activity of the regimen.[106, 107] Mechanistically, venetoclax was found to lead to MCL-1 stabilization and up-regulation through increased phosphorylation of ERK2, phenomena that were quickly reversed by p53 activation by idasanutlin.[107]

In combination with BCR-ABL TKIs, which partially down-regulate MCL-1 and BCL-X_L, venetoclax synergistically targets quiescent stem/progenitor cells in CML-BC patient samples and *BCR-ABL* transgenic mice.[108]

Since fms-like tyrosine kinase 3 (FLT3) signals downstream to regulate multiple members of the BCL-2 family, e.g., via the PI3K/AKT/mTOR and MEK/ERK pathways, and sorafenib, besides inhibiting FLT3, also down-regulates MCL-1 through inhibition of translation[109] and induction of endoplasmic reticulum (ER) stress,[110] there is sound preclinical rationale to combine sorafenib with venetoclax in *FLT3*-mutated AML, and a clinical trial is planned. Synergism between sorafenib and ABT-737 in AML cell lines and primary AML samples, accompanied by up-regulation of BIM and down-regulation of MCL-1, X-linked inhibitor of apoptosis (XIAP) and survivin has already been documented.[56] Recently reported benefits of adding sorafenib to intensive chemotherapy in all patients with AML, regardless of *FLT3* mutational status,[111] may extend the applicability of this approach beyond *FLT3*-mutated AML.

Preclinical studies using siRNA against the major anti-apoptotic proteins showed that in AML cell lines, silencing of BCL-X_L and MCL-1, but not BCL-2, resulted in varying degrees of synergism with azacitidine.[112] These findings were recapitulated by studies using ABT-737 and venetoclax, where the former sensitized most myeloid cell lines and primary patient samples to azacitidine more potently compared with venetoclax, which synergized with azacitidine mostly at higher doses.[112] Silencing of BCL-X_L and MCL-1 increased the activity of venetoclax.[112] In other studies, the combination of azacitidine and ABT-737 has been shown to synergistically induce apoptosis in primary AML cells in a p53-independent fashion, accompanied by azacitidine-induced down-regulation of MCL-1.[113] Highly promising results have been reported in an ongoing phase IB clinical trial (NCT02203773) of the combination of venetoclax with azacitidine or decitabine in patients 65 years of age or older with newly diagnosed AML unfit for intensive chemotherapy.[6] An ORR of 76% was reported for the first 39 patients; this was 88% and 82%, respectively, among patients with poor-risk cytogenetics and *IDH1/2* mutations.[6] Median time to CR/CRi was 29.5 days.[6]

Glutamine levels control mitochondrial oxidative phosphorylation in AML cells, and pharmacologic inhibition (e.g., by CB-839) of glutaminase, the enzyme that catalyzes the rate-limiting conversion of glutamine to glutamate, induces arrest of proliferation and activation of mitochondrial apoptosis, which is synergistically enhanced by venetoclax.[114] AML with mutant IDH enzymes, which produce the oncogenic metabolite, 2-hydroxyglutarate (2-HG) instead of the normal alpha-ketoglutarate (α-KG), creating glutamine addiction, may be particularly susceptible to glutaminase inhibition,[115] as it is to BCL-2 inhibition.[101]

Finally, given the critical importance of MCL-1 in AML,[53] pharmacologic strategies to activate/up-regulate NOXA, such as induction of ER stress, may be therapeutically useful.[116] Pevonedistat, a first-in-class small-molecule inhibitor of protein neddylation with single-agent activity in AML,[117, 118] up-regulates NOXA by causing c-Myc accumulation and synergizes with both venetoclax and navitoclax in AML.[119] ONC201, a

first-in-class imipridone that induces p53-independent apoptosis in primary MCL and AML samples and targets AML stem and progenitor cells, has been shown to increase translation of the transcription factor, ATF4, by triggering an atypical integrated stress response (ISR) not dependent on increased phosphorylation of the translation initiation factor, eIF2α.[120] ONC201 prominently down-regulates MCL-1, and BCL-2 overexpression was found to protect against ONC201-induced apoptosis.[120] Accordingly, ONC201 and venetoclax synergistically triggered apoptosis in AML and MCL cell lines.[120]

Venetoclax resistance in solid tumors

Venetoclax displaces BIM from BCL-2 and induces apoptosis through the mitochondrial pathway in neuroblastoma cells, but up-regulation of MCL-1 and sequestration of BIM by MCL-1 represent a major mechanism of resistance.[121] In colorectal carcinoma, targeting MCL-1 overcomes resistance to both ABT-737 and venetoclax, and gene therapy approaches have been studied *in vitro* and *in vivo*.[122] In both docetaxel-sensitive and -resistant prostate cancer, BCL-X_L seems to be a key determinant of cell survival, as navitoclax, but not venetoclax, significantly augments the anti-tumor efficacy of docetaxel in human prostate cancer cells lines and xenograft mouse models.[123] Conversely, in estrogen receptor-expressing breast cancer, BCL-2 appears to be the crucial target as similar efficacy is observed with venetoclax or ABT-737 in xenografts, despite abundant BCL-X_L expression.[124] Furthermore, BH3-mimetics have been shown to counteract tamoxifen-induced endometrial hyperplasia, as well as enhance its anti-tumor efficacy, and to synergize with dual PI3K/mTOR inhibitors in apoptosis induction in this setting.[124]

Novel compounds that inhibit BCL-X_L or MCL-1

The search for selective inhibitors of the anti-apoptotic functions of BCL-X_L and MCL-1 has been ongoing for many years, although none of these compounds is in the clinic yet. Early efforts to identify selective antagonists of BCL-X_L led to the description of the small-molecule, A-385358[125] and 072RB, a peptide derived from the BH3 domain of BIM.[126] Maritoclax was reported to be a selective MCL-1 antagonist able to disrupt the BIM-MCL-1 interaction and promote proteasomal degradation of MCL-1 that markedly enhanced the efficacy of ABT-737 against multiple hematologic malignancies,[127] but subsequent studies have shown that the compound likely also acts through mechanisms independent of MCL-1.[128] Of the early putative MCL-1 inhibitors, TW-37 is one that induces apoptosis in a BAK-dependent manner in cells that require MCL-1 for survival, but apoptosis induced by TW-37 is also, in part, dependent on NOXA.[129] (-)BI97D6 is a pan-BCL-2 family inhibitor that was shown to disrupt Mcl-1/Bim and Bcl-2/Bax interactions and induce BAX/BAK-dependent apoptosis in AML cells, overcoming MCL-1-mediated resistance as well as extrinsic, microenvironmental resistance.[130]

UMI-77 is a novel, selective MCL-1 inhibitor that blocks the heterodimerization of MCL-1 with BAX and BAK by binding to the BH3-binding groove of MCL-1, and induces BAX/BAK-dependent apoptosis in pancreatic cancer cells; it also effectively inhibited tumor growth in a xenograft model of pancreatic cancer.[131] These investigators also reported the discovery of a 3-Substituted-N-(4-hydroxynaphthalen-1-yl)arylsulfonamide compound that

binds selectively to MCL-1, disrupts the interaction between endogenous MCL-1 and biotinylated NOXA-BH3 peptide and causes BAX/BAK-dependent cell death in human leukemia cell lines and Emu-myc lymphomas overexpressing MCL-1, but not in those overexpressing BCL-2.[132] The development and optimization of potent and selective BCL-X_L inhibitors also continues to be pursued.[133, 134] In particular, BCL-X_L may be a more relevant therapeutic target than BCL-2 in lung cancer, and the BCL-X_L-selective inhibitors, BXI-61 and BXI-72, were found to exhibit greater potency against human lung cancer than ABT-737 with less thrombocytopenia *in vivo*.[135]

BCL-2 is important for neutrophil survival,[136] and neutropenia is therefore an important on-target adverse effect of venetoclax.[1, 2] Navitoclax, which inhibits both BCL-2 and BCL-X_L, causes significant worsening of neutropenia when combined with docetaxel in patients, but this problem is avoided in preclinical studies using the BCL-X_L-selective inhibitors, A-1155463 and A-1331852, in combination with docetaxel against a range of solid tumors, while maintaining high efficacy.[137]

A-1210477 is a potent and selective inhibitor of MCL-1 that binds to MCL-1 with sufficient affinity to disrupt BIM-MCL-1 complexes and induce apoptosis via the mitochondrial pathway in MM and non-small cell lung cancer (NSCLC) cell lines shown to be MCL-1-dependent by BH3 profiling or using siRNA.[138] A-1210477 synergizes with navitoclax to kill a variety of cancer cell lines.[138] A-1210477 synergizes with venetoclax in BCL-2-high non-Hodgkin's lymphoma (NHL) cell lines, and with navitoclax in BCL-2-low NHL cell lines, and chemical segregation studies using venetoclax or A-1155463 show that BCL-X_L inhibition is the principal driver of synergy in the latter.[139] S63845 is a more recently described MCL-1 inhibitor that potently induces BAX/BAK-dependent mitochondrial apoptosis in MM, leukemia and lymphoma cells, and exhibits potent *in vivo* anti-tumor activity against a number of different tumor types with good tolerability in mice.[140] Other compounds that inhibit MCL-1 at picomolar concentrations and cause MOMP and caspase activation in engineered cell lines, as well as inhibition of proliferation of primary MM and AML cells, have been discovered, laying the foundation for the development of clinical candidates.[141]

Thus, the (preclinical) availability of potent and selective small-molecule antagonists of all three major pro-survival BCL-2 family proteins now allows us to carefully dissect the anti-apoptotic protein dependencies of individual tumor types and select optimal drug combinations to test in the clinic. In the case of kinase inhibitors, the oncogenic kinase pathway dependence of leukemia cells can be identified in 70% of specimens in just three days using a rapid inhibitor screen.[142] Once other BH3-mimetics targeting BCL-X_L and MCL-1 become available for clinical use, it is likely that BH3-profiling and similar assays will be widely used to predict tumor susceptibilities to the individual agents.

Conclusions

Venetoclax is the first BH3-mimetic to receive regulatory approval and its use is likely to expand to additional tumor types in the coming years. Predictably, high levels of BCL-X_L and MCL-1, the two major anti-apoptotic proteins of the BCL-2 family not inhibited by

venetoclax, are key determinants of both acquired and intrinsic resistance to venetoclax. This paves the way for a vast array of rational combinations as outlined in this article, both with emerging, selective antagonists of BCL-X_L and/or MCL-1, and with other classes of targeted agents. Table 1 lists clinical trials combining venetoclax with both conventional and other novel agents in diverse hematologic tumor types. At present, there are no clinical trials evaluating this agent in solid tumors. Finally, improved techniques to assess apoptotic priming (e.g., mitochondrial profiling) and a more robust toolkit of potent and selective BH3-mimetics will help us better understand the pro-survival protein addiction patters of individual tumors and tailor therapy accordingly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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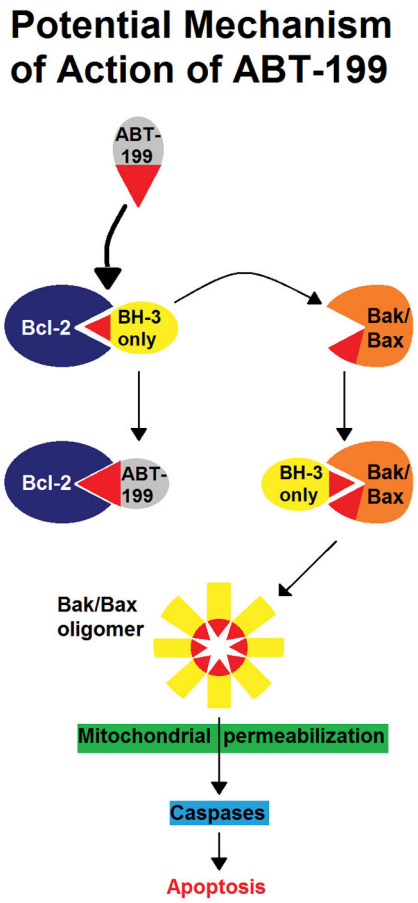

Figure 1.

Potential mechanism of action of ABT-199 (venetoclax). Venetoclax binds to the BH3-binding groove of BCL-2 and displaces Bim and other BH3-only proteins that are normally sequestered by this pro-survival (anti-apoptotic) protein. These BH3-only proteins are thus freed to activate the apoptosis effectors, Bax and Bak. Bax/Bak activation then leads to their oligomerization and mitochondrial outer membrane permeabilization. This commits the cell to apoptosis through the mitochondrial pathway, which is triggered by the activation of caspases. Modified, with permission, from ref. [51].

Table 1

Ongoing and planned combination clinical trials involving venetoclax.

Clinicaltrials.gov identifier	Disease state(s)	Partner drug(s)	Phase
NCT03000660	R/R AL Amyloidosis	Dexamethasone	I
NCT02756897	CLL; R/R or high risk, treatment-naive	Ibrutinib	II
NCT02951117	R/R MM	ABBV-838 (ADC against CS-1); dex	IB
NCT02899052	R/R MM	Carfilzomib; dex	II
NCT02966782	MDS (HMA failures)	Azacitidine	IB
NCT02993523	AML (elderly; treatment-naïve)	Azacitidine (vs. aza plus placebo)	III
NCT02942290	MDS (previously untreated)	Azacitidine (vs. aza alone)	II
NCT02877550	FL, treatment-naive	Obinutuzumab	I
NCT02758665	Previously untreated CLL with *TP53* deletion and/or mutation	Ibrutinib plus obinutuzumab	II
NCT02992522	R/R B-NHL	Lenalidomide plus obinutuzumab	I
NCT02670044	R/R AML	Cobimetinib (MEK inhibitor) or idasanutlin (HDM2 inhibitor)	IB/II
NCT02611323	R/R FL or DLBCL	Obinutuzumab plus polatuzumab vedotin (ADC against CD79b)	Ib/II
NCT02987400	R/R DLBCL	Obinutuzumab	II
NCT02910583	Treatment-naïve CLL/SLL	Ibrutinib	II
NCT02755597	MM (proteasome inhibitor candidates)	Bortezomib plus dex (vs. bortezomib plus dex plus placebo)	III
NCT02956382	R/R FL	Ibrutinib	I/II
NCT01671904	CLL; R/R or previously untreated	BR or bendamustine plus obinutuzumab	IB
NCT02950051	Fit patients with previously untreated CLL without del17p or *TP53* mutation	Rituximab or obinutuzumab or obinutuzumab plus ibrutinib (vs. FCR/BR without venetoclax)	III
NCT02427451	CLL; R/R or previously untreated	Ibrutinib plus obinutuzumab	I/II
NCT02287233	AML (elderly; treatment-naïve)	LDAC	I/II
NCT02419560	R/R MCL	Ibrutinib	I/IB

Abbreviations: R/R, relapsed/refractory; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; ADC, antibody-drug conjugate; MDS, myelodysplastic syndrome; HMA, hypomethylating agent; AML, acute myeloid leukemia; FL, follicular lymphoma; B-NHL, B-cell non Hodgkin's lymphoma; DLBCL, diffuse large B-cell lymphoma; MEK, mitogen activated protein kinase kinase; HDM2, human double minute 2; SLL, small lymphocytic lymphoma; LDAC, low dose cytarabine; MCL, mantle cell lymphoma; BR, bendamustine plus rituximab; FCR, fludarabine, cyclophosphamide and rituximab; dex, dexamethasone; aza, azacitidine.