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Targeting Bcl-2-IP_3 receptor interaction to treat cancer: A novel approach inspired by nearly a century treating cancer with adrenal corticosteroid hormones

Clark W. Distelhorst

Case Western University School of Medicine, Case Comprehensive Cancer Center, University Hospitals Cleveland Medical Center, Cleveland, Ohio 44106

Abstract

Bcl-2 inhibits cell death by at least two different mechanisms. On the one hand, its BH3 domain binds to pro-apoptotic proteins such as Bim and prevents apoptosis induction. On the other hand, the BH4 domain of Bcl-2 binds to the inositol 1,4,5-trisphosphate receptor (IP_3R), preventing Ca^{2+} signals that mediate cell death. In normal T-cells, Bcl-2 levels increase during the immune response, protecting against cell death, and then decline as apoptosis ensues and the immune response dissipates. But in many cancers Bcl-2 is aberrantly expressed and exploited to prevent cell death by inhibiting IP_3R-mediated Ca^{2+} elevation. This review summarizes what is known about the mechanism of Bcl-2's control over IP_3R-mediated Ca^{2+} release and cell death induction. Early insights into the role of Ca^{2+} elevation in corticosteroid-mediated cell death serves as a model for how targeting IP_3R-mediated Ca^{2+} elevation can be a highly effective therapeutic approach for different types of cancer. Moreover, the successful development of ABT-199 (Venetoclax), a small molecule targeting the BH3 domain of Bcl-2 but without effects on Ca^{2+}, serves as proof of principle that targeting Bcl-2 can be an effective therapeutic approach. BIRD-2, a synthetic peptide that inhibits Bcl-2-IP_3R interaction, induces cell death induction in ABT-199 (Venetoclax)-resistant cancer models, attesting to the value of developing therapeutic agents that selectively target Bcl-2-IP_3R interaction, inducing Ca^{2+}-mediated cell death.

Keywords

Bcl-2; inositol 1,4,5-trisphosphate receptor (IP_3R); calcium (Ca^{2+}); endoplasmic reticulum; apoptosis; adrenal corticosteroid hormones; dexamethasone; prednisone

Corresponding Author: Clark W. Distelhorst, MD, Department of Medicine, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106, cwd@case.edu, Phone: 216-368-4546.

Conflict of Interest

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1. Introduction

The inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular Ca^{2+} channel located on the endoplasmic reticulum (ER). Its diverse roles in normal physiology, cell survival and death, and diseases including cancer have been the focus of excellent reviews published in just the last four years (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). Here we concentrate on Bcl-2 regulation of IP_3R function in normal development and how cancer cells exploit Bcl-2-IP_3R interaction to promote their own survival. We start with an historical perspective summarizing how discoveries during the first half of the twentieth century led to cancer treatments mediated by corticosteroid hormones (prednisone, dexamethasone). We then review how Bcl-2 regulates Ca^{2+} to prevent cell death, and potential ways to target Bcl-2's regulation of Ca^{2+} as a novel therapeutic approach for Bcl-2-positive malignancies.

Much of this review centers around normal T-cell development in the immune system, where Bcl-2 plays a critical role, and on B-cell malignancies which exploit Bcl-2 to remain alive despite adverse environmental circumstances and cancer treatments intended to kill them. The diagram in Figure 1 is directed at two audiences: (i) the basic scientist unfamiliar with Bcl-2-expressing hematologic malignancies and how Bcl-2-IP_3R interaction plays a role; and (ii) the clinical scientists who are expert in testing and applying novel therapeutic agents but are not yet familiar with the potential value of treating cancer by targeting the Bcl-2-IP_3R interaction.

The most common hematologic malignancies include chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and multiple myeloma. The right half of Figure 1 is modified after several reviews by leading experts, summarizing recent advances in the treatment of CLL (13, 14, 15). In this cancer, chronic active B-cell receptor signaling promotes prolonged cell survival, aided by high levels of the anti-apoptotic protein Bcl-2. Exciting new therapeutic approaches have dramatically changed the therapeutic approach to CLL by targeting B-cell receptor signaling pathways, for example Bruton tyrosine kinase which is targeted by ibrutinib (16, 17) and the anti-apoptotic Bcl-2 protein which is targeted at one of its mechanisms of action by ABT-199/Venetoclax (18, 11). The left half of Figure 1 summarizes recent advances in targeting the interaction of Bcl-2 with IP_3Rs, using peptide inhibitors or small molecules to induce IP_3R-mediated Ca^{2+}-elevation and cell death as a novel therapeutic approach (19, 20, 21, 22).

2. The path of discovery: From adrenal corticosteroids to IP_3R-mediated Ca^{2+} elevation and apoptosis.

The Nobel Prize in Physiology or Medicine in 1950 was awarded jointly to Edward Kendall, Tadeus Reichstein and Philip Showalter Hench *"for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects"*. In the mid-1930s Kendall and Reichstein isolated and analyzed the composition of a number of similar hormones derived from the adrenal cortex. These became the basis for cortisone preparations that, with input from Kendall and Philip Hench, were first used at the end of the 1940s to treat rheumatoid arthritis and other inflammatory disorders. Also, in the 1940's was the landmark discovery that cortisone preparations have a "lympholytic effect" (23, 24) and

therefore have remarkable therapeutic activity in lymphoid malignancies (25, 26). Throughout the ensuing decades corticosteroid hormones were developed through clinical trials as extraordinarily effective anti-cancer agents, contributing to the cure of children with ALL (27, a type of cancer derived from an early developmental stage of B-lymphocytes. Corticosteroids (prednisone, dexamethasone) continue today to be essential components of treatment regimens for certain B-cell malignancies, including ALL (28, 29, 30) and multiple myeloma (31, 32, 33). Corticosteroids are still useful in treatment of refractory CLL (34, 35, 36).

Thirty years transpired between the initial use of corticosteroids in cancer treatment and recognition that Ca^{2+} plays an important role in cell death induction by these agents (37, 38, 39, 40), involving Ca^{2+}-mediated endonuclease activation (39) and apoptotic DNA fragmentation (41, 42). Numerous refinements to the role of Ca^{2+} in endonuclease activation were to follow (43, 39, 44, 45, 46, 47) along with an expansion of knowledge regarding roles of Ca^{2+} in other pathways including interleukin-1b activation in response to corticosteroid treatment (48) and mechanisms of Ca^{2+} involvement in T-cell receptor (TCR)-mediated apoptosis (49, 50, 51, 52).

But the signaling pathways through which corticosteroids mediate effects on lymphocytes were poorly understood. One reason was that corticosteroid-induced cell death in rat lymph node lymphocytes was found to be independent of extracellular Ca^{2+} uptake (38). Further studies of corticosteroid-induced apoptosis were instrumental in revealing the crucial link between IP_3R-mediated Ca^{2+} release from the ER and apoptosis induction (53, 54, 55, 56).

Now IP_3R-mediated Ca^{2+} elevation is widely recognized as being a ‘double-edged sword’ that on the one hand promotes cell survival and on the other hand induces cell death (57, 58). Whereas physiological Ca^{2+} elevations are generally oscillatory in nature, Ca^{2+} elevations inducing cell death are the result of sustained transfer of high levels of Ca^{2+} from the ER to mitochondria, inducing Ca^{2+} mediated loss of mitochondrial membrane potential, cytochrome *c* release and apoptosis (59, 60, 61). Other mechanisms of Ca^{2+}-mediated cell death include (i) elevation of the pro-apoptotic family member Bim (58); (ii) activation of Ca^{2+}-sensitive proteases and endonucleases; and, activation of calcineurin (CaN), which in turn dephosphorylates and thus activates another pro-apoptotic Bcl-2 family member, Bad. Ca^{2+} mediated cell death mechanisms are extensively summarized in a recent review by two major contributors to understanding how Ca^{2+} fluxes mediate cell death (62, 2).

Because of the delicate balance between its functions in both cell survival and cell death, IP_3R-mediated release of Ca^{2+} from the ER must be carefully balanced. This regulation is produced by a number of factors including kinases and phosphatases that bind to the IP_3R, regulating channel opening and Ca^{2+} release (63, 64, 65, 66, 67, 68, 69, 70). In addition to their function as Ca^{2+} channels, IP_3Rs serve as scaffolds and signal integrators, bringing proteins and protein complexes within close proximity to the ER and mitochondria (71, 72, 1). This is facilitated by the large tetrameric IP_3R structure located on the cytoplasmic side of the ER (73, 67, 66, 72). The role of these regulatory mechanisms is particularly important in pathways involving phospholipase C activation and IP_3 synthesis (74). These signaling

hubs are important for control of pro-apoptotic and anti-apoptotic Bcl-2 family members, oncogenes and tumor suppressors in regulating cell death and cell survival (75, 76).

3. Prevention of excessive Ca^{2+} elevation and cell death by Bcl-2 during T-cell development

It has been over thirty years since the Bcl-2 protein was discovered and initially characterized (77, 78, 79, 80), twenty-five years since the first indication that Bcl-2 regulates intracellular Ca^{2+} dynamics (81, 82, 83, 84), and fourteen years since an interaction of Bcl-2 with the IP_3R was reported (85). Bcl-2 is a 26 kDa integral membrane protein that normally resides on the outer mitochondrial membrane and endoplasmic reticulum (ER). It is anchored on these membranes by a C-terminal hydrophobic region and is mainly cytoplasmic in its location. Bcl-2 elicited widespread interest when it was found to promote cell survival by inhibiting apoptosis (86). From a functional standpoint, members of the Bcl-2 protein family generally fall into two opposing groups: anti-apoptotic proteins and pro-apoptotic proteins. Anti-apoptotic members such as Bcl-2 typically have four Bcl-2 homology (BH) domains (BH1–4). Pro-apoptotic members fall into two groups: those with three BH domains (BH1–3) and those with only a BH3 domain, the 'BH3-only proteins'. These distinctions are useful from an operational standpoint but are undergoing revision and clarification based on recent findings (87).

One of the most remarkable features of Bcl-2 is its lack of any obvious inherent function. Sequence analysis does not reveal any recognizable functional domains. Without any inherent activity of its own, enzymatic or otherwise, Bcl-2 and its anti-apoptotic relatives nevertheless exert widespread influence over various cell functions, ultimately influencing cell survival. Bcl-2 accomplishes this through its well documented interactions with other proteins, and through its localization on the outer mitochondrial membrane and the ER.

It is appealing to study the function of Bcl-2 in a cell type where this protein normally plays an important functional role as opposed to cancer cells where Bcl-2 levels are aberrantly expressed and elevated at abnormally high levels. For this reason, many investigators focused on the role of Bcl-2 in regulating IP_3R-mediated Ca^{2+} signals in T-cells, because IP_3R-mediated Ca^{2+} signals following TCR activation are of critical physiological importance in the immune system (88, 89, 90). Numerous studies highlight the importance of Bcl-2 in lymphocyte development and survival (Figure 2). The Bcl-2 knockout mouse, developed in the laboratory of the late Stanley Korsmeyer, demonstrates fulminant lymphoid apoptosis (91). Enforced expression of Bcl-2 in transgenic mice reduces negative selection, causing excessive accumulation of thymocytes (92, 93, 94). Transgenic Bcl-2 inhibits negative selection by a mechanism independent of its ability to antagonize Bax (95, 96). In addition, studies in hematopoietic cells and pre-lymphomatous B-cells suggest that Bcl-2 may regulate intracellular Ca^{2+} dynamics (82, 84), and findings in our laboratory suggest that positive *versus* negative selection decisions in the thymus are partly encoded by distinct Bcl-2-regulated Ca^{2+} signaling patterns (97).

The developing T-cell passes through successive maturational stages within the thymus (98) and Bcl-2 levels vary considerably throughout these different developmental stages (99, 81,

100, 101, 102) (Figure 2). The earliest precursors from the bone marrow or fetal liver do not express either the TCR or the CD4 and CD8 antigens (*i.e.,* "double negative stage"), but do express Bcl-2: this provides an element of protection upon movement to the thymus gland. In the thymus these cells first express the TCR and both CD4 and CD8 antigens (*i.e.,* "double positive stage"). Bcl-2 levels are down-regulated at this stage, rendering the cells very sensitive to Ca^{2+} induced apoptosis (81). This facilitates a stringent test of whether or not the T-cells respond to self-antigens, with strong responders undergoing apoptosis ("negative selection") and weak responders avoiding apoptosis ("positive selection") (103, 104). During negative selection, apoptosis is induced by Ca^{2+}-dependent up-regulation of the pro-apoptotic Bcl-2 family member Bim (90). Positively selected cells advance to the "single positive stage" (CD4+/CD8-, CD4-/CD8+), where Bcl-2 levels are upregulated, and enter the circulation to mount immune responses to foreign antigens.

Antigen binding to the TCR triggers a signaling cascade that activates PLC-γ, which generates IP_3. IP_3 binds to IP_3Rs, inducing channel opening and ER Ca^{2+} release, thus stimulating T cell proliferation (88, 105, 89, 106). Depending on the strength of TCR activation, a variety of Ca^{2+} response patterns are generated, including a transient Ca^{2+} elevation, sustained Ca^{2+} elevation, or Ca^{2+} oscillations (107). Ca^{2+} oscillations are the most important physiologically as they encode information by their frequency, amplitude and shape (108, 109). Consistent with earlier findings by Donnadieu *et al* (110), we find that strong TCR activation by a high concentration of anti-CD3 antibody induces a large transient elevation of Ca^{2+}, whereas weak TCR activation by a low concentration of anti-CD3 antibody induces sustained Ca^{2+} oscillations (97).

Earlier *in vitro* studies demonstrated that TCR activation by a high concentration of anti-CD3 antibody induces thymocyte apoptosis, whereas lower concentrations of anti-CD3 antibody do not trigger apoptosis (40). TCR activation by physiologically-relevant antigenic peptides produce a similar effect (111): negatively-selecting antigenic peptides induce a strong Ca^{2+} flux in immature thymocytes, whereas positively-selecting peptides induce a smaller Ca^{2+} flux. We find that Bcl-2 selectively inhibits the pro-apoptotic high sustained Ca^{2+} elevations induced by strong TCR activation while enhancing the pro-survival Ca^{2+} oscillations induced by weak TCR activation (97). These Ca^{2+} signaling patterns differ in two important ways (97): high anti-CD3 induces a much longer Ca^{2+} elevation than low anti-CD3 (> 4 min versus < 1 min); and high antiCD3 triggers a much higher peak Ca^{2+} amplitude than low anti-CD3. High amplitude Ca^{2+} elevation, particularly if continuous and sustained, triggers cell death (57.

There was a thirty-year lapse between the initial clinical use of corticosteroids in cancer treatment and recognition that Ca^{2+} plays an important role in corticosteroid-induced lymphocyte cell death (37, 38, 39, 40), involving Ca^{2+}-mediated endonuclease activation (39) and apoptotic DNA fragmentation (43, 42)., 58). The critical determinant of whether or not TCR stimulation induces apoptosis appears to lie in both the duration and amplitude of the Ca^{2+} elevation.

The positive effect of Bcl-2 on Ca^{2+} oscillations and its pro-survival effects are consistent with a number of other findings. For example, Ca^{2+} oscillations regulate thymocyte motility

during positive selection, thereby modulating interactions with stromal cells (112). Ca^{2+} oscillations also lead to a sustained activation of CaN (104), which dephosphorylates and thereby activates nuclear factor of activated T-cells (NFAT). The continuous NFAT activation prevents NFAT nuclear dephosphorylation, allowing NFAT to remain in the nucleus and induce interleukin-2 production (107, 105, 113).

4. Bcl-2 promotion of normal cell survival through its regulation of IP_3R-mediatd Ca^{2+} release.

Studies in which Bcl-2 was selectively targeted to the ER demonstrate that ER-localized Bcl-2 inhibits apoptosis (114, 115, 116). The anti-apoptotic activity of ER-localized Bcl-2 derives both from its binding to pro-apoptotic BH3-only proteins (*e.g.,* Bim) (116) and to its interaction with IP_3Rs to prevent excessive IP_3R-mediated Ca^{2+} elevation. The concept that Bcl-2 regulates IP_3R-mediated Ca^{2+} elevation evolved from evidence that Bcl-2 represses apoptosis by regulating ER-associated Ca^{2+} fluxes (83), ultimately leading to the discovery of an interaction between Bcl-2 and the IP_3R (85), an interaction mediated by binding of the BH4 domain of Bcl-2 to a region located within the regulatory and coupling domain of the IP_3R (117, 118, 119). Interactions between IP_3Rs and other anti-apoptotic Bcl-2 family members, Bcl-xl and Mcl-2 are also reported (120, 121, 122, 123). The reader is referred to an excellent review discussing the full complexity of various Bcl-2 family member interactions with IP_3Rs and their impact on cell survival and cell death (124). Moreover, Vervliet et al (125) discovered that Bcl-2 also binds to ryanodine receptors, dampening Ca^{2+} release from these intracellular channels.

Recent studies indicate that the BH4 domain of Bcl-2 is highly conserved in different classes of vertebrates and can act as a binding partner and inhibitor of IP_3R channels (126). This same region is responsible for Bcl-2's interaction with ryanodine receptors (125). In addition, a region in the BH4 domain of Bcl-2 (Ile14, Val15) has been found critical to stability and function as an inhibitor of Ca^{2+}-mediated apoptosis (127). Furthermore, the significance of this region is further evidenced by findings indicating that the alpha helical nature of Ile14, Val15 region is essential to the function of the BH4 domain in inhibiting IP_3R-mediated Ca^{2+} release (128).

Early analysis of Bcl-2-IP_3R interaction focused on what appeared to be a single interaction site involving BH4 domain interaction with IP_3R domain 3, located in the regulatory and coupling region (Figure 3). Recent studies have expanded understanding of the Bcl-2 interaction to include a region in its C-terminal domain (C-term Dom, a.a. 2512–2749), which is in close proximity of the channel pore. This region was previously identified as critical for the Ca^{2+} regulatory functions of the Bcl-2 homologue Bcl-xl (129). This is particularly enlightening as earlier studies using synthetic peptides targeting the BH4 domain of Bcl-2 (BH4-Bcl-2), revealed this domain is necessary and sufficient to bind to the IP_3R and to suppress its activity (117, 118, 119). Nevertheless, the relatively low affinity of inhibition by the BH4 domain (measured *in vitro* IC50=30μM) (118, Monaco, 2012 #5128) does not appear to explain the potent inhibitory effect of Bcl-2 full-length protein under physiological conditions. Using genetic and pharmacological approaches, Ivanova *et al* (22)

implicate the C-terminal IP_3R1 domain in Bcl-2 binding and cell death regulation. Furthermore, they demonstrated a direct interaction between a peptide corresponding to the transmembrane domain of Bcl-2 (TMD-Bcl-2) and the purified C-terminal fragment of IP_3R1. This peptide was able to suppress IP_3-induced Ca^{2+} release (IICR) when applied at high concentrations. These results suggest that the C-terminal region of Bcl-2 not only serves as an anchor for tethering Bcl-2 to membranes, but also an important functional regulator of IP_3R activity.

A recent report also indicates that Bcl-2 may not interact with IP_3Rs across all circumstances or cell types (130), raising the important question of what regulates the Bcl-2-IP_3R interaction in different types of cells. Also, it has been suggested that Bcl-2 may also regulate ER Ca^{2+} release through additional mechanisms besides its interaction with the IP_3R. One proposed mechanism involves Bcl-2 interaction with Sarco/Endoplasmic Reticulum-associated Ca^{2+}-ATPases (SERCA). These proteins pump Ca^{2+} ions from the cytoplasm into the ER lumen, maintaining large ER luminal Ca^{2+} stores. This steep Ca^{2+} concentration gradient from ER lumen to cytoplasm propels Ca^{2+} movement upon IP_3R channel opening, leading to pro-apoptotic calcium spikes. Bcl-2's interaction with SERCA attenuates ER Ca^{2+} filling, indirectly diminishing IP_3R-mediated Ca^{2+} release and Ca^{2+}-mediated apoptosis (131). Interestingly, Bcl-2 alone completely inhibits SERCA *in vitro*, which can trigger apoptosis by increasing cytosolic Ca^{2+} and inducing store-operated Ca^{2+} entry (132). Recent findings indicate that HSP70 regulates the Bcl-2-SERCA interaction, maintaining SERCA in an active state that may be essential for apoptosis regulation (132). Accordingly, an earlier report of the Bcl-2-SERCA interaction finds that Bcl-2 increases the ER Ca^{2+} pool, promoting the high luminal Ca^{2+} concentration required for normal cell function (133).

Recent work provides insight into how Bcl-2-IP_3R interaction controls IP_3R-mediated Ca^{2+} elevation, preventing Ca^{2+}-induced cell death. Oakes *et al* (121) show that Bcl-2 regulates IP_3R phosphorylation at serine 1755 within the regulatory and coupling domain of the IP_3R. Protein kinase A (PKA) phosphorylates serine 1755 and serine 1589, increasing IP_3-mediated channel opening and Ca^{2+} release (134, 135, 136). We previously reported that Bcl-2 decreases IP_3R phosphorylation, although a specific phosphorylation site was not identified (85). In further work, we find that Bcl-2 inhibits IP_3R phosphorylation at serine 1755, correlating with its inhibition of anti-CD3-induced Ca^{2+} elevation.

PKA-mediated protein phosphorylation is typically regulated by PP1α (137), and an IP_3R-PP1α complex has been implicated in Bcl-2-mediated suppression of ER Ca^{2+} release in breast cancer cells (138). Bcl-2 also binds CaN (139) and increases the association of CaN with IP_3Rs (140, 141, 120); this has a neuroprotective effect in primary neuronal cells (141). However, Bultynck *et al* predicted that CaN's IP_3R effects are indirect and may be secondary to PP1α acting with DARPP-32 (dopamine- and c-AMP-regulated phosphoprotein of 32 kDa) (142). DARPP-32 is a PKA-activated and CaN-deactivated PP1α inhibitor studied extensively in the brain (143). Tang *et al* (137) discovered a direct association between PP1α and IP_3R-1 and established that the association with PP1α reverses PKA-mediated IP_3R-1 phosphorylation. AKAP9, a multifunctional PKA anchoring protein, docks both PKA and PP1α to IP_3R-1 (144). In experiments with medium spiny

neurons from DARPP-32 knock-out mice, DARPP-32 was shown to regulate dopamine-induced Ca^{2+} oscillations (145). However, very little is known about the role of DARPP-32 in peripheral tissues, including lymphocytes, although DARPP-32 has been shown to increase the phosphorylation and activity of various ion channels (146). We report that Bcl-2 docks DARPP-32 and CaN in a complex on the IP_3R, preventing exaggerated IP_3R-mediated Ca^{2+} elevation in T-cells by decreasing PKA-mediated IP_3R phosphorylation (147).

A very interesting report for the first time has implicated Bcl-2 in regulating store-operated Ca^{2+} channels, involving the BH1 domain of Bcl-2 (148). This work indicates that a triplicate amino acid substitution in the BH1 domain creates a mutant form of Bcl-2 that enhances thapsigargin-induced Ca^{2+} elevation, whereas these investigators find wild type Bcl-2 dampens thapsigargin-induced Ca^{2+} elevation. Their evidence implicates an effect of the mutant Bcl-2 on store-operated Ca^{2+}, producing massive Ca^{2+} influx contributing to caspase activation and apoptosis.

5. Cancer cell exploitation of Bcl-2-IP_3R interaction and the strategy of targeting this interaction to treat cancer.

Bcl-2-expression levels are elevated in many different malignancies (http://broadinstitute.org/ccle/home). Bcl-2 levels are invariably elevated in chronic lymphocytic leukemia (CLL) and follicular lymphoma (FL) (15, 13). Comparable levels of Bcl-2 are present in multiple myeloma (MM) (149). Bcl-2 levels are also elevated in acute myelogenous leukemia (AML) (150) and small cell lung cancer (SCLC) (151), in which novel therapeutic approaches are desperately needed. Cancer cells exploit Bcl-2 to stay alive in the stressful microenvironment and resist immunotherapy, chemotherapy and radiation therapy. Therefore, agents that inhibit Bcl-2 have the potential of dramatically improving outcomes in multiple types of cancer.

In support of targeting Bcl-2 for cancer treatment there is a distinct difference between normal cells and cancer cells in terms of Bcl-2 function. As summarized earlier in this review, Bcl-2 expression levels are carefully regulated in normal cells, such as in T-cells, where Bcl-2 levels increase during the immune response to prevent inadvertent cell death in response to proliferative Ca^{2+} signals, and then decline once the immune response wanes. The difference in Bcl-2 over-expressing cancer cells is that the Bcl-2 level does not decline and remains elevated over time, preventing normal, physiological cell death responses. Thus, Bcl-2 preserves Ca^{2+} homeostasis in normal cells by preventing excessive Ca^{2+} elevation, but cancer cells exploit Bcl-2 to stay alive under adverse growth conditions that would generally lead to cell death.

The Bcl-2 protein is expressed at abnormally high levels in a wide variety of cancers. The classic example of this is follicular lymphoma, where Bcl-2 levels are abnormally elevated by a *t*(14;18) chromosomal translocation (77, 78, 79). Bcl-2 elevation involves other mechanisms in a variety of cancers, and in many types of cancer the mechanism of Bcl-2 elevation may not be recognized (152). For example, Bcl-2 is elevated in CLL due to loss of

microRNAs that normally repress Bcl-2 gene expression (153). Because of its role in preventing cell death, Bcl-2 has become a major therapeutic target in cancer.

Bcl-2 promotes cell survival by two major mechanisms, illustrated in Figure 4. In mitochondria, Bcl-2 preserves cell survival by binding and inhibiting the function of pro-apoptotic proteins, illustrated by Bim in the figure. This function of Bcl-2 is mediated by its hydrophobic cleft, involving the BH3 domain which is responsible for binding pro-apoptotic proteins. On the ER, Bcl-2 promotes survival through its interaction with IP_3Rs, mediated by its BH4 domain, preventing excessive Ca^{2+} elevation.

Small molecules that bind to the hydrophobic cleft formed by the BH1–3 domains of Bcl-2 displace pro-apoptotic proteins from Bcl-2 and thus trigger apoptosis (154, 155, 156, 157, 158, 18). These molecules, including the Bcl-2 selective and platelet-sparing ABT-199 (Venetoclax), are already in clinical use to treat CLL, and undergoing clinical trials to test efficacy in other types of cancer (154, 155, 156, 157, 158, 18, 159, 3). For an in-depth understanding of how Bcl-2 interacts with its pro-apoptotic relatives and preserves outer mitochondrial membrane integrity, the reader is referred to a recent review by Llambi *et a*l (160).

However, these agents are only effective in types of cancer that have elevated levels of pro-apoptotic proteins such as Bim, rendering these cancer cells addicted to Bcl-2 interaction with pro-apoptotic proteins for their survival. Another limitation is that ABT-199/Venetoclax responsiveness varies among cancers (161). For example, CLL is highly responsive to ABT-199, although resistance is reported (162). On the other hand, ABT-199/Venetoclax response rates are 28% in Diffuse Large B-cell Lymphoma (DLBCL) and 31% in Follicular Lymphoma (FL) (163). Although Bcl-2 is commonly expressed in multiple myeloma at levels comparable to CLL and FL (149), responses to ABT-199/Venetoclax are limited to a small subset of myeloma lines (164) and patients with the CCND1/IGH translocation(164, 163). Also, AML is a Bcl-2-positive malignancy, but ABT-199 is effective in only a fraction of AML patients (165, 150). Main reasons for ABT-199/Venetoclax resistance include: (i) low expression levels pro-apoptotic proteins so the cancer cells are not primed to respond to ABT-199/Venetoclax (161); (ii) expression of Mcl-1 or Bcl-xl, which bind and inhibit pro-apoptotic proteins released from Bcl-2 by ABT-199 (166).

In collaboration with Jan Parys and Geert Bultynck in Belgium, we developed a synthetic peptide corresponding to the IP_3R binding site for Bcl-2 (117, 118). This IP_3RDerived Peptide (IDP), more recently termed BIRD2 (Bcl-2 IP_3R Disruptor-2), inhibits the Bcl-2-IP_3R interaction by binding to the BH4 domain of Bcl-2, destabilizing Bcl-2's alpha-helical structure (117, 118, 119). By inhibiting Bcl-2-IP_3R interaction, BIRD2 attenuates Bcl-2's control over IP_3R-mediated Ca^{2+} elevation, induces marked Ca^{2+} elevation and Ca^{2+}-mediated apoptosis in primary human CLL cells, with minimal if any effect on the viability of normal human lymphocytes (19). BIRD-2 also induces apoptosis in diffuse large B-cell lymphoma lines (DLBCL) (119, 167) and in multiple myeloma cells, both *in vitro* and in an *in vivo* xenograft mouse model (20). BIRD-2 also induces apoptosis in small cell lung cancer, a Bcl-2 positive solid tumor (168), and in ovarian cancer (169).

In each of the known ABT-199/Venetoclax resistance mechanisms, agents that induce apoptosis by disrupting Bcl-2-IP_3R interaction are expected to have value. First, these agents may increase the sensitivity of unprimed cancer cells to ABT-199/Venetoclax by increasing Bim expression levels. As proof of principle, we have reported that BIRD-2-induced Ca^{2+} elevation increases Bim levels in CLL and multiple myeloma cells (170, 20). Second, in cells resistant to ABT-199/Venetoclax due to increased expression of Mcl-1 and Bcl-xl, or decreased levels of Bax and Bak, agents that disrupt Bcl-2-IP_3R interaction are still expected to induce Ca^{2+}-mediated apoptosis. This is mainly because Ca^{2+} elevation induces apoptosis by multiple mechanisms not employed by ABT-199, including by activating Ca^{2+}-sensitive proteases (calpains) that trigger caspase-independent apoptosis. As proof of principle, we have demonstrated that BIRD-2 induces this apoptotic mechanism in ABT compound-resistant multiple myeloma and SCLC cells (20, 168). Also, ABT-199 and BIRD-2 demonstrate synergy inducing cell death in cancer cells (20, 168). BIRD-2 synergizes with ABT-199 by inducing Ca^{2+}-mediated elevation of the pro-apoptotic protein Bim (20). Thus, agents that target Bcl-2-IP_3R interaction may not only be useful as single agents, but also be useful in combination with ABT- 199, perhaps facilitating ABT-199/Venetoclax use at lower, less toxic doses. Also, ABT199-/Venetoclax does not inhibit Bcl-2-IP_3R interaction and does not trigger Ca^{2+} elevation, confirming that ABT-199 and BIRD-2 work by separate mechanisms (171, 22). Moreover, ABT199-/Venetoclax and BIRD-2 demonstrate a reciprocal relationship in terms of their ability to induce cell death (20, 172), confirming they work by independent mechanisms.

The utility of targeting the Bcl-2-IP_3R interaction may be dependent upon a number of factors. Perhaps the most obvious of these factors is the level of Bcl-2 in different types of cancer cells and their reliance on Bcl-2 for survival (173). Although Bcl-2 is typically overexpressed in lymphoid malignancies, other Bcl-2 family members are expressed in non-lymphoid malignancies, including Mcl-1. Individual pro-apoptotic family members may also differ in their interaction with IP_3Rs. For example, the BH4 domain of Bcl-2 interacts with IP_3Rs, but the BH4 domain of Bcl-xL does not (119). As such, BIRD-2 is likely to be more effective in Bcl-2 positive malignancies than in Bcl-xl positive malignancies. Additionally, the anti-apoptotic Bcl-2 family member Mcl-1 contributes to apoptosis inhibition in lymphoid malignancies and is reported to interact with IP_3Rs (129); we do not know where Mcl-1 interacts on the IP_3R and if it would be inhibited by BIRD-2. Another factor is the IP_3R isoform itself. There are three IP_3R isoforms, which vary in both tissue distribution and in sensitivity to Ca^{2+} and IP_3 regulation (174). A recent study discovered that the sensitivity of lymphoma cells to BIRD-2-induced apoptosis correlated best with IP_3R isoform 2 (167), whereas numerous studies in other cell types demonstrated a stronger correlation with IP_3R isoform 3 (54).

In B-cell malignancies like CLL (Figure 1), constitutive B-cell receptor signaling drives cell proliferation and survival *via* Bruton's tyrosine kinase (BTK) (175, 176, 177). The immediate downstream target of BTK is phospholipase Cγ. (PLCγ), which catalyses the synthesis of IP_3 from PIP_2, provoking IP_3R channel opening and Ca^{2+} release (17, 177). Recent findings indicate that BIRD-2-triggered Ca^{2+} rises and cell death are critically dependent on the increase in basal IP_3 signaling that occurs downstream of the B-cell receptor in B-cell malignancies (178). As such, inhibition of PLC activity or buffering IP_3

reduces BIRD-2-induced cell death in a variety of DLBCL and primary CLL cells, both in unsupported short-term cultures and in prolonged co-cultures with CD40L-expressing fibroblasts. Therefore, we postulate that the pro-apoptotic Ca^{2+} elevation we observe in BIRD-2-treated CLL cells is driven by constitutive signaling *via* BTK. Moreover, we find that BIRD-2 induces cell death in Ibrutinib-resistant cells, suggesting it may have therapeutic value in patients who relapse while taking ibrutinib (20).

Recently, a small molecule antagonist to the BH4 domain of Bcl-2, BDA-366, has been developed and demonstrated to have activity in lung cancer and multiple myeloma models (21, 179). BDA-366 binds the BH4 region of Bcl-2 with high affinity and selectivity. This development is very interesting as it suggests a different mode of action than BIRD-2, even though like BIRD-2 it was reported to elevate cytoplasmic Ca^{2+} levels. The proposed mechanism of action is that BDA-366 induces a conformational change in Bcl-2 that abrogates its anti-apoptotic function by converting Bcl-2 from a survival molecule to a cell death inducer. The authors find that BDA-366 suppresses growth of lung cancer xenografts derived from cell lines and patients without significant normal tissue toxicity at the effective doses (21, 179). Although the findings of this report are most intriguing, BDA-366 is toxic to a wide range of cell types, apparently regardless of Bcl-2 expression level, raising the possibility that Bcl-2 may not be the only target.

6. Summary and Future Directions

An inherent weakness in virtually any review is the potential exclusion of topics as important or even more important than the one covered. This certainly is the case with regard to the role of mitochondria in Ca^{2+} function, including metabolism and both cell survival and cell death. Indeed, one of the most important functions of IP_3R-mediated Ca^{2+} signaling is in promoting cell survival by increasing mitochondrial Ca^{2+} uptake and metabolism. The close proximity of ER-localized IP_3Rs to mitochondria facilitates Ca^{2+} transfer from the ER lumen into mitochondria (180, 181, 182). This promotes mitochondrial ATP production by activating multiple Ca^{2+}-sensitive enzymes in the citric acid cycle and catalyzing the conversion of pyruvate to acetyl-CoA (183, 184). Insufficient ER-mitochondrial Ca^{2+} transfer results in autophagy, a survival mechanism through which cells digest intracellular components in order to produce ATP (185); conversely, excessive transfer of Ca^{2+} to mitochondria induces Ca^{2+} overload, resulting in loss of membrane potential, cytochrome *c* release and apoptosis (59, 60, 61).

Particularly exciting, novel concepts have been introduced in the field of Ca^{2+} signaling, deserving careful attention as these concepts are likely to guide future directions of research. One of these relates directly to cancer metabolism and is based on the important discovery that mitochondrial bioenergetics is positively regulated by constitutive, low level IP_3R Ca^{2+} transfer from the ER to mitochondria (185, 186, 7). Another concept has to do with how we view anti-apoptotic proteins such as Bcl-2. The discerning reader with surely note that in this review we focus in a singular manner on the Bcl-2 protein, in isolation with only brief mention of other anti-apoptotic family members. A recent paper by Carrington et al (187) has analyzed the requirements of multiple immune cell subsets (*e.g.,* naïve T cells require Bcl-2, regulatory T cells require Mcl-1), supporting a novel model in which survival is

determined by participation of multiple anti-apoptotic proteins rather than a single anti-apoptotic protein. It will be important to determine if a similar model fits cancer cells. For example, does survival of a cancer cell depend just on Bcl-2, or does it depend on the participation of multiple anti-apoptotic family members. This may be important in terms of efforts to target Bcl-2 for cancer treatment. Perhaps it does and multiple anti-apoptotic proteins may need to be targeted to achieve optimal effect.

While the current review is focused on Bcl-2's interaction with IP_3R's and its regulation of intracellular Ca^{2+} signaling, there are many other important dimensions of Bcl-2 protein family function, including its phosphorylation. Particularly interesting is the widespread distribution of Bcl-2 family members, beyond the ER and mitochondria. As recently reviewed elsewhere (188), these include the Golgi apparatus, nucleus and peroxisomes. The consequences broaden the Bcl-2 protein family impact to include not only Ca^{2+} homeostasis, but also cell cycle control and cell migration, topics of great relevance to the understanding and treatment of cancer.

Finally, the work summarized in the present review may provide a modest lesson for those interested in development of novel cancer treatments. Many in the current era seem to believe that understanding basic mechanism is an absolute pre-requisite for novel therapeutic development. It is therefore humbling to realize that the mechanism of corticosteroid-induced cell death is only partially understood, yet this hormone has produced many cancer cures over the past fifty years and still contributes to cures today.

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Highlights

- Bcl-2 is an anti-apoptotic protein elevated in many different types of cancer.
- Bcl-2 interacts with inositol 1,4,5-trisphosphate receptors, regulating calcium release from the endoplasmic reticulum and inhibiting apoptosis.
- Bcl-2 interaction with inositol 1,4,5-trisphosphate receptors and control over calcium release can be blocked using synthetic peptides.
- Blocking Bcl-2 interaction with inositol 1,4,5-trisphosphate receptors is proposed as a novel therapeutic target for Bcl-2-positive malignancies.

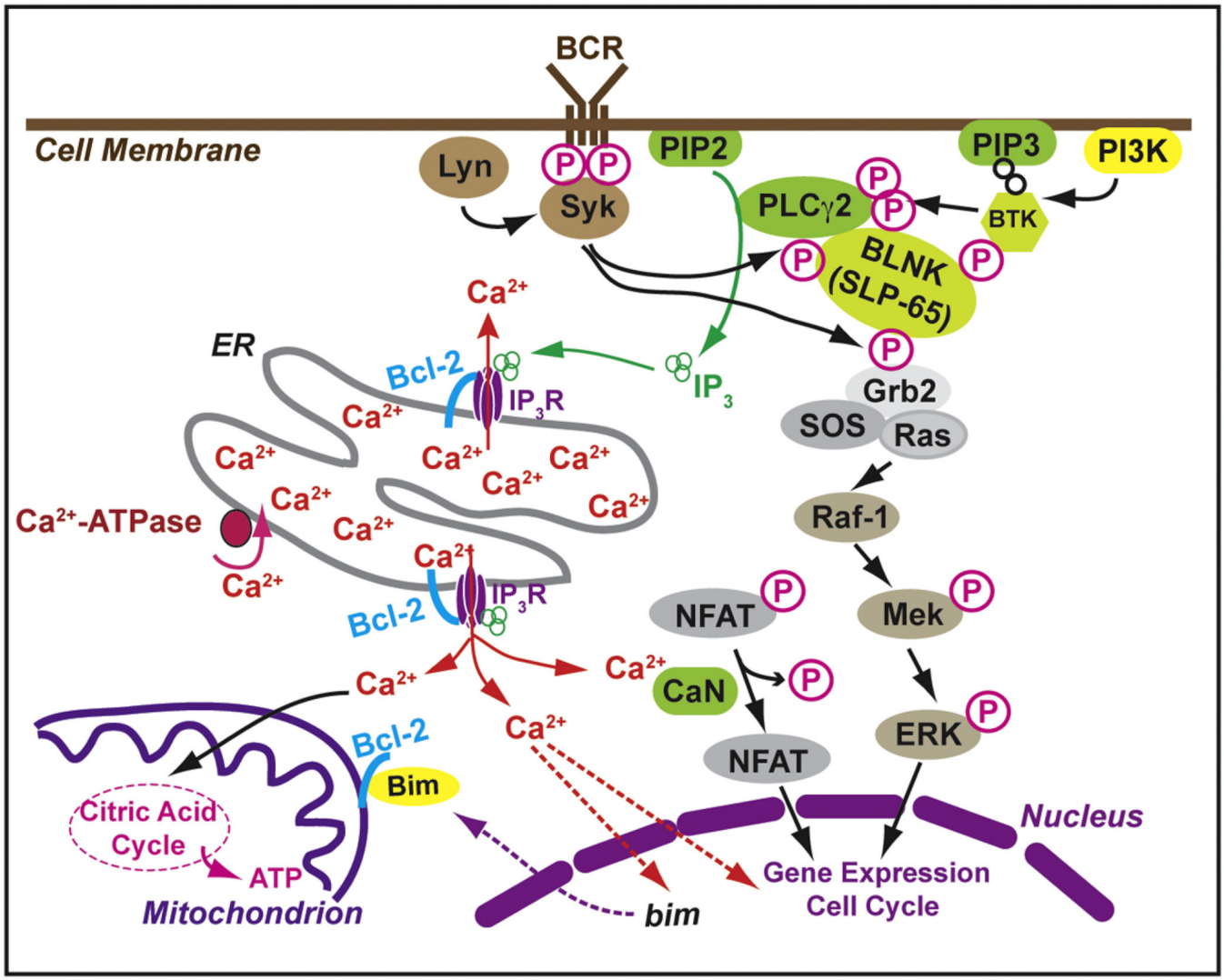

Figure 1. B-cell receptor (BCR) signaling pathways important to the pathophysiology and treatment options for chronic lymphocytic leukemia (CLL).

A critical step in BCR signaling is Bruton's tyrosine kinase (BTK) which feeds forward into IP_3 receptor-mediated Ca^{2+} release from the ER (left side of figure) and through the Ras-Raf-Mek-Erk pathway (right side of figure). The Bcl-2 protein is located on both the ER and mitochondria where it regulates Ca^{2+} signals important in generating a variety of light and death decisions.

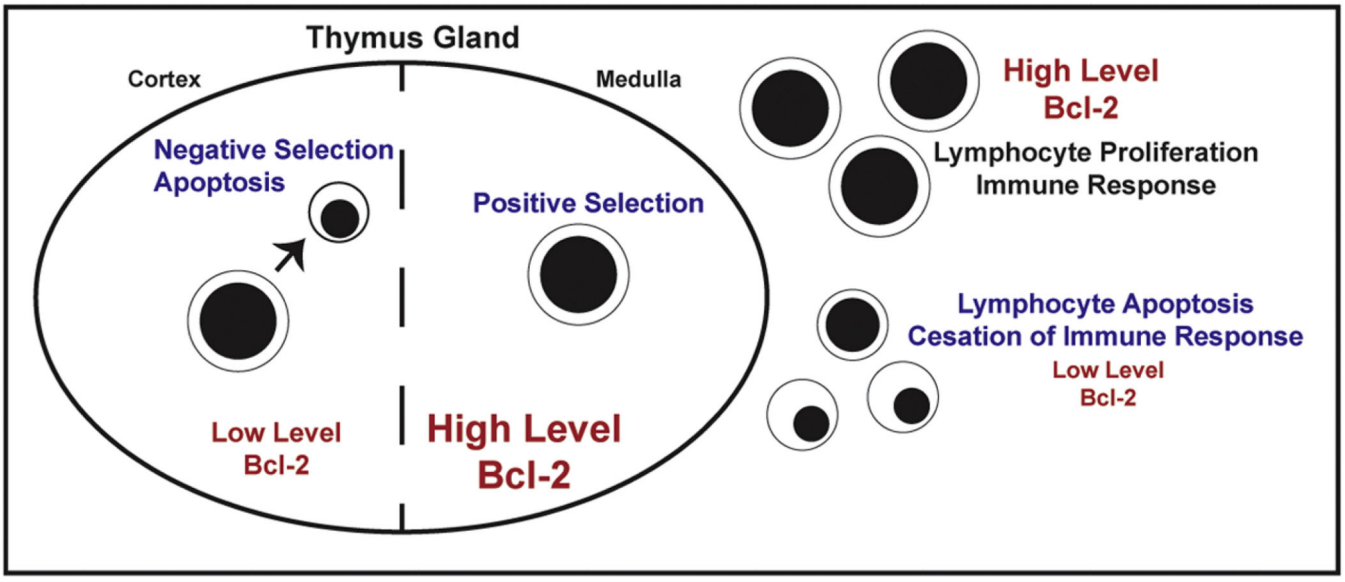

Figure 2. Bcl-2 and T-cell responses in the immune response.

T-cell levels of Bcl-2 vary during T-cell development, allowing for negative selection in the thymic cortex and facilitating positive selection in the thymic medulla. High levels of Bcl-2 insure cell survival during the immune response, then decline as apoptosis allows the immune response to decline.

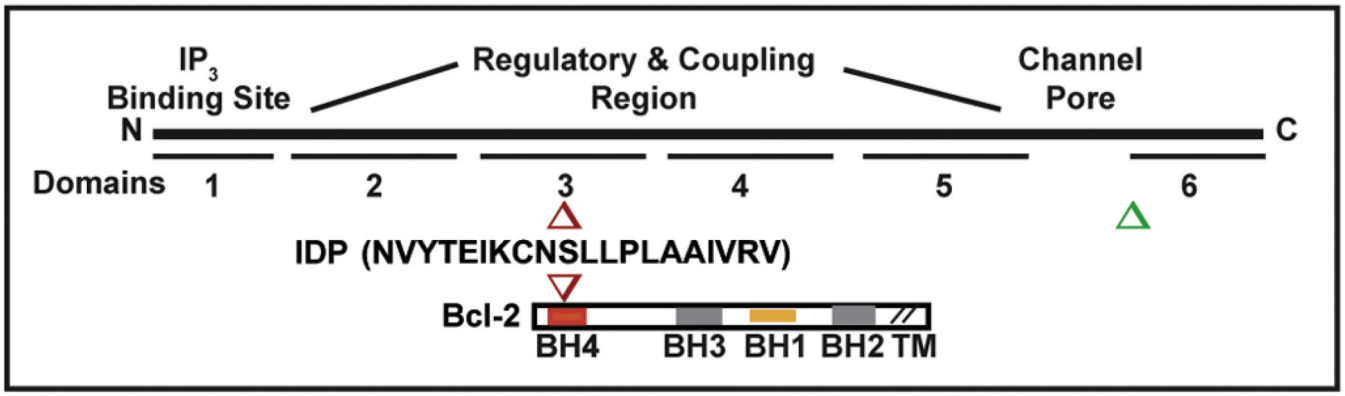

Figure 3. Bcl-2-IP$_3$R interacting sites and derivation of BIRD-2.

The BH4 domain of Bcl-2 binds to IP$_3$R domain 3 (red) and a region near domain 6 (green). BIRD-2 is a 20 amino acid synthetic peptide based on a coiled coil region in IP$_3$R domain 3, with a DD/AA mutation introduced to block a protease cleavage site. BIRD-2 binds to the BH4 domain of Bcl-2 and functions as a decoy peptide, inhibiting Bcl-2-IP$_3$R interaction and inducing cell death in Bcl-2-positive malignancies.

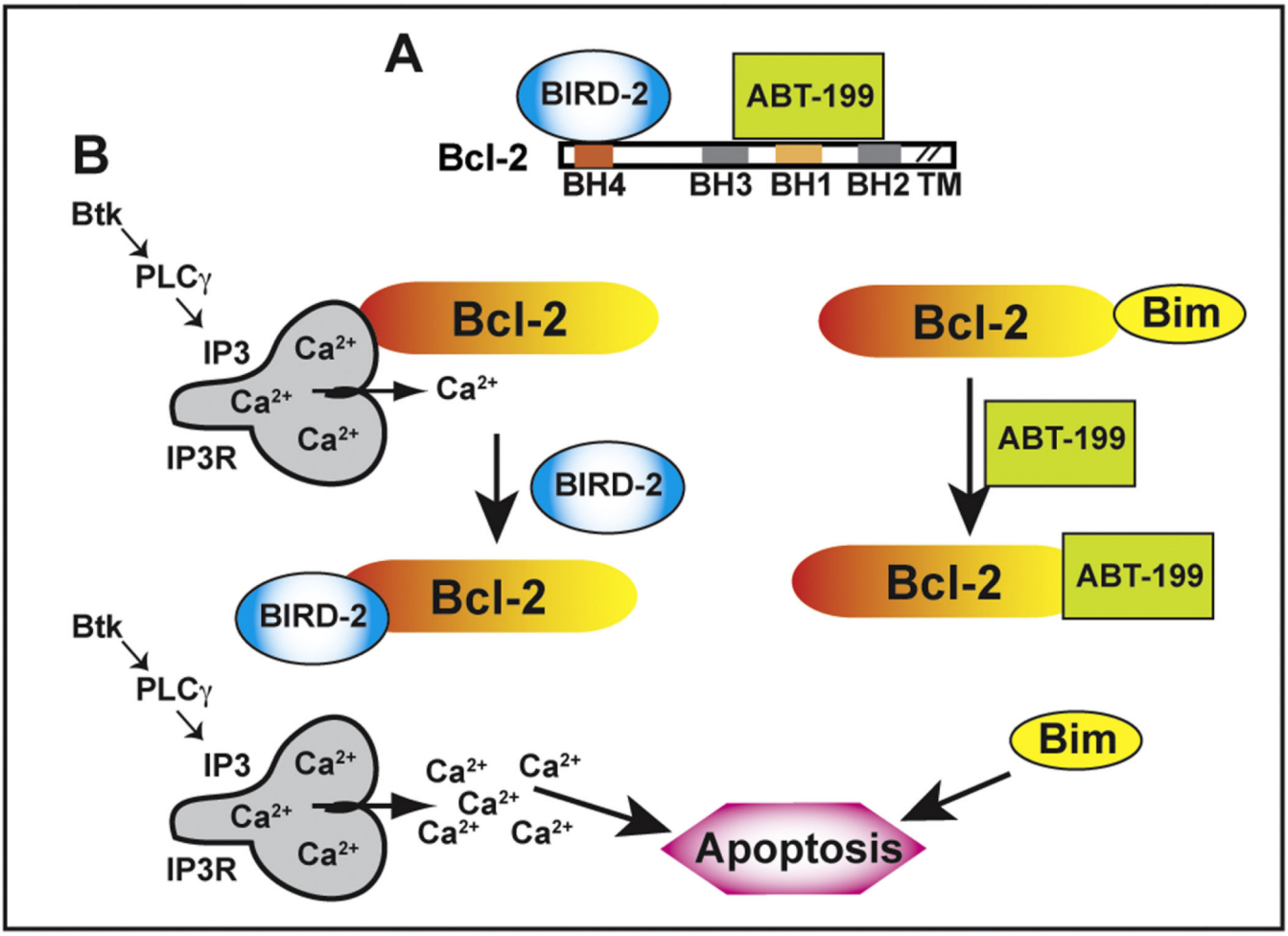

Figure 4. Targeting Bcl-2's dual anti-apoptotic mechanisms.

(A) The decoy peptide BIRD-2 binds to the BH4 domain of Bcl-2, whereas the small molecule ABT-199/Venetoclax binds to the BH3 domain of Bcl-2. (B) *Left:* Bcl-2 binds *via* its BH4 domain to IP_3Rs, preventing excessive IP_3R-mediated Ca^{2+} elevation, thereby inhibiting Ca^{2+} induced apoptosis. BIRD-2 binds to the BH4 domain of Bcl-2, disrupting Bcl-2-IP_3R interaction and thus inducing high amplitude Ca^{2+} elevation that triggers apoptosis. *Right:* The BH3 region of Bcl-2 binds and sequesters the pro-apoptotic protein Bim, preventing Bim-mediated apoptosis. ABT-199 (Venetoclax) displaces Bim, thus triggering Bim-mediated apoptosis.