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Review

Cancer Research

Finally, An Apoptosis-Targeting Therapeutic for Cancer

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Abstract

Resistance to cell death represents one of the hallmarks of cancer. Various genetic and epigenetic changes in malignant cells afford cytoprotection in the face of genomic instability, oncogene activation, microenvironment stress, chemotherapy, targeted anticancer drugs, and even immunotherapy. Central among the regulators of cell life and death are Bcl-2 family proteins, with the founding member of the family (B-cell lymphoma/leukemia-2) discovered via its involvement in chromosomal translocations in lymphomas. The quest for therapeutics that target cell survival protein Bcl-2 represents a long road traveled, with many dead-ends, disappointments, and delays. Finally, a Bcl-2–targeting medicine has gained approval as a new class of anticancer agent. *Cancer Res; 76(20); 5914–20. ©2016 AACR.*

Only 32 years – that's how long it took from discovery of the gene encoding antiapoptotic protein Bcl-2 in 1984 to the delivery a Bcl-2–targeting therapeutic for cancer. This year, 2016, the FDA approved the Bcl-2 inhibitor venetoclax for the treatment of an aggressive form of chronic lymphocytic leukemia (CLL). The approval of venetoclax constitutes a milestone in medicine, representing the first apoptosis-targeting therapeutic available to patients and tackling (at last) one of the six hallmarks of cancer (resisting cell death; ref. 1).

The relevance of Bcl-2 as a cancer drug target was immediately obvious, because the *BCL-2* gene was identified via its involvement in t(14;18) chromosomal translocations commonly found in non-Hodgkin B-cell lymphomas (B-cell NHL; refs. 2–4). These chromosomal translocations place the *BCL-2* gene at 18q21 into juxtaposition with the immunoglobulin heavy-chain (*IGH*) locus at 14q32, where powerful enhancers associated with the *IGH* locus dysregulate the transcription of *BCL-2* (Fig. 1A). The majority of follicular small-cell NHLs contain the t(14;18) and highly express *BCL-2*. These neoplasms are called "indolent" NHLs because they have very low mitotic rates and tend to progress slowly, compared with aggressive forms of NHL such as diffuse large B-cell lymphomas (DLBCL) that rapidly proliferate. The association of Bcl-2 expression with indolent neoplasms was further strengthened when it was found that *BCL-2* mRNA levels are equally high in most CLLs (5), although only occasional CLLs contain chromosomal translocations involving *BCl-2* (generally occurring with Ig light-chain gene loci (Fig. 1B; ref. 6). CLL in its early stages also is typified by a very low mitotic index, where the leukemic mature B-lymphocytes gradually accumulate in the patient. Gene amplification is another documented mechanism for *BCL-2* gene activation (Fig. 1C).

Experimentally confirming the causal role of Bcl-2 in neoplasia, transgenic mice were produced that contained *BCL-2* minigenes under the control of IgH gene elements, causing massive expansion of B-lymphocytes *in vivo* without impacting the cell cycle (7). Moreover, transfecting plasmids overexpressing the human Bcl-2 protein into murine 3T3 fibroblasts did not lead to morphologic transformation in culture, in contrast to well-known oncogenes such as Ras and Src, but it did result in higher frequencies of tumorigenesis when Bcl-2–expressing cells were injected into mice and given time for secondary genetic events to occur (8). Altogether, these early observations suggested that *BCL-2* defined a novel category of oncogenes.

This indolent behavior of low-grade NHLs and CLLs is now easily explained by the understanding that roadblocks to apoptosis constitute one of the hallmarks of cancer (1), but at the time the notion that pathologic cell accumulation could be the consequence of failed programmed cell death was still not widely acknowledged. Gene transfer experiments proved that Bcl-2 suppresses apoptosis in hematolymphoid cells when deprived of growth factors in culture (9), thus firmly establishing *BCL-2* as the first cell survival gene to be discovered in any organism.

Interest in Bcl-2 as a cancer drug target climbed when the connection between blocking apoptosis and chemoresistance was made. Gene transfer experiments showed, for example, that overexpressing Bcl-2 in lymphoid leukemia cell lines conferred resistance to the cytotoxic actions of essentially every anticancer drug available at that time (10). Conversely, reducing Bcl-2 expression using antisense oligonucleotides (ASO) that bind Bcl-2 mRNA and promote its RNaseH-dependent degradation restored chemosensitivity in leukemia and lymphoma cells (11, 12). Moreover, a myriad of clinical-correlative studies began to show an association between higher Bcl-2 expression and poor responses to chemotherapy in clinical trials (see, for example, ref. 13). Altogether, these observations established a novel mechanism of cancer chemoresistance caused by a terminal block to cellular pathways for apoptosis, thus differentiating it from classical chemoresistance mechanisms such as altered drug metabolism, drug efflux, and cellular DNA repair. It also suggested that the

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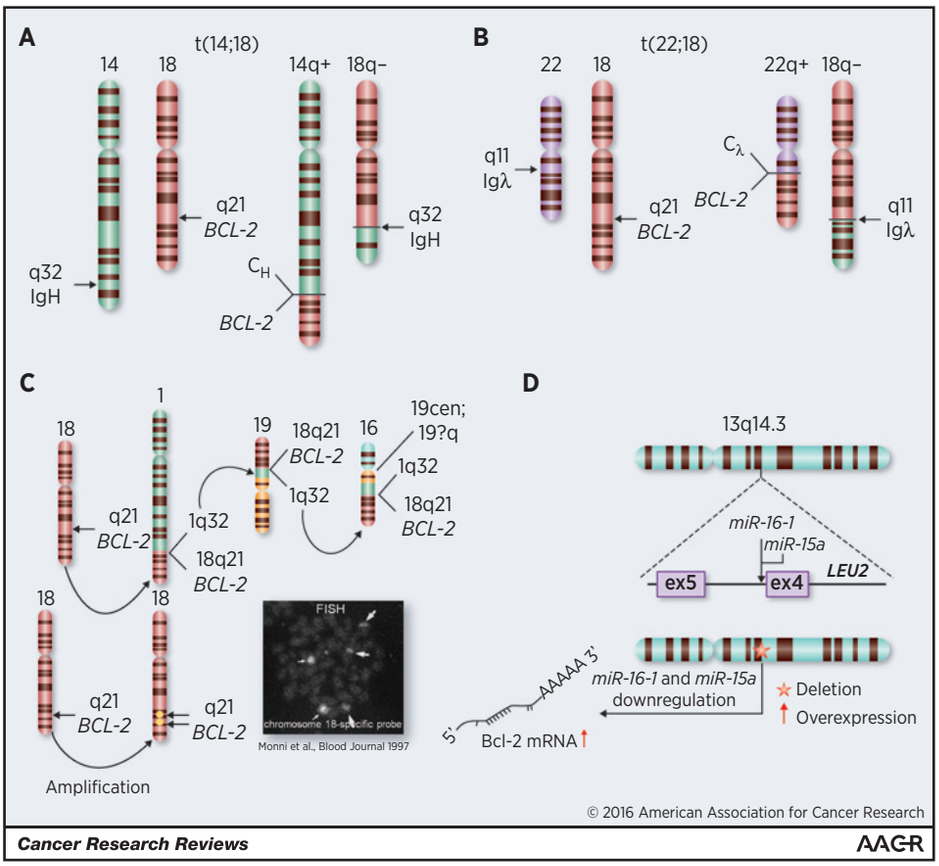

Figure 1.
Genetic lesions accounting for dysregulation of *BCL-2* gene expression in malignancies. **A** and **B,** the t(14;18) (**A**) and the t(22;18) (**B**) reciprocal chromosome translocations are depicted. The translocations juxtapose the *BCL-2* gene to enhancer elements of the Ig loci, causing deregulation of expression of *BCL-2*. **C,** *BCL-2* gene amplification schemes. Top, chromosome 18q–derived sequences are depicted with translocation to chromosome 1q32, which was further translocated to chromosomes 19 and 16. FISH using a chromosome 18-specific probe shows *BCL-2* amplification, three labels (large arrows) in addition to normal chromosomes (small arrows). Reproduced with permission of American Society of Hematology from Monni et al., Blood Journal 1997. **D,** bottom, *BCL-2* gene amplification without chromosome rearrangements. The 13q14 genomic region is deleted in most CLLs. The genes encoding miR-15a and miR-16-1 lie within a 30-kb deleted region between exons 2 and 5 of the *DLEU2* gene. The deletion of miR-15a and miR-16-1 locus leads to Bcl-2 mRNA overexpression.

cellular damage induced by various anticancer drugs, (regardless of whether targeting DNA synthesis, DNA repair, nucleotide pools, or microtubules), converged on a final common pathway that was governed by Bcl-2.

While there was plenty of excitement about Bcl-2, it was still unclear how this oncoprotein promotes cancer cell survival at the molecular level. Even to this day, the Bcl-2 protein remains a bit of a mystery, but it was particularly perplexing in the early days of apoptosis research because Bcl-2 was unlike any other human oncoprotein previously studied. The 26-kDa Bcl-2 protein contains a stretch of hydrophobic amino acids at its carboxyl-terminus and associates with intracellular membranes (14). However, closer inspection revealed that Bcl-2 was not associated with plasma membranes (unlike Ras, Src, Erb, and other membrane-associated oncoproteins) but rather with mitochondrial membranes (15). Cell-free systems provided evidence that mitochondria played an essential role in apoptosis and that the Bcl-2 protein could block these mitochondria-derived death signals (16). Subsequently, it was discovered that Bcl-2 blocks the release from mitochondria into the cytosol of apoptogenic proteins, most prominently cytochrome *c* (17), which causes assembly of "apoptosomes", multiprotein macromolecular assemblies that initiate cascades of cell death protease activation (caspases; ref. 18).

Because Bcl-2 is an intracellular protein, mAb (large molecule) therapeutics are not an option. At the time, a robust small-molecule strategy was challenging to devise because of lack of an understanding of the molecular details of how Bcl-2 controlled cell death. Consequently, an alternative approach to generating Bcl-2–targeting therapeutics was tried by targeting the Bcl-2 mRNA using nuclease-resistant oligonucleotides (19), with at least 3 such compounds taken into the clinic. Oblimersen sodium (Genasense) got the farthest and was tested in pivotal phase III trials [sponsored by Genta, Inc and Aventis (now Sanofi-Aventis)], but failed to convince the FDA of therapeutic benefit. Nevertheless, those early forays into the clinic with RNA-targeting compounds helped to further validate Bcl-2 as an attractive cancer target. For instance, the randomized phase III study of oblimersen in relapsed or refractory CLL compared treatment with chemotherapy alone (cyclophosphamide plus fludarabine) with chemotherapy plus Bcl-2 antisense (20). Complete response (CR) rates were significantly higher when adding Genasense to chemotherapy and durations of responses (DoR) were longer. There was also a progression-free survival (PFS) benefit for a subset of relapsed (but not refractory) patients found by *post hoc* analysis. In retrospect, CLL was a superb context for testing the attributes of Bcl-2 mRNA-targeting therapeutics, because it was later discovered that most CLLs suffer deletions of genes encoding miRNAs that suppress Bcl-2 expression (Fig. 1D), thus explaining why Bcl-2 mRNA and protein levels are so high in these leukemias (21).

The dream of producing small molecules targeting Bcl-2 was greatly aided when the laboratory of the late Stanley Korsmeyer

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reported that Bcl-2 binds a proapoptotic protein Bax (22). The notion emerged that dimerization of antiapoptotic Bcl-2 with proapoptotic Bax suppresses apoptosis, and thus it was reasoned that chemicals that disrupt this protein–protein interaction could provide an approach to overcoming blocks to cell death in cancer. Within a short time, a diversity of both anti- and proapoptotic *BCL-2* family genes were discovered (n = at least 18 in humans) and shown to dimerize with themselves and/or other members of the rapidly expanding family. Structure–function mutagenesis studies dissected a minimal domain within proapoptotic Bcl-2 family proteins, termed the Bcl-2 homology-3 (BH3) domain, which was shown to mediate interactions with and functionally neutralize antiapoptotic family members (23–26).

The quest for small-molecule inhibitors of antiapoptotic Bcl-2 family proteins leaped forward when investigators at Abbott Laboratories (now AbbVie) determined the 3D structure of the antiapoptotic protein Bcl-XL alone (27) and then later in a complex with a BH3 peptide from a proapoptotic protein Bak (28). Similar data were generated for Bcl-2 (29). It was revealed that Bcl-XL and Bcl-2 have a hydrophobic crevice on their surface that binds proapoptotic amphipathic, α-helical BH3 peptides (Fig. 2). However, the structural information also revealed that this was a very challenging protein–protein interaction (PPI) that would not easily yield to small-molecule drug discovery. Bcl-2/Bcl-XL-binding BH3 peptides were demonstrated to be approximately 16 amino-acids in length, making multiple contacts with the crevice on Bcl-2 and Bcl-XL. Thus, to mimic a BH3 peptide with a small molecule having drug-like properties was a tall order, which would require a unique strategy and a novel technology. Indeed, in support of this notion, high-throughput screens were conducted by many groups, revealing molecules with only weak (micromolar) inhibitory activity. Among these was obatoclax (30), which went as far as phase II trials.

At the same time that the Abbott team was determining the structures of the Bcl-2 family of proteins and their complexes, they were also pioneering an approach for targeting challenging proteins such as Bcl-2 using chemical fragment-based methods. The approach involved the use of high-field solution NMR to detect interactions of small organic compounds (typically 150-250 MW) with protein targets (31). The technique was dubbed "SAR by NMR" (32), because it provided structure–activity relationships using nuclear magnetic resonance. Applying this approach to the Bcl-2 family of proteins produced hits and useful starting points for generating potent molecules. Using this innovative approach of NMR-based chemical fragment screening, structure-based drug design, and medicinal chemistry, the Abbott group generated extremely potent inhibitors of Bcl-2 and Bcl-XL. In a collaborative effort with academic and biotech partners, the first prototypical compound (ABT-737) was described, a dual inhibitor of Bcl-2 and Bcl-XL that served as an informative tool compound for further testing concepts about apoptosis-targeting cancer therapeutics (33).

Although a useful tool, a limitation of ABT-737 was the lack of oral bioavailability. In an effort to circumvent this problem, iterative cycles of compound optimization were performed by the Abbott group and resulted in the first clinical candidate, Navitoclax (ABT-263), an orally bioavailable and potent, dual inhibitor of Bcl-2 and Bcl-XL (34). In the clinic, navitoclax displayed excellent pharmacology and showed promising signals of efficacy in hematologic malignancies (35–37). However, a mechanism-based side-effect of Navitoclax emerged, thrombocytopenia, which was attributed to Bcl-XL inhibition (38, 39). Analysis of Bcl-XL knockout mice showed that this antiapoptotic protein is required for the longevity of platelets (40).

Navitoclax may ultimately prove to be useful against tumors that upregulate both Bcl-2 and Bcl-XL (and still undergoes testing in clinical trials), but thrombocytopenia can be problematic, especially for patients treated with chemotherapy whose platelet counts are already low. Thus, an effort was initiated to dial out Bcl-XL inhibition, while retaining potent Bcl-2 inhibition. After several years effort involving a collaborative effort of Abbvie with Genentech (member of the Roche Group), and academic partners, the Bcl-2–selective inhibitor venetoclax (ABT-199/GDC-0199) was discovered using structure-based design (Fig. 3) and taken into the clinic with the idea to avoid thrombocytopenia and the hope that clinical activity would be maintained even through the molecule inhibits only Bcl-2 (41). In this regard, preclinical

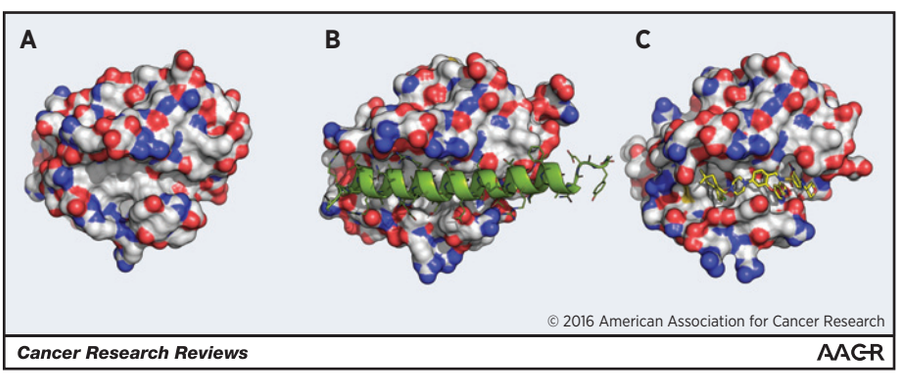

Figure 2.

Three-dimensional structure of antiapoptotic Bcl-2 family members. The 3D structure of the human Bcl-XL protein is depicted with an empty groove (**A**; PDB accession code: 1MAZ) and in complex with the BH3 peptide from Bim (**B**; PDB accession code: 1PQ1). The human Bcl-2 protein is represented in complex with a modeled structure of venetoclax based on the crystal structure of (4-(4-{[4-(4-chlorophenyl)-5,6-dihydro-2H-pyran-3-yl]methyl}piperazin-1-yl)-N-{[3-nitro-4-(tetrahydro-2H-pyran-4-ylamino)phenyl]sulfonyl}benzamide), a close analog (**C**; PDB accession code: 4MAN). The Connolly surface of the proteins is colored by mapped atom type (carbon, white; nitrogen, blue; oxygen, red; sulfur, yellow).

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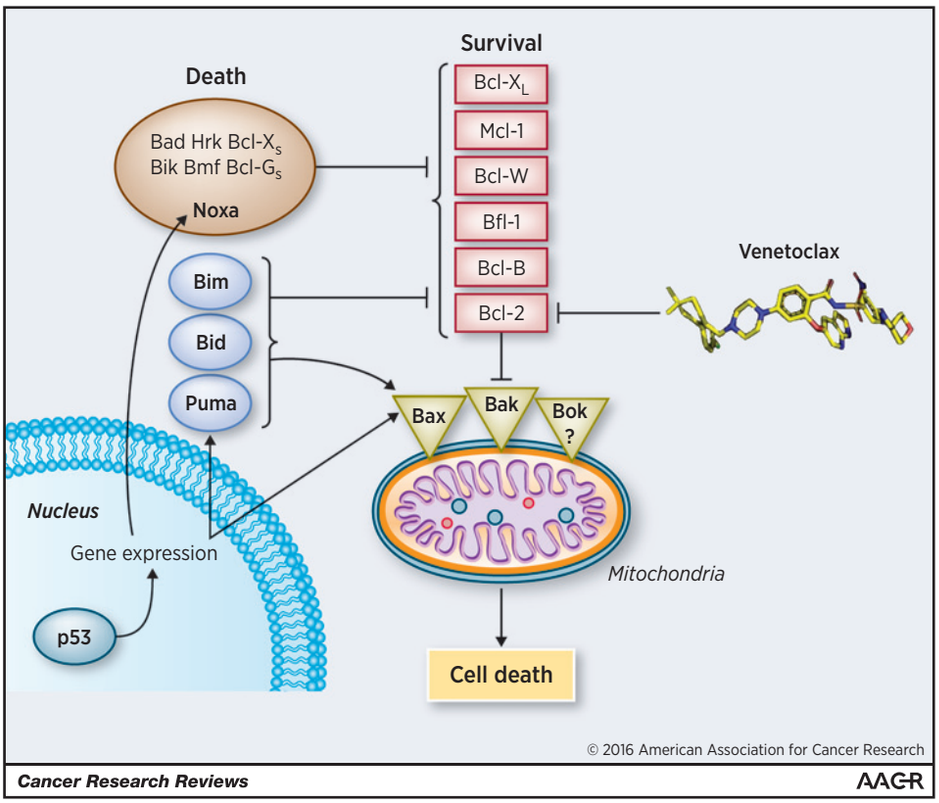

Figure 3.
Interactions among Bcl-2 family proteins. The categories of the Bcl-2 family are represented, including: (i) antiapoptotic proteins, Bcl-2, Bcl-XL, Mcl-1, Bcl-W, Bfl-1, and Bcl-B (red); (ii) the multi-domain proapoptotic, Bax, Bak, and possibly Bok (yellow), which permeabilize the outer mitochondrial membrane; (iii) BH3-only proteins that operate as both agonists of proapoptotic Bax/Bak and antagonists of antiapoptotic Bcl-2 members (pink); and (iv) BH3-containing proapoptotic members that operate as antagonists of the antiapoptotic proteins (orange). Tumor suppressor p53 plays important roles in responses to chemotherapy and stimulates transcription of specific proapoptotic members of the family (*BAX, PUMA, BID, NOXA*). Venetoclax is a selective antagonist of Bcl-2.

studies of the dual Bcl-2/Bcl-XL inhibitor navitoclax (or the analogous tool compound ABT-737) had taught the research community that inducing apoptosis of malignant cells was often stymied by coexpression of other antiapoptotic Bcl-2 family members such as Mcl-1 and Bfl-1 (42, 43). Concerns therefore existed that the narrow spectrum of venetoclax might reduce efficacy if malignant cells coexpressed Bcl-XL and other antiapoptotic Bcl-2 family proteins. Table 1 summarizes the critical steps involved on the road to venetoclax.

The studies of venetoclax conducted thus far in a codevelopment partnership of Abbvie and Genentech have focused on hematopoietic malignancies, generating highly encouraging results, with venetoclax garnering thus far three Breakthrough Designations from the FDA. Promising clinical activity of venetoclax monotherapy has been reported for CLL, mantle cell lymphoma, and Waldenstrom's macroglobulinemia, and to a lesser extent for follicular NHL and diffuse large B-cell NHL (see, for example, ref. 44). Arguably the most impressive single-agent activity has been seen in relapsed/refractory (R/R) CLL, where venetoclax resulted in response rates of nearly 80% (45). In fact, responses were so brisk in CLL that early patients experienced tumor lysis syndrome (TLS), unfortunately fatal in some cases. By gently titrating the dose upwards, TLS was subsequently avoided (46). Moreover, similar response rates were achieved in an aggressive, chemoresistant subtype of CLL in which chromosome 17p13 deletions are found. In the clinical study that formed the basis for the NDA (NCT01889186), venetoclax monotherapy generated a response rate of 79% in these 17p-deleted R/R CLL patients ($n = 107$).

Table 1. Milestones in history of Bcl-2 research

1984	14; 18 breakpoint cloned
1985–1986	Bcl-2 cDNA cloned; sequenced
1988	Apoptosis suppression
1989	Bcl-2 poor progress in NHL
1990	Bcl-2 localized to mitochondria
1992	Chemoresistance
1993	Bax dimerizes with Bcl-2
1993	Bcl-2 over expressed in CLL
1993	ASO reverses chemoresistance
1994	Mitochondria required
1996	BH3 mediates dimerization
1996	Bcl-X 3D structure
1997	Bcl-2 ASO (Ph3 CLL)
1997	Bcl-XL + BH3 3D structure
1997	Bcl-2 gene amplified (DLBCL)
1999	SAR by NMR
2001	Bcl-2 3D structure
2002–2005	miR-15/16 deletion (CLL)
2005	ABT 737 discovered
2007	Bcl-XL required for platelets
2007	Obatoclax discovered
2008	Navitoclax discovered
2009	Obatoclax Ph 1
2011	Navitoclax Ph 1
2013	Venetoclax discovered
2016	Venetoclax impressive activity in R/R CLL
2016	FDA approval

NOTE: Some of the milestones are listed chronologically in the journey from discovery of the gene encoding Bcl-2 to the FDA approval of the first Bcl-2 inhibitor, venetoclax.

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The biology underlying this 17p-deleted subgroup of CLLs relates to loss of tumor suppressor p53. In p53-wild-type leukemias, levels of p53 protein rise in response to DNA-damaging chemotherapeutic agents, causing apoptosis. The p53 protein is a DNA-binding transcription factor that directly stimulates the expression of several proapoptotic *BCL-2* family genes, including *BAX*, *NOXA*, *BID*, and *PUMA* (47–49). Thus, leukemias lacking p53 have lost their endogenous mechanism for counteracting Bcl-2 when exposed to chemotherapy.

More impressive than its single-agent activity, response rates > 90% have been achieved in R/R CLL when combined with anti-CD20 mAbs Rituxan/MabThera (rituximab) or Gazyva (obinutuzumab; ref. 50). The basis for the apparent synergy between venetoclax and anti-CD20 is potentially multifactorial, but it is noteworthy that treatment of CLL patients with rituximab stimulates reductions of Mcl-1 protein within hours after infusion (51), raising the possibility that anti-CD20 sensitizes CLLs to Bcl-2 inhibition by removing one of the Bcl-2 family proteins implicated in resistance to venetoclax.

Beyond CLL, venetoclax has also shown promise for treatment of other types of hematopoietic malignancies, particularly when combined with appropriate partner agents. For instance, in acute myelogenous leukemia (AML) occurring in adults, venetoclax monotherapy has delivered response rates of <20%. (52). However, when combined with DNA methyltransferase (DMTase) inhibitors azacitidine or decitabine, response rates of >70% were reported recently (53). The reason for this synergy with DNA hypomethylating agents is hypothesized to relate to changes in expression of other *BCL-2* family genes, tipping the balance in favor of apoptosis. Recognizing that the vast majority of AMLs retain wild-type p53, and following impressive preclinical data (54), clinical trials are also underway in AML to explore the combination of venetoclax with p53 activator idasanutlin (Roche), a small-molecule inhibitor of Mdm2 (E3 ligase that mediates degradation of p53 protein; ref. 55). However, as Bcl-2 inhibition causes neutropenia as well as lymphopenia, it remains to be seen whether a p53 stimulator such as idasanutlin can be safely combined given the potential for overlapping hematotoxicity. Finally, for multiple myeloma, another promising drug combination involves proteasome inhibitors such as bortezomib, which acts by multiple mechanisms including reducing degradation of proapoptotic Bcl-2 family members (e.g., Bim, Noxa), stimulating expression of proapoptotic proteins induced via the "unfolded protein response" (e.g., Bim), and interfering with transcription factors such as NF-κB that promote expression of antiapoptotic Bcl-2 family members (e.g., Bcl-XL, Bfl-1, Bcl-B; ref. 56). Altogether, the emerging data suggest that venetoclax has the promise to become a major weapon in the treatment not just of CLL but also a variety of hematologic malignancies. Further exploration is needed of predictive biomarkers that identify those leukemia and lymphoma patients that are most likely to benefit from venetoclax-containing therapeutic regimens. In addition, the potential of Bcl-2–selective inhibitors for treatment of solid tumors remains to be defined, but opportunities (as monotherapy or particularly when combined with synergistic agents) could include *BCL-2* gene–amplified small-cell lung cancers, estrogen receptor–positive breast cancers, and neuroblastomas.

The road to venetoclax was paved with many other sidetracks and dead-ends not elaborated on here. Efforts to discover and develop broad-spectrum inhibitors of Bcl-2 family proteins, as well as selective inhibitors of other family members such as Mcl-1, are currently under pursuit. That work continues and may yet provide additional weapons for oncologists, which will surely be needed when venetoclax resistance mechanisms emerge. Nevertheless, the approval of venetoclax marks a successful milestone in a long-sought quest, and the beginning of a new journey to learn how to optimize the opportunity provided by Bcl-2–targeting therapeutics. At last, we have a medicine that addresses one of the fundamental hallmarks of cancer, suppression of apoptosis (1).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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