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Finally, an apoptosis-targeting therapeutic for cancer

Carlo M. Croce

Department of Cancer Biology and Genetics The Ohio State University and Comprehensive Cancer Center, Columbus, Ohio, USA

John C. Reed

Roche Pharmaceutical Research and Early Development, Basel, Switzerland CH 4070; ETH Zürich, Department of Biology, Zürich, Switzerland CH 8093

Summary

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.

Only thirty-two years -- that's how long it took from discovery of the gene encoding anti-apoptotic protein Bcl-2 in 1984 to the delivery a Bcl-2-targeting therapeutic for cancer. This year, 2016, the Food & Drug Administration (FDA) of the USA approved the Bcl-2 inhibitor Venetoclax for 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)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's B-cell lymphomas (B-cell NHL)^{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* (Figure 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 to 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 CLLs⁵, though only occasional CLLs contain chromosomal translocations involving *BCL-2* (generally occurring with Ig light-chain gene loci⁶ (Figure 1b). 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 (Figure 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 cycle⁷. Moreover, transfecting plasmids over-expressing the human Bcl-2 protein into murine 3T3 fibroblasts did not lead to morphological 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 occur⁸. 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 cancer¹, but at the time the notion that pathological 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 culture⁹, 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 over-expressing Bcl-2 in lymphoid leukemia cell lines conferred resistance to the cytotoxic actions of essentially every anticancer drug available at that time¹⁰. Conversely, reducing Bcl-2 expression using antisense oligonucleotides (ASOs) 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 example¹³). 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 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 membranes¹⁴. 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 membranes¹⁵. 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 signals¹⁶. Subsequently, it was discovered that Bcl-2 blocks the release from mitochondria into the cytosol of apoptogenic proteins—most prominently cytochrome c¹⁷, which causes assembly of “apoptosomes”, multiprotein macromolecular assemblies that initiate cascades of cell death protease activation (caspases)¹⁸.

Because Bcl-2 is an intracellular protein, monoclonal antibody (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 oligonucleotides¹⁹, with at least 3 such compounds taken into the clinic. Oblimersen sodium (Genasense) got the farthest and was tested in pivotal phase 3 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 3 study of oblimersen in relapsed or refractory CLL compared treatment with chemotherapy alone (cyclophosphamide plus fludarabine) with chemotherapy plus Bcl-2 antisense²⁰. 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 microRNAs that suppress Bcl-2 expression (Figure 1d), thus explaining why Bcl-2 mRNA and protein levels are so high in these leukemias²¹.

The dream of producing small molecules targeting Bcl-2 was greatly aided when the laboratory of the late Stanley Korsmeyer reported that Bcl-2 binds a pro-apoptotic protein Bax²². The notion emerged that dimerization of anti-apoptotic Bcl-2 with pro-apoptotic 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 pro-apoptotic 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 pro-apoptotic Bcl-2 family proteins, termed the Bcl-2 Homology-3 (BH3) domain, which was shown to mediate interactions with and functionally neutralize anti-apoptotic family members^{23–26}.

The quest for small molecule inhibitors of anti-apoptotic Bcl-2 family proteins leaped forward when investigators at Abbott Laboratories (now AbbVie) determined the 3D-structure of the anti-apoptotic protein Bcl-XL alone²⁷ and then later in a complex with a BH3 peptide from a pro-apoptotic protein Bak²⁸. Similar data were generated for Bcl-2²⁹. It was revealed that Bcl-XL and Bcl-2 have a hydrophobic crevice on their surface that binds pro-apoptotic amphipathic, α -helical BH3 peptides (Figure 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 ~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 obatoclax³⁰, which went as far as phase 2 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 targets³¹. 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 therapeutics³³.

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 was 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-XL³⁴. In the clinic, Navitoclax displayed excellent pharmacology and showed promising signals of efficacy in hematological 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 anti-apoptotic protein is required for the longevity of platelets⁴⁰.

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 (Figure 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-2⁴¹. In this regard, preclinical 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 co-expression of other anti-apoptotic 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 co-expressed Bcl-

XL and other anti-apoptotic Bcl-2 family proteins. Table 1 summarizes the critical steps involved in the development of Venetoclax.

The studies of Venetoclax conducted thus far in a co-development partnership of Abbvie and Genentech have focused on hematopoietic malignancies, generating highly encouraging results, with Venetoclax garnering thus far 3 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 example⁴⁴). 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 avoided⁴⁶. 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).

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 pro-apoptotic 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 monoclonal antibodies Rituxan/MabThera (rituximab) or Gazyva (obinutuzumab)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 infusion⁵¹, 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 recently⁵³. 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 data⁵⁴, 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)⁵⁵. However, since 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 may improve sensitivity to Venetoclax acts by multiple mechanisms including reducing degradation of pro-apoptotic Bcl-2 family members (e.g., Bim, Noxa), stimulating expression of pro-apoptotic proteins induced via the “unfolded protein response” (e.g. Bim), and interfering with transcription factors such as NF- κ B that promote expression of anti-apoptotic Bcl-2 family members (e.g. Bcl-XL, Bfl-1, Bcl-B)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 hematological 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. Additionally, 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 side-tracks 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 apoptosis¹.

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Figure 1. Genetic lesions accounting for dysregulation of *BCL-2* gene expression in malignancies (A):The t(14;18) and the (B) t(22;18) 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. Fluorescence in situ hybridization using a chromosome 18-specific probe shows *BCL-2* amplification, three labels (large arrows) in addition to normal chromosomes (small arrows). (Bottom) *BCL-2* gene amplification without chromosome rearrangements (D) 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.

Figure 2. 3D-structure of anti-apoptotic 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 analogue (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).

Figure 3. Interactions among Bcl-2 family proteins

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

Table 1

Croce & Reed

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
2005	MiR15-16 deletion (CLL)
2005	ABT 737 development
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