BH3 Mimetics in AML Therapy: Death and Beyond?

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B cell lymphoma 2 (BCL2) homology domain 3 (BH3) mimetics are targeted therapeutic agents that allow response prediction and patient stratification. BH3 mimetics are prototypical activators of the mitochondrial death program in cancer. They emerged as important modulators of cellular mechanisms contributing to poor therapeutic responses, including cancer cell stemness, cancer-specific metabolic routes, paracrine signaling to the tumor microenvironment, and immune modulation. We present an overview of the antagonism between BH3 mimetics and antiapoptotic BCL2 proteins. We focus on acute myeloid leukemia (AML), a cancer with reduced therapeutic options that have recently been improved by BH3 mimetics.

BH3 Mimetics Act beyond Mitochondrial Cell Death Commitment?

BCL2 family proteins are crucial cell-survival modulators that are aberrantly expressed in several forms of cancer and contribute to disease progression and therapeutic resistance. The development of synthetic small-molecule inhibitors, specifically BH3 mimetics, provides a successful strategy to re activates cell death signaling in cancer cells. Their mechanism of action is conceptually simple. BH3 mimetics imitate short sequences derived from the BH3 domain of the canonical binding partners of prosurvival BCL2 proteins (the proapoptotic BH3-only proteins), thus preventing BH3-only/prosurvival BCL2 protein family interaction, and eventually inhibiting cell death modulation (Box 1).

Approval of the first selective BCL2 inhibitor, venetoclax (ABT-199, Venclexta®), by the FDA in 2016 provided an important impulse to the personalized therapeutic use of BH3 mimetics. From a clinical perspective, BCL2 protein dependency became a marker of therapeutic response, thus allowing patient stratification (see Glossary; Box 2). Mechanistically, its emergence fostered the discovery of additional natural and synthetic BH3 mimetics that are selective for other BCL2 family members. Despite the generally positive response to these drugs, primary or secondary forms of resistance have been described. Indeed, pre-existing clones could be dependent on alternative or multiple BCL2 members (BCL2 codependency), and therapy-induced selection of such clones cannot be excluded. Identifying and administrating appropriate BH3 mimetics alone or in combination is currently under preclinical and clinical investigation as a treatment strategy to overcome these forms of resistance.

BH3 mimetics became a suitable tool to explore the regulatory functions of BCL2 family proteins beyond their role in BCL2 resistance; in addition, they helped to extend the network of BCL2 protein binding partners. These research efforts allowed the discovery of modulatory mechanisms that are potentially relevant for innovating therapeutic approaches by targeting processes such as cancer cell stemness, metabolic functions, signaling to the microenvironment, and immune interactions. Elucidation of these mechanisms, whether interconnected or not with the canonical function of BCL2 family proteins in the modulation of mitochondrial outer-

Highlights

Acute myeloid leukemia (AML) is efficiently targeted by combinatorial regimens including B cell lymphoma 2 (BCL2) protein antagonists.

BCL2 homology domain 3 (BH3) mimetics selectively target leukemic stem cells.

Strategies that inhibit BCL2 proteins impair metabolic routes that are preferentially exploited in AML.

BCL2 protein antagonists prevent BCL2 family member-dependent paracrine signaling to the AML tumor microenvironment.

Inhibition of MCL1 expression/activity modulates the antitumor immune response.

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Box 1. The BCL2 Protein Family in Cancer

Cancer cells cope with permanent cellular stress and can withstand a higher stress threshold before dying. Their refractoriness to cell death commitment is a crucial hallmark. The mitochondria are the stage for this pro-survival strategy, and BCL2 family proteins are the major players. In this context, the antiapoptotic members of the BCL2 family, including BCL2L1 (also known as BCLXL), BCL2L2, MCL1, and BCL2-associated protein A1 (BCL2A1), antagonistize the activation of the proapoptotic members BCL2-associated X apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1) that are essential for triggering the mitochondrial apoptotic pathway (Figure I). BH3-only proteins act as liaisons between pro- and antiapoptotic family members. Arising in stress conditions, they are functionally different, where the activators (including BCL2-like 11/BCL2L11, also known as BIM), truncated BH3 interacting-domain death agonist (tBID), and BCL2-binding component 3 (BBC3, also known as PUMA) directly bind to BAK1 and BAX to promote their activation, and the sensitizers (including BCL2-associated agonist of cell death, BAD; BCL2-interacting killer, BIK; phorbol-12-myristate-13-acetate-induced protein 1, PMAIP1, also known as NOXA; BCL2-modifying factor, BMF; and harakiri or BCL2-interacting protein, HRK), interact with antiapoptotic BCL2 members to displace them and enable the activating BH3-only proteins and BAK1/BAX to trigger mitochondrial outer membrane permeabilization (MOMP). BH3 mimetics mimic the sensitizers (Figure I). They interact with antiapoptotic BCL2 proteins in a competitive manner, eventually reactivating the death signal. The ability of BH3 mimetics to induce MOMP in the absence of other stimuli is proof of concept that most cancer cells are primed to die and depend on antiapoptotic BCL2 proteins for survival. This vulnerability can be exploited for therapeutic purposes. There are preferential patterns of interaction between anti- and proapoptotic BCL2 family members. Cancer cells derived from different patients (intratumoral heterogeneity), or belonging to distinct subpopulations within the same patient (intratumoral heterogeneity), may rely on different BCL2 proteins for survival. Furthermore, the patterns of BCL2 protein dependency can vary, and therapeutic treatments can change these patterns through the clonal expansion of specific malignant subpopulations. Approaches to monitor the BCL2 dependency of cancer cells could be used to predict responders and to personalize therapies, and these strategies could also be applied after therapy to monitor the potential onset of unresponsiveness (Box 2).

Glossary

- Allogeneic hematopoietic stem cell transplantation (HSCT): transplantation of hematopoietic cell progenitors from compatible donors. HSCT may follow the consolidation phase as a curative approach.
- Complete remission (CR): the disappearance of any signs of disease in individuals under treatment (absence of circulating leukemic blasts, recovery of lymphocytes and platelets, <5% bone marrow (BM) blast detection). CR can be achieved after a small number of induction phase cycles, or within a range of months.
- Consolidation therapy: the second standard therapeutic phase of acute myeloid leukemia (AML) treatment that follows the induction phase. The consolidation (maintenance) phase targets residual cancer cells to prevent recurrence. The consolidation phase involves additional chemotherapeutic cycles incorporating the same agents used during the induction phase or other combinations.
- Glutaminolysis: reactions by which glutamine is converted into glutamate.
- Immunogenic cell death (ICD): a type of cell death accompanied by exposure to, or the release of, stress factors collectively termed damage-associated molecular patterns (DAMPs) that trigger immunostimulatory functions by promoting cancer cell clearance by the immune system.
- Induction therapy: the first standard therapeutic phase of AML treatment. The goal of the induction phase is the eradication of the bulk AML cell population from the blood and BM of patients with primary AML (i.e., remission). Conventionally, the induction phase consists of the combined administration of cytarabine (ARA-C) and anthracyclines (e.g., daunomycin) in a consolidation regimen (‘7 + 3’ that is based on the days of administration.
- Intratumoral heterogeneity: different genetic backgrounds of cancer cells derived from different patients that highlight individual vulnerabilities relevant for personalized therapy and patient stratification.

membrane permeabilization (MOMP), will provide new rationales for the therapeutic use of BH3 mimetics. In this context, AML, a disease exhibiting dismal survival rates, has become an important model for investigation.
Box 2. Diagnostic Tools To Monitor BCL2 Protein Dependency

BH3 profiling uses peptides derived from the BH3 domains of BH3-only proteins that recapitulate all possible interaction patterns between BH3-only proteins and antiapoptotic BCL2 members (Box 1). In the original protocol, mitochondria are isolated and probed using the entire panel of peptides. The release of cytochrome c, detected by enzyme-linked immunosorbent assay (ELISA), indicates MOMP and identifies effective peptides derived from sensitizing BH3-only proteins. The concomitant use of peptides derived from BH3-only protein activators confirms the presence of functional BAK1 and BAX in the isolated mitochondria [74,75]. More recently, this technique has been adapted to primary cells to serve as a therapeutic biomarker for BCL2 dependency. Patient cells undergo partial permeabilization to preserve mitochondrial functionality while allowing the intracellular diffusion of BH3 peptides. MOMP is directly monitored through the use of fluorescent probes (JC-1,5,6-tetrachloro-1,1′,3,3′-tetrathylbenzimidazolocarbocyanine iodide or tetramethylrhodamine, TMRE) [76]. BH3 profiling contributed to the elucidation of the mechanisms underlying BCL2 protein dependency. In contrast to lymphoid malignancies, AML cells do not rely exclusively on unique antiapoptotic BCL2 members to survive, and instead frequently develop codependencies [75,77] or turnover of BCL2 proteins [78]. BH3 profiling also allows monitoring of resistance that arises from the selection and expansion of pre-existing minor clones bearing different patterns of BCL2 dependency [79]. This outcome suggests that a combination of agents targeting diverse dependencies (not limited to BCL2 family proteins) is an essential strategy to improve CR rates and OS in AML patients. The concept of BCL2 dependency has inspired several approaches aimed at engineering BCL2 addiction to test the efficacy and selectivity of new BCL2 inhibitors. Cells are engineered to coexpress specific pairs of pro- and antiapoptotic BCL2 proteins and become ‘mito-prime’ [80–82]. The proapoptotic member is tagged with GFP, allowing the monitoring of its localization to mitochondria and any perturbation of this condition by immunofluorescence. In addition to assessing the selectivity and potency of new inhibitors, mito-prime profiles any preferential interactions between BH3-only proteins and their effectors BAX and BAK1. Importantly, this technique can be combined with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing to corroborate mechanistic aspects. CRISPR/Cas9 screening can also be used in combination with drug screening approaches. For example, combining this methodology with a BCL2 inhibitor screen on a broad panel of cell models helped to identify the epithelial–mesenchymal transition (EMT) as a novel mechanism influencing BCL2 dependency in solid tumors [83].

AML is a hematological type of cancer characterized by inhibition of myeloid differentiation, with consequent clonal expansion and accumulation of immature myeloid blasts within the bone marrow (BM) and blood [1]. This disease typically affects adults (80% of cases) and is particularly prevalent among elderly individuals. AML is one of the most aggressive forms of blood cancer, and patients exhibit poor prognosis and limited 5 year overall survival (OS; 27.8%). The ineffectiveness of a subset of patients to undergo intensive cycles of chemotherapy, a high rate of relapse even in patients with apparent complete remission (CR) after therapy, and limited access to allogeneic hematopoietic stem cell transplantation (HSCT) are the major reasons for these unfavorable outcomes.

AML was treated for many years using a standard ‘one-size-fits-all’ therapeutic regimen. Since 2017, new drugs, including targeted agents, have been approved for the treatment of AML. These new drugs have substantially replaced standard therapies during the induction therapy and/or consolidation therapy phases, ultimately allowing patient stratification before the implementation of novel personalized therapeutic approaches [1]. Venetoclax is one such newly approved therapeutic agent. The public availability of data regarding patients who were enrolled into clinical trials has progressively shed light on the mechanisms responsible for the high AML relapse rate and allowed researchers and clinicians to highlight new targetable mechanisms, including BCL2 protein inhibition.

**Natural and Synthetic BCL2 Protein Antagonists**

**BCL2 Antagonists**

Venetoclax (ABT-199; GDC-0199) was approved in 2018 in combination with the hypomethylating agent azacitidine or low-dose cytarabine (LDAC) for the treatment of newly diagnosed AML patients who are unfit for intensive chemotherapy (Table 1 and Box 3). It is also under evaluation for the treatment of myelodysplastic syndrome [2]. Initially developed to
Table 1. Chemical Structures of BCL2 Antagonists (Direct Inhibitors and Downregulators)\textsuperscript{a}

| Compound name                | Structure | Targets | Comment                                                                                           | Clinical status                                                                                                     | Refs          |
|------------------------------|-----------|---------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|---------------|
| BH3 mimetics targeting BCL2  |           |         |                                                                                                   |                                                                                                                    |               |
| Venetoclax (ABT-199, GDC-0199) | ![Structure](image1) | BCL2    | Potent and selective BCL2 inhibitor; BAD-like BH3 mimetic                                          | Approved since 2017 in combination with the hypomethylating agent azacitidine or LDAC for the treatment of patients with AML who are unfit for chemotherapy | [2,4,5,10,67] |
|                              |           | K\textsubscript{i} 0.01 nM | It is associated with a fast response in elderly AML patients that are ineligible for other therapies. Good tolerability | Under evaluation for the treatment of MDS                                                                       |               |
|                              |           |         | Suitable for AML with IDH1/2 mutations or developing BCL2 dependency in response to other therapeutic regimens | In clinical trials involving combinational regimens                                                              |               |
|                              |           |         | Limitations: fast but limited durability of response owing to outgrowth of resistant AML clones; tumor lysis syndrome now controlled with dose escalation administration and suitable prophylaxis |                                                                                                                    |               |
| Navitoclax (ABT-263)         | ![Structure](image2) | BCL2, BCL2L1, and BCL2L2 K\textsubscript{i} \textless{} 0.5–3.3 nM | Oral selective inhibitor, BAD-like BH3 mimetic, orally available derivative of ABT-737 | In clinical trials involving solid tumors or hematological malignancies other than AML (e.g., ALL) (NCT03592576) (NCT03181126) | [96,97]       |
|                              |           |         | Limitations: on-target effects on platelets of thrombocytopenia, currently manageable with suitable prophylaxis; more recently found to impact on healthy blood progenitors depending on BCL2L1 |                                                                                                                    |               |
| ABT-737                      | ![Structure](image3) | BCL2, BCL2L1, and BCL2L2 K\textsubscript{i} \textless{} 0.1 nM | Prototypical BH3 mimetic                                                                                     | In clinical trials involving solid tumors or hematological malignancies other than AML (e.g., ALL) (NCT03592576) (NCT03181126) | [97,98]       |
|                              |           |         | Induces the disruption of BCL2/BAK1 complexes by mimicking BAD                                       |                                                                                                                    |               |
|                              |           |         | Binds with much lower affinity to BCL2A1 and MCL1 (K\textsubscript{i} 0.46 \textmu{}M)                |                                                                                                                    |               |
|                              |           |         | Not orally available; intraperitoneal administration in mouse models                                |                                                                                                                    |               |
| BH3 mimetics targeting MCL1 |           |         |                                                                                                   |                                                                                                                    |               |
| A-1210477                    | ![Structure](image4) | MCL1    | First direct MCL1 binder and inhibitor with relevant selective potency                               | Research tool                                                                                                      | [99]          |
|                              |           | K\textsubscript{i} 0.45 nM |                                                                                                       | Precluded from further clinical consideration                                                                |               |
| Compound name | Structure | Targets | Comment | Clinical status | Refs |
|---------------|-----------|---------|---------|-----------------|------|
| Derived from indole-2-carboxylic acids, disrupts both MCL1–BCL2L11 and MCL1–PMAIP1 complexes. Synergy with venetoclax and navitoclax | | | Limitations: bioavailability and potency compared to more recent MCL1 inhibitors; lacks in vivo studies | | |
| AZD5991 | ![AZD5991 structure](image1.png) | MCL1 | $K_i$ 0.13 nM | Macrocyclic molecule; particularly active against hematological malignancies including MM and AML. Cytotoxic activity against sensitive cell lines, synergy in combination with venetoclax in AML (and bortezomib in MM); tumor regression in vivo (in murine and rat models), with evidence of good tolerability even at high doses | In clinical trial (NCT03218883) | [100] |
| AMG-176 | ![AMG-176 structure](image2.png) | MCL1 | $K_i$ 0.13 nM | Spiromacrocyclic compound Activity and no systemic toxicity validated in a knock-in mouse model where murine Mcl1 was replaced with its human ortholog to bypass the known resistance of murine MCL1 Decreased numbers of MCL1-dependent blood cells (B cells, neutrophils, and monocytes) in both the BM and peripheral sites in mouse models could be used as markers to monitor future clinical applications Synergy with standard treatments (cytarabine, decitabine, and doxorubicin) and with venetoclax in AML; 100% tumor regression in combination with venetoclax in mouse xenografts and on a panel of human AML specimens Orally available | In clinical trial (NCT03797261) | [101] |

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| Compound name | Structure | Targets | Comment | Clinical status | Refs |
|---------------|-----------|---------|---------|-----------------|------|
| AM-8621       | Undisclosed | MCL1    | $K_i$ 0.06 nM | Sporomacrocyclic compound; analog of AMG-176; same features as AMG-176 | Experimental [101] |
| S63845        |           | MCL1    | $K_i$ <1.2 nM | Synthetic MCL1 inhibitor with a 20-fold higher binding affinity for MCL1 compared to that of A-1210477 | Experimental [73] |
|               |           |         |         | Anticancer activity against a panel of cancer cell lines, including both hematological and solid tumors and in in vivo mouse models, with good tolerability even at high doses | |
|               |           |         |         | High potency against patient samples of lymphoid and myeloid origin, including an AML cohort, without affecting healthy CD34+ myeloid cells | |
|               |           |         |         | Synergy with standard therapies and venetoclax | |
| S64315 (MIK665) |           | MCL1    | $K_i$ 0.048 nM | Analog of S63845 | In clinical trials in AML (NCT03672696) MDS (NCT022973966) Also in other blood malignancies including multiple myeloma (MM) and lymphoma (NCT02992483) [102] |
| VU661013      |           | MCL1    | $K_i$ 97 pM | Derived from indole-2-carboxylic acids | In vitro and in vivo anticancer effects; synergy in combination with venetoclax in AML models | Experimental [79] |
|               |           |         |         | Efficacy of VU661013/venetoclax combination in venetoclax/LDAC-resistant AML specimens, confirming that MCL1 is a crucial factor in therapeutic failure | |
|               |           |         |         | Limitations: switch from MCL1 to BCL2 dependency as a mechanism of resistance | |
| Compound name                        | Structure | Targets | Comment | Clinical status | Refs |
|-------------------------------------|-----------|---------|---------|-----------------|------|
| BH3 mimetics targeting BCL2L1       |           |         |         |                 |      |
| WEHI-539                            | BCL2L1    | $K_d$ 0.6 nM | Isolated through high-throughput screening and structure-guided development. Binds to BCL2L1 in a different manner than ABT-737, exhibiting both faster association to and dissociation from BCL2L1. Preferentially induces BAK1-mediated apoptosis and kills cells whenever MCL1 is absent or blocked, thus indicating MCL1 as a resistance factor. | Experimental | [103] |
|                                     | A-1155463 | BCL2L1 $K_i$ <0.01 nM | More potent than WEHI-539, with validated anticancer effects in lung cancer models in vitro and in vivo; however, it is associated with on-target thrombocytopenia. | Experimental | [104] |
|                                     | A-1331852 | BCL2L1 $K_i$ <0.01 nM | Orally available derivative of A-1155463. Exhibits the highest performance in terms of in vivo tumor regression, either as a single agent or in combination with other investigational drugs. | Experimental | [97] |
| Selected groups of inhibitors of MCL1 protein expression | | | | |
| Roscovitine (seliciclib)             | CDKs      | IC$_{50}$ 0.2–0.8 nM | Second-generation orally available CDK inhibitor impacting on short-lived protein expression (including MCL1 and TP53). | Experimental in AML | [43] |
| Dinaciclib (MK7965)                 | CDKs      | IC$_{50}$ 1–4 nM | Small-molecule CDK inhibitor. Currently investigated in combination with venetoclax, with the rationale of impairing MCL1 expression at the transcriptional level in R/R AML settings. | In clinical trial (NCT03484520) | [43,44] |
| Alvocidib                           | CDKs      | A flavonoid alkaloid CDK9 | | In clinical trials | [44] |

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Table 1. (continued)

| Compound name | Structure | Targets | Comment | Clinical status | Refs |
|---------------|-----------|---------|---------|-----------------|------|
| (flavopiridol) | ![Structure](image) | $K_d$ 3.0–7.1 nM | inhibitor | Currently investigated in combination with venetoclax in R/R AML patients or with standard therapies in R/R AML patients following treatment with venetoclax combination therapy | (NCT03441555) (NCT03969420) |
| PHA-767491 | ![Structure](image) | CDKs $IC_{50}$ 10–34 nM | Currently investigated in combination with standard chemotherapy and with other BH3 mimetics, with the rationale of impairing MCL1 expression at the transcriptional level in AML models | Experimental | [105] |
| Omacetaxine mepesuccinate (homoharringtonine; Synribo) | ![Structure](image) | Large ribosome subunit | Cephalotaxine-derived ester derived from extract of leaves of the plant Cephalotaxus harringtonia | Reversible and transient inhibitor of the elongation phase | Approved for CML treatment | [106,107] |
| | | Protein synthesis | | First protein synthesis inhibitor approved by the FDA for the treatment of TK inhibitor-unfit patients with CML | In clinical trials in AML in FLT3-ITD AML patients (NCT03170895) (NCT03135054) | |
| 1(R), 6(S), 1′(R), 6′(S), 11(R), 17(S)-fistularin-3 | ![Structure](image) | DNMT1 $IC_{50}$ 10 to >50 nM | Brominated tyrosine derivative from the marine sponge Suberea clavata | Induces ER stress and downregulation of MCL1 expression | Experimental | [108] |
Table 1. (continued)

| Compound name | Structure | Targets | Comment | Clinical status | Refs |
|---------------|-----------|---------|---------|-----------------|------|
| Silvestrol    | ![Silvestrol](image) | eIF4A   | Protein synthesis | Rocaglate derivative; Translation inhibitor isolated from the fruits and twigs of the tropical plant *Aglaia foveolata*; Synergy with ABT-737; Limitations: complex synthesis; analogs required | Experimental | [109] |
| Nutlin        | ![Nutlin](image) | MDM2 (TP53) | Cis-imidazoline analog stabilizing TP53 via MDM2/TP53 interaction/inhibition | | Experimental | [8] |
| Idasanutlin   | ![Idasanutlin](image) | TP53    | Binds to and reactivates p53, in turn leading to MCL1 phosphorylation at T163 and subsequent proteasomal degradation by GSK3β upon MAPK pathway activation in AML cell models | In clinical trials in combination with venetoclax (NTC02670044) | | [8] |
| UNBS1450      | ![UNBS1450](image) | NKA     | Hemisynthetic cardenolide (CG) derived from 2’-oxovoruscharin extracted from *Calotropis procera* | MCL1 protein downregulation within 4–6 h in vitro, whereas mRNA levels remain unchanged in multiple cancer cell models of different origins; Synergy with venetoclax against AML cell models and a panel of primary AML samples in vitro and in vivo | Experimental | [15,26] |
| Digitoxin     | ![Digitoxin](image) | NKA     | A cardenolide (CG) extracted from the plants *Digitalis lanata* and *purpurea* | Clinically used against heart failure and arrhythmias; Exerts antiproliferative and cytotoxic activities in different cancer cell models; Downregulates MCL1 protein | Approved to treat heart alterations; Experimental in cancer | [26] |

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overcome the on-target side effects of thrombocytopenia caused by the broader BCL2/BCL2-like 1 (BCL2L1)/BCL2-like 2 (BCL2L2) inhibitor navitoclax (ABT-263; Table 1) [3], its safety and pharmacokinetics have been improved through the use of suitable dosing protocols and prophylaxis to avoid its most dangerous side effects [4–6]. Recent clinical developments in regard to venetoclax allow this drug to target BCL2-dependent AML subgroups, including those possessing isocitrate dehydrogenase 1/2 (IDH1/2) mutations [7], or patients with a dependency in response to other therapeutic regimens (synthetic lethality [8]). Although this drug achieves excellent CR levels and fast responses, a limitation of venetoclax is the durability of its clinical response [9,10]. The outgrowth of AML clones expressing other BCL2 family members that replace BCL2 as prosurvival factors is a major reason for this limitation. In particular, MCL1 (apoptosis regulator, BCL2 family member) is the Achilles heel of venetoclax-based therapy, and this has driven research efforts to develop selective MCL1-targeting approaches.

**MCL1 Antagonistic Strategies**

**Selective MCL1 Inhibitors and MCL1-Downregulating Compounds**

MCL1 is an important factor in therapeutic resistance and plays a causative role in the relapse of several hematological malignancies, including AML. Therefore, considerable research effort has been made in regard to the development of selective MCL1 inhibitors (Table 1). However, the structural complexities of this protein and the multistep regulation of its expression make this goal challenging [11], and the first truly selective direct MCL1 inhibitors were only recently developed. Despite some differences, all MCL1 inhibitors share several similarities. They rapidly induce typical mitochondrial apoptotic pathway activation in MCL1-dependent cell models within 1–2 h of administration. Their binding stabilizes MCL1, thus causing a reversible increase in its intracellular levels. They also exhibit higher binding affinity for human versus murine MCL1.
owing to species-dependent conformational differences [12], and this must be accounted for when performing in vivo tolerability studies in mouse models. Hematological malignancies are common sensitive targets. In many instances, BCL2L1 expression is a robust predictor of resistance. All MCL1 inhibitors exhibit important synergistic effects when combined with standard therapies or with the BCL2-selective inhibitor venetoclax. Preclinical studies in vitro and in vivo have demonstrated favorable profiles for some of these inhibitors in terms of potency and safety, thus making them interesting candidates for clinical evaluation (Table 1).

Modulating MCL1 expression is an alternative strategy to direct inhibition. MCL1 is a peculiar antiapoptotic BCL2 family member. It undergoes a complex regulatory program, and its expression is controlled at all steps of gene expression, from transcription to post-translational modification and protein half-life. The recent use of venetoclax has further implicated MCL1 as an AML resistance factor, and selected combinations, including compounds inhibiting MCL1 expression, may provide promising treatment regimens (Table 1).

**BCL2L1 Antagonists**

BCL2L1 contributes to chemoresistance in a manner similar to that of other antiapoptotic BCL2 family members, and it has been investigated extensively for its roles in the resistance of solid cancers to treatment. BCL2L1 also impacts on the prognosis of hematological malignancies [13,14] and is overexpressed in subgroups of AML [15]. Three selective BCL2L1 antagonists have emerged for investigational and clinical purposes (Table 1). The conserved Arg139 residue present in the BCL2L1 groove is essential for its interaction with selective BH3 mimetics. Common side effects of these agents include thrombotoxicity that occurs due to platelet BCL2L1 dependency and healthy progenitor cell toxicity [16].

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**Box 3. What We Are Learning from the First Clinically Approved BH3 Mimetic Therapies**

Venetoclax offers a therapeutic opportunity for AML patients who are unfit for intensive chemotherapy. This heterogeneous group includes individuals exhibiting patient-related factors of vulnerability that predict poor chemotherapy tolerability (concomitant pathologies, advanced age, and reduced physical performance according to scoring systems) and/or adverse prognostic disease-related factors (complex karyotypes, high-risk mutations, and refractoriness/resistance to previous therapies).

The first clinical trials on newly diagnosed AML patients showed a high overall response rate and tolerability, even in elderly patients. In addition, dose escalation and prophylaxis can mitigate side effects (tumor lysis syndrome and neutropenia) [5]. Response to venetoclax-based combinations is mutation-agnostic, where 72% and 36% patients, respectively, carrying high-risk FLT3 and TP53 alterations reached CR/CRI over historical outcomes [84]. Venetoclax increased the efficacy of HSCT in elderly patients [85]. Venetoclax-based therapy discontinuation does not worsen the risk of relapse [86]. More recently, a venetoclax-based combination study has been launched for pediatric relapsed/refractory AML [87]. Overall, the trial settings show a favorable scenario.

Despite the overall high CR/CRI, the OS of treated patients remains relatively comparable to standard therapies, suggesting a limited effect on relapse prevention [5,88]. The VALE-A Phase III clinical trial (NCT02993523) announced improved OS in venetoclax/azacytidine regimens in untreated ineligible patients [89]. A recent comparison revealed lower rates of CR/CRI (63.3% vs 84.9%), OS (381 vs 880 days), and median response (321 days vs not reached within the follow-up) for off-trial versus in-trial AML patients [90]. Furthermore, recent studies report that ~30% of the cohort corresponds to azacytidine/venetoclax relapses, and these patients exhibit clonal enrichment of monocytic subpopulations [86]. Correlations between molecular features and response in two combined clinical trials (NCT02203773 and NCT02287233) identified expansion of clones bearing FLT3 and TP53 defects as factors for adaptive resistance under therapy pressure [87]. The clonal selection occurs early and displays polyclonal features. These findings highlight the need to establish new markers of risk stratification and monitor clonal evolution to associate the most efficient targeted therapies [91,92]. A dynamic scheme of combinational strategies appears to provide a feasible approach. Interestingly, both AML monocyte subclones and FLT3-positive cell models overexpress BCL2 protein members (mostly MCL1) [66,67] and, furthermore, TP53 reactivation synergizes with venetoclax by promoting MCL1 degradation [8]. Taken together, combinational strategies that concomitantly target BCL2 family proteins may provide a means to target convergent cancer vulnerabilities that occur downstream from several malignant alterations with negative prognosis.

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Mechanistically, the anticancer effects of BCL2 protein antagonists rely on restoring mitochondrial priming through MOMP and cytochrome c release (Box 1). The generally accepted model of mitochondrial apoptotic commitment involves oligomerization of BAX and BAK1 on the outer mitochondrial membrane (OMM). This triggers early defects in the electron transport chain (ETC), and leads to reactive oxygen species (ROS) generation and subsequent cleavage of the long form of OPA1 (OPA1 mitochondrial dynamin-like GTPase). OPA1 maintains the tightness of the cristae at the inner mitochondrial membrane (IMM); its processing contributes to cristae widening that allows massive redistribution of cytochrome c into the intermembrane space, subsequently facilitating its release. This model implies that early perturbation of the mitochondrial respiration is downstream from BAX/BAK1 activation/oligomerization. More recently, several studies have highlighted the importance of the modulation of metabolic pathways, particularly oxidative metabolism, in the context of mitochondrial dysfunction and cancer progression. Figure 1. Regulation of Mitochondrial Metabolism in Acute Myeloid Leukemia (AML). Overview of metabolic reprogramming in AML. Black arrows indicate metabolic intermediates redirected towards biosynthetic pathways to support anabolic demands (cataplerosis). Blue arrows indicate alternative sources exploited to sustain the tricarboxylic acid (TCA) cycle (anaplerosis). Green and red dashed arrows indicate stimulation and inhibition, respectively. Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; αHG, hydroxyglutarate; αKG, α-ketoglutarate; CoQ, coenzyme Q; CS, citrate synthase; Cyt c, cytochrome c; CPT-1, carnitine palmitoyl transferase-1; FADH₂, flavin adenine dinucleotide (reduced form); FAS, fatty acid synthesis; FFA, free fatty acid; GAC, glutamine, arginine carnitine; GDH, glutamate dehydrogenase; GLS1, glutaminase 1; IDH1/2, isocitrate dehydrogenase 1/2; LCAD, long-chain acyl-CoA dehydrogenase; LDH, lactate dehydrogenase; ME, malic enzyme; mutIDH1, mutant IDH1; NADH, nicotinamide adenine dinucleotide (reduced form); PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; SCO2, cytochrome c oxidase assembly factor; UCP2, uncoupling protein 2. The figure was created using ScienceSlides (https://visiscience.com/).

Targeting Cancer Stemness by Antagonizing Antia apoptotic BCL2 Proteins

Mechanically, the anticancer effects of BCL2 protein antagonists relying on restoring mitochondrial priming through MOMP and cytochrome c release (Box 1). The generally accepted model of mitochondrial apoptotic commitment involves oligomerization of BCL2-associated X apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1) on the outer mitochondrial membrane (OMM). This triggers early defects in the electron transport chain (ETC), and leads to reactive oxygen species (ROS) generation and subsequent cleavage of the long form of OPA1 (OPA1 mitochondrial dynamin-like GTPase). OPA1 maintains the tightness of the cristae at the inner mitochondrial membrane (IMM); its processing contributes to cristae widening that allows massive redistribution of cytochrome c into the intermembrane space, subsequently facilitating its release. This model implies that early perturbation of the mitochondrial respiration is downstream from BAX/BAK1 activation/oligomerization. More recently, several studies have highlighted the importance of the modulation of metabolic pathways, particularly oxidative
phosphorylation (OXPHOS), that is associated with the use of BH3 mimetics and BCL2 protein targeting, for the selective eradication of AML stem cells and chemoresistant cells (Figure 1).

A multicenter, nonrandomized Phase Ib clinical trial on elderly patients with AML reported that venetoclax combined with hypomethylating agents (decitabine or azacytidine) induced fast and durable CR and increased OS with good tolerability [5]. A durable response required the selective eradication of leukemic stem cells (LSCs) [6]. BCL2 inhibition preferentially eradicates LSCs by affecting their dependency on OXPHOS [17]. LSCs (identified by a ROS-low phenotype) exhibited increased BCL2 expression at the mRNA and protein levels. Chemical inhibition of BCL2 (ABT-263 and obatoclax) induced rapid OXPHOS impairment (within minutes) that was followed by ATP depletion, mitochondrial oxidation, and glutathione (GSH) depletion (4–6 h), and eventually
decreased LSC viability (after 18 h) [17]. In agreement with these observations, AML cells undergoing BCL2 depletion (by BCL2 short hairpin RNA) exhibited reduced OXPHOS. This study did not monitor MOMP or the use of BAX/BAK1 knockout cells. Accordingly, early OMM permeabilization could be the cause of the observed early metabolic alterations.

The venetoclax/azacytidine combination impacts on the two major pathways responsible for replenishing OXPHOS, specifically amino acid (AA) metabolism and the tricarboxylic acid (TCA) cycle (Figure 1). OXPHOS reduction was only achieved by the combination of both agents, such that this mechanism resembled synthetic lethality [6,18]. Primary AML samples treated with this combination reduced AA uptake after inhibition of AA transporter gene expression in vivo and in vitro [18], leading to forced AA starvation. Again, neither venetoclax nor azacytidine alone induced any modulation of AA transporter gene expression. Treating AML cells in the presence of higher extracellular AA levels prevented the venetoclax/azacytidine combination from impacting on OXPHOS and LSC viability, thus excluding mechanisms other than AA uptake. AA availability, in turn, controlled the efficiency of the TCA cycle. The hypothetical model assumes a cascade of events in which forced AA starvation impairs the TCA cycle, ultimately reducing the efficiency of the OXPHOS electron transport chain (ETC) [18]. Of note, although the metabolic changes were detectable 1 day post-therapy, a substantial reduction of the leukemic cell burden was only apparent after 2–6 days, suggesting that the observed metabolic alterations took place much earlier, preceding the onset of overt leukemic cell death in patients [18].

OXPHOS impairment is associated with mitochondrial ROS generation and GSH depletion. Preventing GSH depletion through the use of the antioxidant thiol donors N-acetyl cysteine and GSH monooethyl ester partially reversed the reduction of both OXPHOS and viability, suggesting a role for GSH in a potential later amplification loop [6]. This study indicates that the suppression of ETC II activity is a crucial event for cell death sensitization. A more recent study indicates that a specific inhibitory activity of venetoclax on ETC I is a crucial step in the OXPHOS impairment observed in AML [19]. In this instance, the enzymatic activity of ETC I immunocaptured from bovine heart mitochondria further confirmed that this complex is centrally affected. In addition, the expression of yeast NADH dehydrogenase (NDI1) to circumvent the mammalian ETC defects restored mitochondrial respiration, that also occurred in venetoclax-resistant cell models. It is conceivable that the observed ETC defects may be a consequence of MOMP. Their early occurrence, however, has not been assessed or functionally investigated in these studies. An investigation of lymphoid malignancies revealed that venetoclax-mediated impairment of OXPHOS occurs independently of caspase activation (i.e., in the presence of zVAD); however, this process depends on OMM permeabilization because venetoclax-treated Bak1/Bax double-knockout cells did not exhibit OXPHOS impairment [20]. Interestingly, a small molecule (compound A) capable of preserving ETC II functions was found to protect ETC and prevent cytochrome c release and apoptosis, although mitochondrial BAX translocation and oligomerization were maintained [21]. These findings corroborate the model that an early OMM perturbation and consequent MOMP are events that modulate OXPHOS; however, we could speculate that MOMP per se does not represent a point of final commitment to death, and that OXPHOS inhibition would potentially not be a passive consequence.

Recent findings support a role for MCL1 in maintaining stemness while delaying differentiation [22]. Its high expression in human embryonic pluripotent stem cells correlates with high expression of stemness biomarkers such as the transcriptional factors Nanog homeobox (NANOG) and POU class 5 homeobox 1 (POU5F1), and with the presence of a highly fragmented mitochondrial network. By contrast, genetic depletion or chemical inhibition of MCL1 leads to cell differentiation that is marked by decreased expression of NANOG and POU5F1, and by the
accumulation of elongated mitochondria. Among the antiapoptotic BCL2 family members, MCL1 is the only member that is able to localize to both the mitochondrial matrix and the OMM [23]. Both MCL1 pools play crucial roles in the maintenance of pluripotency. OMM-associated MCL1 interacts with dynamin 1-like (DNM1L) to promote mitochondrial fragmentation. Matrix-associated MCL1 prevents mitochondrial fusion by interacting with the IMM-localized protein OPA1. Importantly, both interactions are sensitive to MCL1 inhibitors. The selective small-molecule inhibitors MIM-1 and S63845 decrease NANOG and POU5F1 expression. Furthermore, S63845 disrupts the interactions between MCL1 and both DNM1L and OPA1, thus reducing their stability [22]. This prompts us to speculate about the accessibility of MCL1 inhibitors to both MCL1 pools. Moreover, the BH3 domain-binding groove of MCL1 could be involved.

BCL2L1 also supports stemness. Studies in adherent cell models overexpressing BCL2L1 revealed a direct interaction with F1F0-ATP synthase [24]. In neuronal models, destabilization of this interaction leads to F1F0-ATP synthase inhibition, with consequent oxidative damage of complex I and IMM depolarization that leads to cell death [24]. In lung cancer cell models, the BCL2/BCL2L1/BCL2L2 inhibitor ABT-737, but not the BCL2-specific drug venetoclax (ABT-199), selectively induced the death of LSCs exhibiting a quiescent/slow-proliferating phenotype [25]. Subsets of AML primary samples overexpress BCL2L1 [26]. Furthermore, chemical inhibition of BCL2L1 by the BH3 mimic A-1331852 suppresses the clonogenic potential of hematopoietic progenitors in chronic myeloid leukemia (CML) [14]. Taken together, these findings imply a similar role for BCL2L1 in BCL2L1-dependent AML subsets. The recent development of selective BCL2L1 inhibitors (Table 1) will allow researchers to further elucidate their therapeutic potential and side effects (inhibition of healthy progenitors and platelets).

Disruption of Energy Metabolism and BCL2 Protein-Targeting Strategies

There is evidence that (i) OXPHOS is a determinant of chemoresistance in AML, (ii) BCL2 protein inhibition/downregulation eradicates AML LSCs, and (iii) chemoresistant AML cells (including LSCs) may elude OXPHOS inhibition by exploiting energy alternative routes. To sensitize AML cells to death and to improve the odds of CR, combination strategies that prevent the anaplerotic refueling of the TCA cycle/OXPHOS and inhibit the antiapoptotic functions of BCL2 proteins provide a promising therapeutic option.

Glutaminolysis is an important anaplerotic source for the TCA cycle. Glutaminases that are encoded by the glutaminase and glutaminase 2 (GLS and GLS2) genes catalyze the conversion of glutamine to glutamate (Figure 1). Both GLS depletion and pharmacological inhibition using CB-389 induce proliferative arrest and apoptosis in AML cells; in addition, these treatments impair AML engraftment in mice. GLS inhibition using CB-389 further sensitizes AML cells to venetoclax [27].

Fatty acid (FA) metabolism is another important anaplerotic route for TCA cycle/OXPHOS function, and this pathway also contributes significantly to mitochondrial reprogramming (Figure 1). Increased FAs are associated with a proinflammatory phenotype and BCL2 protein upregulation, and these contribute to the resistance of AML to accumulating ROS levels and to inducers of cell death, including therapeutic agents. Combination treatment using etomoxir (an inhibitor of the leukemia cell scavenger receptor CD36 that is required for internalization of BM adipocyte-supplied fatty acids) with the pan-BCL2 inhibitor ABT-737 exerts synergistic effects on AML cell death [28], suggesting a similar effect in combination with the selective and FDA-approved venetoclax.
There is evidence that MCL1 supports OXPHOS and FA metabolism, a function that is associated with its unique distribution in the mitochondria [23,29] and its ability to modulate mitochondrial dynamics [22,30,31]. IMM-associated MCL1 is a truncated isoform of the protein (36 kDa). Its expression sustains OXPHOS levels; however, it plays no role in promoting MOMP, which is instead controlled by the 40/38 kDa OMM-associated isoforms. Accordingly, both Mcf1 deletion and the expression of MCL1 constructs with defective matrix localization signals impair OXPHOS, ATP production, and oxygen consumption rate (OCR), ultimately triggering the accumulation of mitochondrial superoxide species. In murine cell models, this regulatory pattern cannot be compensated for by the expression of MCL1 constructs confined to the OMM or by BCL2 expression. Mcf1 deletion and the expression of matrix localization-defective MCL1 are associated with an inability to properly assemble large mitochondrial complexes and with defective mitochondrial fusion, processes that are essential to maintain mitochondrial DNA (mtDNA) [23].

Rasmussen et al. provide evidence that the selective MCL1 inhibitor S63845 interacts with both the IMM and OMM MCL1 pools, and subsequently modulates mitochondrial dynamics in human pluripotent stem cells [22], but not in cardiomyocytes where the action of S63845 remains confined to the OMM MCL1 pool [30]. These findings imply that compounds inhibiting MCL1 expression possess the potential to modulate mitochondrial metabolism and structure, thus contributing to their cell death-inducing effects. However, further studies will be necessary to elucidate the actual accessibility to the IMM and role of MCL1 inhibitors on the IMM. A hypothetical model could involve different MCL1 domains in modulating effects at the level of the IMM; alternatively, the destabilization of MCL1–DRP1 complexes by MCL1 inhibitors on the OMM could generate a perturbation of mitochondrial dynamics that would also destabilize the function of the IMM MCL1 pool. In addition, we could speculate about differential accessibility to the IMM or a differential role of MCL1 inhibitors on the IMM depending on the cellular context or level of differentiation.

Given the importance of a functional ETC and OXPHOS in AML subsets that exploit mitochondrial uncoupling for anabolic processes or that rely on an OXPHOS-high phenotype as a major source of ATP generation, respectively, approaches leading to ETC perturbation may synergize with BCL2 antagonists. Results presented by Konopleva and colleagues at the 39th American Hematology Society (ASH) annual meeting [32] indicated that sequential treatment of AML cell models with venetoclax and the ETC complex I inhibitor IACS-010759 dramatically impacts on cell survival [33]. Although single treatments already modulate mitochondrial respiration per se, their combination exerts a synergistic effect. Immunoprecipitation studies suggest that a direct interaction between BCL2 and the voltage-dependent anion channel (VDAC) prevents the loss of coupled mitochondrial respiration during apoptosis, and that this functions as a contributing regulatory event.

Alternatively, cancer cells could be forced to rely exclusively on OXPHOS to survive by impairing metabolic flexibility. Murine models of aggressive forms of AML develop general insulin resistance at the expense of adipose tissue (AT) and muscle, two major glucose-consuming tissues [34]. This modulation leads to increased glucose availability in other tissues, and this glucose is promptly used by rapidly dividing AML cells. Interference with this process may lead to AML starvation. A combination involving BCL2 protein targeting may trigger synthetic lethality, ultimately potentiating AML cell eradication. Interestingly, synergistic effects were observed when OXPHOS was impaired with the ETC complex I inhibitor metformin in colorectal and melanoma tumor-bearing mice subjected to feeding/fasting cycles to reduce glucose availability. Mechanistically, the modulation was caused by MCL1 protein downregulation mediated by the activation of the glycogen synthase kinase 3α (GSK3α) pathway [35]. Although this is an emerging field that remains largely unexplored in AML, concomitant interference with systemic glucose metabolism may present new therapeutic avenues [34].
BCL2 Protein Inhibition versus the Microenvironment

In coculture models of AML cells and BM-derived adipocytes or mesenchymal stromal cells (MSCs) mimicking the BM niche, AML cells exacerbate fatty acid oxidation (FAO) [28]. FAO improves AML cell resistance against death inducers by upregulating antiapoptotic factors, including BCL2 [28]. BCL2 inhibition using ABT-737 combined with pharmacological inhibition of FA-related pathways (e.g., with the CD36 inhibitors etomoxir or ranolazine) synergistically impairs AML cell survival in AML/MSC coculture models, supporting the anticancer therapeutic potential of this combination [28].

Extracellular vesicles (EVs) may act as carriers of cancer progression factors. In this context, antiapoptotic factors may be directly transferred to/from cancer cells. Moreover, their expression can also be induced through the transfer of regulatory clusters that include nuclear and cytoplasmic regulatory proteins as well as mRNA encoding such factors (Figure 2). EVs propagate the potential of BCL2 protein upregulation from selected AML cells to other AML cells or to lymphocytes in the BM of AML patients [36]. Isolated exosomes derived from stromal cell components appear to be enriched in MCL1 [37]. Furthermore, BCL2L1 has been described to be associated with the outer leaflet of the exosomal membrane [37]. Exosomal BCL2L1 is a cleaved form of the protein that is processed by caspase-3. The use of BH3 mimetics targeting BCL2L1 (e.g., ABT-737) or the expression of uncleavable BCL2L1 mutants reduces the uptake of stromal cell-derived exosomes by hematopoietic malignant cells, thereby impacting on their growth. Incubation of EVs with a caspase-3 inhibitor results in the same effect [37]. These first published results allow us to speculate about a role for EVs/exosomes in cancer cells (including AML) where they would act as carriers of BCL2 protein-mediated resistance mechanisms. Further investigations will be necessary to validate the association of BCL2 proteins with EV membranes/lumen to ascertain whether (specific) BCL2 proteins (or related regulatory clusters) might actively contribute to the efficient delivery of EV/exosome cargo to cancer cells.

However, specific microenvironmental agonists could also exacerbate BCL2 expression. In a clinical trial involving patients with CLL (chronic lymphocytic leukemia) and MCL (mantle cell lymphoma), unexpected resistance developed against the combination of venetoclax and the tyrosine kinase ibrutinib owing to robust nuclear factor κB1 (NF-κB1) pathway activation in cancer cells [38]. Importantly, the BCL2 promoter contains NF-κB1-responsive elements. Resistance arises from an accumulation of BCL2 protein in amounts that exceed the inhibitory capacity of venetoclax. This likely also occurs in AML, but because of dose-limiting side effects it cannot be solved by dose escalation. The same study found that the prosurvival factors MCL1, BCL2L1, and survivin were also upregulated, suggesting shifts to other protein dependencies. Monitoring the tumor microenvironment (TME) secretome may provide useful indications regarding suitable combinatorial treatments to block TME-induced gene expression. In CLL/MCL cells, the NF-κB1 inhibitor BMS-345541 blocked cell resistance. Similarly, inhibitors of MCL1 (A-1210477 and UMI-77), BCL2L1 (BH3I-1 and ABT-737), and survivin (YM155) induced synergistic effects on cell death [38]. This evidence provides several combinatorial strategies to pursue to allow clinicians to bypass TME-mediated resistance to venetoclax and potentially to other BH3 mimetics in clinical development.

Immunomodulatory Activities of BCL2 Protein Antagonizing Strategies

Potentiating the immunogenicity of cancer cells is an attractive emerging area in the development of new anticancer agents. The ability of some cytotoxic compounds to induce immunogenic cell death (ICD) in cancer cells could provide a feasible strategy to achieve this therapeutic goal [39,40].
Critical reading of the literature evokes differential correlations between the targeting of specific antiapoptotic BCL2 family members and the occurrence of ICD. Overall, selective targeting of BCL2 does not increase the immunogenicity of dying cancer cells because venetoclax essentially induces tolerantogenic cell death through mitochondrial apoptosis. By contrast, treatments leading to MCL1 regulation act also as potent ICD inducers. The prototypical ICD inducers doxorubicin and daunorubicin downregulate MCL1 protein levels [41,42]. Cyclin-dependent kinase (CDK) inhibitors are ubiquitous MCL1-downregulating agents [43–46] that have also recently been identified as ICD inducers [47,48]. Similarly, the ICD-inducing cardiac glycosides (CGs) [49,50] ubiquitously inhibit MCL1 protein expression [15,26]. Similar considerations may be drawn for other described ICD inducers, including the hydroxycoumarin OT-55 [51] and the flavone wogonin [14,52]. The induction of ER stress, generally described as a determinant of ICD, promotes the downregulation of short-lived proteins, including MCL1 [53]. Moreover, for many of the compounds indicated above, there is strong evidence of synergistic effects with venetoclax or anti-PD-1 antibodies (e.g., dinaciclib [43]). MCL1 exhibits a particular mitochondrial distribution and regulates specific mitochondrial functions, and MCL1 targeting might contribute to ICD induction by inhibiting these activities. Mitochondria represent an important source for damage-associated molecular patterns (DAMPs) [54,55], including mtDNA, cardiolipin, transcription factor A, mitochondrial (TFAM), cytochrome c, and formyl-methionine-labeled peptides [56]. MCL1 is a crucial factor in the maintenance of mitochondrial fusion and mtDNA [23], where it affects the release of DAMPs. Recently, the autophagy inhibitor spautin-1 was described as a novel ICD inducer [57]. Mechanistically, spautin-1 induces mitochondrial apoptosis associated with mitochondrial TFAM release and subsequent immunostimulation of dendritic cells (DCs) and T cells. Notably, spautin-1 also inhibits MCL1 expression [58]. The first selective MCL1 inhibitors were developed only recently. Investigation of the regulatory effects of these new compounds will shed light on the role of MCL1 in promoting the release of DAMPs and ICD.

Although venetoclax is not immunogenic per se, its combination with immune therapies could improve durable response and allogeneic BM transplantation [59]. Patients with venetoclax-resistant CLL harbored CD274 (or programmed cell death ligand 1, PD-L1)+ clones that were targetable by anti-programmed cell death (PD-1) strategies [60]. Consistent with these results, a murine model of MYC-driven breast cancer treated with ABT-737 and receiving adjuvant anti-PD1 or PD-1 exhibited reduced post-treatment tumor regrowth [61]. PD-L1 is expressed in patients with AML, and higher expression correlates with mutations that lead to unfavorable prognosis [62,63]. Currently, various clinical trials in AML that involve immune checkpoint inhibitors in combination with standard/targeted treatments are underway (NCT03867682, NCT03932318, and NCT03390296). Together, these findings provide a rationale for the investigation of sequential and combinatorial AML treatments that include BH3 mimetics and immune checkpoint inhibitors.

Antiapoptotic BCL2 proteins regulate the survival of several immune cell types [64]. However, it is unknown whether BCL2 protein targeting can impair specific immune cell populations, particularly when combined with other immunomodulatory treatments. Findings presented at the 2018 ASH annual meeting by Raval and colleagues demonstrated the resistance of natural killer and T cells to venetoclax alone or in combination with anti-PD-1 in a murine lymphoma model [65]. Although additional studies are required, this suggests the feasibility of combining venetoclax and anti-PD-1 for the treatment of hematological and solid cancers.

Conclusions and Future Perspectives
The BH3 mimetic venetoclax has improved tolerability compared to intensive standard therapies; however, despite the higher response rate to this drug, the OS remains limited
Furthermore, venetoclax-relapsed AML forms are currently emerging [66,67]. The characterization of the anticancer action of BH3 mimetics presents potential alternative strategies to overcome cancer cell refractoriness and resistance (Figure 2). This goal will necessitate further investigations to delineate important mechanistic and therapeutic implications (see Outstanding Questions).

OXPHOS impairment is documented in AML cells treated with single-agent or combinatorial regimens including venetoclax. It is generally accepted that OXPHOS impairment may be mechanistically the consequence of the canonical functions ascribed to the BH3 mimetics leading to MOMP. Alternatively, we may speculate about effects on metabolism that could at least partly be the outcome of non-apoptotic or target-independent roles of BCL2 proteins. Very recent findings show that venetoclax inhibits mitochondrial respiration irrespective of BCL2 expression [68]. A second relevant aspect is to establish the active role that OXPHOS impairment, and not MOMP, might play in cell death commitment even if downstream from MOMP following the treatment of AML with single or combinatorial regimens including BH3 mimetics. Recent studies highlight that sublethal concentrations of venetoclax induce mitochondrial ultrastructural perturbations that include loss and/or widening of IMM cristae [19,69]. These alterations resemble those induced by conditions that inhibit the expression or the function of factors controlling mitochondrial structural and functional integrity (respectively, the mitochondrial chaperone casinolytic peptidase B protein homolog CLPB [69] and the mitochondrial protein synthesis inhibitor tedizolid [69]). Chen, Aifantis, and colleagues [69] have documented that venetoclax-resistant cell models exhibit a well-preserved and even tighter organization of the cristae, a modulation that would prevent the redistribution of cytochrome c into the intermembrane space of mitochondria and ultimately impair efficient cytochrome c release into the cytosol. OPA1 has a determinant role in this process. Although CLPB-silenced cells generate a similar pattern of ultrastructural mitochondrial alterations and impact on OXPHOS, we have no direct information regarding whether OXPHOS is affected by venetoclax depending on the level of the tightness of the cristae. Sharon, Chan, and colleagues [19,69] revealed that venetoclax-resistant cell models also exhibit ultrastructural changes and OXPHOS impairment upon single-agent venetoclax treatment, and this occurs independently of apoptosis induction. The actual mechanisms determining cristae remodeling by venetoclax deserve further investigation, although a role for BAX/BAK1 activation/oligomerization in mitochondrial dynamics could be expected. The monitoring of OXPHOS in venetoclax-resistant cell models, the possibility of concomitantly tracking the association of MMP loss with the appearance of mitochondrial alterations, the timing between the OXPHOS impairment (generally immediately occurring) versus the occurrence of the cristae remodeling, and the use of double-knockout BAK1/BAX cell models may represent important future experimental strategies that could be used to corroborate this mechanistic aspect.

Preserving ETC dysfunction downstream from mitochondrial BAX translocation/oligomerization protects against apoptosis; it also maintains the proliferative potential of the cell [21]. Preventing AA starvation or redox modulation induced by a combination of venetoclax and azacytidine protects AML cells from death [6,18]. Strategies to preserve ETC integrity under conditions where BAX translocation/oligomerization or MOMP regularly take place will provide new mechanistic and therapeutic insights into the importance of the additional modulatory activities of BH3 mimetics on metabolic pathways, beyond the canonical involvement in MOMP/cytochrome c release. Higher OXPHOS levels are a characteristic of a resistant phenotype against venetoclax as well as to other therapeutic agents [20,70]. Accordingly, combinatorial strategies targeting mitochondrial integrity and OXPHOS show promising therapeutic synergistic effects [19,69].
Several mechanisms of resistance to venetoclax are emerging. Combinatorial treatments appear to provide an extremely promising strategy [71]. These could co-target primary forms of addiction (e.g., co-targeting BCL2 and MCL1 [15]), or alternatively they could promote dependency upon specific BCL2 proteins (e.g., the synthetic lethality induced by p53 regulators [8]). Notably, the abilities of several BCL2 targeting agents to affect cancer cell metabolism and stemness [6,17,18,70,72] or to promote ICD [99,40,48,49] offer further rationales to test specific combinations, thus impacting on nonredundant cancer dysfunctions. Second, targeted agents such as BH3 mimetics could be used as tools to track the trajectories taken by coexisting subclones, eventually providing predictive biomarkers for clonal expansion under therapeutic conditions [67,73] (see Outstanding Questions).

We stand at the beginning of a new area in AML treatment because targeted therapies have only become a reality in the past few years. The emerging nonredundant functions of BCL2 proteins, combined with the development of selective MCL1 and BCL2L1 inhibitors beyond venetoclax, provide a multitude of experimental and therapeutic opportunities to explore and exploit.

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Outstanding Questions
What is the best approach/marker to identify AML stem cells and monitor their metabolic targeting with BH3 mimetics?

How to inhibit the metabolic flexibility of AML cells (blasts and stem components) that are resistant to the anti-metabolic effects of venetoclax and other BH3 mimetics?

Are there specific AML subgroups based on cytogenetic classification (FAB) or genetic/epigenetic properties (WHO) that are more prone to eluding the effects of combinational therapies including BH3 mimetics by changing their metabolic behavior?

What is the impact of AML (de)differentiation (conversion of blasts to stem-like cells and vice versa) on the anticancer potential of BH3 mimetics in single/combinational strategies?

How to efficiently monitor clonal heterogeneity as a cause of resistance to venetoclax and other BCL2 protein antagonists?

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