Creating a New Cancer Therapeutic Agent by Targeting the Interaction between Bcl-2 and IP₃ Receptors

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Bcl-2 is a member of a family of proteins that regulate cell survival. Expression of Bcl-2 is aberrantly elevated in many types of cancer. Within cells of the immune system, Bcl-2 has a physiological role in regulating immune responses. However, in cancers arising from cells of the immune system Bcl-2 promotes cell survival and proliferation. This review summarizes discoveries over the past 30 years that have elucidated Bcl-2's role in the normal immune system, including its actions in regulating calcium (Ca²⁺) signals necessary for the immune response, and for Ca²⁺-mediated apoptosis at the end of an immune response. How Bcl-2 modulates the release of Ca²⁺ from intracellular stores via inositol 1,4,5-trisphosphate receptors (IP₃R) is discussed, and in particular, the role of Bcl-2/IP₃R interactions in promoting the survival of cancer cells by preventing Ca²⁺-mediated cell death. The development and usage of a peptide, referred to as TAT-Pep8, or more recently, BIRD-2, that induces death of cancer cells by inhibiting Bcl-2's control over IP₃R-mediated Ca²⁺ elevation is discussed. Studies aimed at discovering a small molecule that mimics BIRD-2's anticancer mechanism of action are summarized, along with the prospect of such a compound becoming a novel therapeutic option for cancer.

A principal focus of this review is on Bcl-2's physical interaction with, and regulation of, inositol 1,4,5-trisphosphate receptors (IP₃R), and the prospect of targeting this interaction for cancer treatment. IP₃Rs are Ca²⁺ channels located on intracellular organelles including the endoplasmic reticulum (ER) (Mak and Foskett 2015; Prole and Taylor 2016). Numerous extrinsic stimuli can lead to activation of the phosphoinositide signaling pathway, production of the intracellular messenger IP₃, and the consequent

activation of IP₃Rs (Berridge 2016). Bcl-2 (anti-apoptotic B cell lymphoma 2) is a major therapeutic target because its expression is elevated in many types of cancers, in which it contributes to malignant cell survival and chemotherapy resistance (Nougarède et al. 2018). Malignancies associated with elevated Bcl-2 expression include T cell and B cell acute lymphoblastic leukemia, acute myeloid leukemia (AML), chronic myelogenous leukemia, diffuse large B cell lymphoma, Burkitt lymphoma, Hodgkin lympho-

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ma, multiple myeloma, neuroblastoma, melanoma, meningioma, Ewing's sarcoma, and small-cell lung cancer (see broadinstitute.org/ccle/home).

Currently, the only approved anti-Bcl-2 drug for use in cancer treatment is ABT-199/Venetoclax (Place et al. 2018), and this approval is limited to chronic lymphocytic leukemia (CLL) with some additional approvals expected to come (Fig. 1). We posit that developing an additional therapeutic agent, namely, one directed at the Bcl-2/IP₃R interaction, will be a considerable advance because it would work by a totally different mechanism than that of ABT-199/Venetoclax, and may therefore be effective in malignancies already found to be resistant to ABT-199/Venetoclax.

The mechanisms leading to an increase in Bcl-2 expression are known for some cancer cell types. The classic example is follicular lymphoma (FL), in which Bcl-2 levels are abnormally high because of a chromosomal translocation,

t(14;18) (Tsujimoto et al. 1984; Bakshi et al. 1985; Cleary et al. 1986). A variety of mechanisms account for Bcl-2 elevation in other malignancies (Yip and Reed 2008). For example, Bcl-2 elevation in CLL, one of the most common malignancies associated with Bcl-2 elevation, is because of a loss of microRNAs that normally repress Bcl-2 gene expression (Cimmino et al. 2005).

Whatever mechanism is involved in an elevated expression of Bcl-2, numerous studies have shown that part of the ensuing increased cell survival arises because of a modulation of cellular Ca²⁺ signaling by Bcl-2 (Distelhorst and Bootman 2011; Greenberg et al. 2014; Vervliet et al. 2016; Distelhorst 2018). This review proceeds in a chronological manner through the elucidation of Bcl-2/IP₃R interaction, the modulation of Ca²⁺ signaling by Bcl-2, and ends with a discussion of the current development of small molecules that can modify the Bcl-2/IP₃R interaction and thereby possess therapeutic potential for the treatment of cancer.

Figure 1. Targeting Bcl-2's dual antiapoptotic mechanisms. (A) BIRD-2 binds to the BH4 domain of Bcl-2. ABT-199 binds to the hydrophobic domain of Bcl-2 located in BH domains 1–3. (B) (Left) By binding to Bcl-2's BH4 domain, BIRD-2 inhibits Bcl-2–IP₃R interaction, inducing cytoplasmic Ca²⁺ elevation and apoptosis. (Right) By binding to Bcl-2's hydrophobic groove, ABT-199 displaces proapoptotic proteins, such as Bim, triggering apoptosis. But Bcl-2 relatives such as Mcl-1 can sequester Bim, and thereby be responsible for ABT-199 resistance.

THE Bcl-2 PROTEIN FAMILY

Bcl-2 is the founding member of a protein family involved in maintaining cellular homeostasis. Bcl-2 family members can play opposing roles; on the one hand, some family members promote cell survival, whereas others can trigger apoptotic cell death. Thus, family members are generally branded either antiapoptotic (e.g., Bcl-2, Bcl-xl, Mcl-1) or proapoptotic (e.g., Bim, Bax). Within cells, antiapoptotic Bcl-2 family members bind to their proapoptotic relatives, and thereby prevent activation of cell death. If there is an increased expression of proapoptotic Bcl-2 family members, or if these proteins escape from the control of their antiapoptotic counterparts, they can then trigger cell death. For example, the proapoptotic protein Bim can be sequestered, and neutralized, by the antiapoptotic Bcl-2 protein. Release of Bim from Bcl-2, or overcoming the ability of Bcl-2 to buffer cellular levels of Bim, leads to the interaction of Bim with the proapoptotic protein Bax, which consequently causes the release of cell death-inducing factors from mitochondria. The expression level, or activity, of these functional classes of Bcl-2 family proteins is critical in determining the balance between cell survival and cell death.

The Bcl-2 protein was discovered more than 30 years ago (Tsujimoto et al. 1984; Bakshi et al. 1985; Cleary et al. 1986; Tsujimoto and Croce 1986). Soon thereafter, Bcl-2 was found to regulate cell survival by inhibiting apoptotic cell death (Vaux et al. 1988). Bcl-2 is a relatively small (26 kDa) integral membrane protein residing on the ER and outer mitochondrial membrane (Youle and Strasser 2008; Chipuk et al. 2010). It is anchored on these membranes by a carboxy-terminal transmembrane domain and is mainly cytoplasmic in its location. Antiapoptotic Bcl-2 family members, such as Bcl-2 itself, typically have four Bcl-2 homology (BH) domains (BH1-4). Proapoptotic Bcl-2 family members fall into two groups: those with three BH domains (BH1-3; the “multidomain proapoptotic Bcl-2 proteins”), and those with only a BH3 domain (the “BH3-only Bcl-2 proteins”). These distinctions are useful from an operational standpoint, although they undergo revision and

clarification over time (Chipuk et al. 2010; Aouacheria et al. 2013).

Bcl-2 AND THE IMMUNE SYSTEM

Bcl-2 is widely expressed in tissues and may play other roles in addition to promoting cell survival, such as altering mitochondrial morphology and energetics (Gross 2016). Within cells of the immune system, Bcl-2 is known for its role in regulating immune responses and self-tolerance. Bcl-2 levels fluctuate widely during T cell development, and during an immune response (Hockenberry et al. 1991; Andjelic et al. 1993; Gratiot-Deans et al. 1993; Veis et al. 1993a; Linette et al. 1994). The Bcl-2 knockout mouse, developed in the laboratory of the late Stanley Korsmeyer, revealed fulminant apoptosis in lymphocytes (Veis et al. 1993b). Enforced expression of Bcl-2 in transgenic mice, on the other hand, reduced negative selection, causing excessive accumulation of thymocytes (Strasser et al. 1991, 1994; Siegel et al. 1992). Bcl-2 levels are low during the process of negative selection in the thymus gland (Hogquist 2001; Neilson et al. 2004). In cells undergoing positive selection, Bcl-2 levels increase, protecting cells from apoptosis during antigenic responses. Then, when the immune response wanes, Bcl-2 levels decline, permitting apoptosis to ensue. During negative selection, low levels of Bcl-2 permit Ca²⁺-dependent up-regulation of Bim, thereby triggering apoptosis (Cante-Barrett et al. 2006). This observation is critically important for the later discussion of the role of Bcl-2 in cancer cells; Bim is a mediator of cell death, and elevated levels of Bcl-2 promote cell survival by sequestering Bim and repressing Bim expression.

Bcl-2's ROLE ON THE ER

As mentioned above, Bcl-2 is located on both the outer mitochondrial membrane and the ER. Because of the central role of mitochondria in cell death processes, interest in the prosurvival action of Bcl-2 generally focused on these organelles and how Bcl-2 supports their function and integrity to prevent Bax-mediated apoptosis. That is, until David Andrews's laboratory stud-

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ied the effects of selectively targeting Bcl-2 to either the mitochondria or the ER (Zhu et al. 1996). These experiments revealed differences among cell types in terms of the efficacy of mitochondria-targeted Bcl-2 versus ER-targeted Bcl-2 in inhibiting Bax-mediated apoptosis. Using similar methods to selectively localize Bcl-2 on the mitochondria or the ER, the Distelhorst laboratory also found that ER-targeted Bcl-2 was effective at inhibiting Bax-mediated apoptosis (Wang et al. 2001; Thomenius et al. 2003). These studies were insightful because the prosurvival action of ER-targeted Bcl-2 raised the possibility of Bcl-2 having functions that were discrete from preventing activation of Bax on mitochondrial membranes. Indeed, these data suggested that Bcl-2 had important actions when localized to the ER membrane.

Numerous studies have shown that Bcl-2 plays a central role in preserving Ca^{2+} homeostasis and preventing Ca^{2+} -mediated apoptosis (Zhang et al. 2001; Pinton et al. 2002; Hanson et al. 2008b; Rong and Distelhorst 2008; Vervloessem et al. 2018). The location of Bcl-2 on the ER membrane places it in close proximity to pumps and channels that are responsible for Ca^{2+} sequestration and the generation of cytosolic Ca^{2+} signals. The ER is the major constitutive Ca^{2+} store in most cell types, and is a source of Ca^{2+} for important signaling events such as gene transcription. Various extrinsic stimuli can trigger the release of Ca^{2+} from IP_3R channels located on the ER membrane, thereby causing an increase in cytosolic Ca^{2+} concentration. Typically, stimulation of cells leads to repetitive cytosolic Ca^{2+} oscillations: rapid, transient, increases in cytosolic Ca^{2+} concentration that are sensed by Ca^{2+} -binding proteins and lead to activation of cellular effectors (Berridge 2009). The diffusion of Ca^{2+} ions within a cell during each Ca^{2+} oscillation delivers information throughout the cytoplasm, and also into mitochondria and the nucleus, thus regulating a wide variety of cellular processes, including cell survival and cell death (Berridge 1997; Lewis 2003; Gallo et al. 2006; Fracchia et al. 2013).

A number of observations were critical in linking Bcl-2 that was localized on the ER to regulation of Ca^{2+} signaling and homeostasis:

1. A flux of Ca^{2+} from the ER lumen into the cytoplasm accompanied cell death induction in glucocorticosteroid hormone-treated lymphoma cell lines (McConkey et al. 1989; Lam et al. 1992, 1993).
2. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line was associated with repartitioning of intracellular Ca^{2+} between organelles, and was blocked by enforced Bcl-2 expression (Baffy et al. 1993).
3. In thymocytes, sensitivity to Ca^{2+} -mediated negative selection was associated with, and enabled by, Bcl-2 down-regulation (Andjelic et al. 1993).
4. Bcl-2 overexpression inhibited apoptosis by reducing Ca^{2+} release from the ER following glucocorticosteroid treatment of a T cell leukemia cell line (Lam et al. 1994; Sobecks et al. 1996; He et al. 1997).
5. Whereas, induction of apoptosis mediated by N-myc required normal Ca^{2+} levels, apoptosis prevention conferred by Bcl-2 correlated with the inhibition of intracellular Ca^{2+} fluxes. This led to the hypothesis that regulation of intracellular Ca^{2+} concentration is an important aspect of the oncogenic cooperation between Bcl-2 and N-myc (Zornig et al. 1995).

Bcl-2/ IP_3R INTERACTION

Novel insight into how Bcl-2 regulates Ca^{2+} homeostasis and Ca^{2+} signaling came from work initiated by the Distelhorst laboratory, some of which was performed during fruitful collaborations with others. Distelhorst and colleagues described, for the first time, a physical interaction between Bcl-2 and IP_3Rs that repressed anti-CD3-mediated Ca^{2+} release from the ER in T cells (Fig. 1; Chen et al. 2004; Hanson et al. 2004, 2008a). These initial findings have been extended to other members of the Bcl-2 protein family by studies showing that not only Bcl-2, but also Bcl-xl and Mcl-1 can modulate Ca^{2+} release from the ER (White et al. 2005; Eckenrode et al. 2010; Vervliet et al. 2016).

Using both cell population measurements and single cell imaging to monitor cytosolic Ca²⁺ levels, it was observed that Bcl-2 inhibited anti-CD3-induced Ca²⁺ elevation in multiple Bcl-2-expressing clones of the WEHI7.2 T cell line (Chen et al. 2004). The inhibitory effect of Bcl-2 on ER Ca²⁺ release was detected when the phosphoinositide signaling pathway mediating anti-CD3-induced IP₃ synthesis was bypassed by adding a cell-permeant IP₃ ester to cells, or by adding IP₃ to digitonin-permeabilized cells. The anti-CD3-induced Ca²⁺ elevation in T cells was not only inhibited by wild-type Bcl-2, which localizes to both the ER and mitochondria, but also by Bcl-2 selectively targeted to the ER membrane. In subsequent studies, summarized in depth below, we further verified Bcl-2/IP₃R interaction using fluorescence resonance energy transfer (FRET)-based assays (Rong et al. 2008). Taken together, these findings indicated that the action of Bcl-2 in inhibiting cellular Ca²⁺ signals resides at the level of the ER, rather than in the upstream signal transduction pathway that mediates IP₃ synthesis. Importantly, a series of control experiments indicated that inhibition of IP₃-induced Ca²⁺ release by Bcl-2 was not a result of decreased ER luminal Ca²⁺ concentration, decreased IP₃R levels, or altered expression of luminal Ca²⁺-binding proteins. Although there is a decrease in the affinity of IP₃Rs for IP₃ in T cells expressing Bcl-2, it has been found that Bcl-2 inhibited IP₃-induced Ca²⁺ release even at saturating IP₃ concentrations (Chen et al. 2004). These observations indicated that the inhibition of Ca²⁺ release by Bcl-2 has at least two components: a decrease in the affinity of IP₃Rs for IP₃ and reduced IP₃R channel opening even under conditions of maximal stimulation.

An intriguing aspect of the action of Bcl-2 is that it differentially regulates Ca²⁺ signals according to the strength of T cell receptor activation (Zhong et al. 2006). High concentrations of anti-CD3 antibodies (used to evoke T cell receptor activation) induce a longer-lasting cytosolic Ca²⁺ elevation, with a greater peak amplitude, than low anti-CD3 antibody concentrations. The large, sustained cytosolic Ca²⁺ elevations caused by strong T cell receptor activation are proapoptotic. Whereas, the lesser cytosolic Ca²⁺

signals caused by weaker T cell activation are prosurvival. These findings are consistent with the work of many investigators regarding the characteristics of Ca²⁺ elevations capable of mediating cell death (Szalai et al. 1999; Hajnóczky et al. 2000, 2003; Lin et al. 2005; Joseph and Hajnóczky 2007).

Strikingly, Bcl-2 was found to inhibit the proapoptotic sustained Ca²⁺ elevations induced by strong T cell receptor activation, while enhancing the prosurvival Ca²⁺ oscillations induced by weaker T cell receptor activation (Zhong et al. 2006). It is not fully clear how Bcl-2 has this duality of action, and it might seem surprising from a simple bimolecular interaction between two constitutively bound proteins. However, continuing studies have shown that Bcl-2 can act to dock additional proteins at IP₃Rs (see below), thereby raising the possibility that the regulation of IP₃Rs by Bcl-2 is dependent on multiple players. Whatever the mechanism, these observations emphasize the point that Bcl-2 functions to enhance cell survival and can have this action by either dampening large cytosolic Ca²⁺ elevations or promoting lesser cytosolic Ca²⁺ signals.

TARGETING Bcl-2/IP₃R INTERACTION WITH A SYNTHETIC PEPTIDE

To develop an experimental tool for use in identifying the functional role of the Bcl-2/IP₃R interaction, we mapped the Bcl-2-binding site to a 20 amino acid sequence in domain 3 of type 1 IP₃R, which is located in a portion of the channel known as the “regulatory and coupling region” (Fig. 1; Rong et al. 2008). A synthetic peptide mimicking the IP₃R sequence within domain 3 binds Bcl-2 and functions as a decoy peptide in that it displaces Bcl-2 from the IP₃R. This peptide, referred to as TAT-Pep2, abrogates Bcl-2’s inhibition of IP₃R-mediated Ca²⁺ release, thereby exaggerating cytosolic Ca²⁺ signals and triggering apoptotic cell death (Rong et al. 2008). These findings were consolidated by showing effects of TAT-Pep2 (or just Pep2 without the TAT conjugate that allows the protein to be membrane-permeating) on IP₃R channel activity in vitro, IP₃-induced ER

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Ca^{2+} release in permeabilized cells, and cell-permeable IP_3 ester-induced Ca^{2+} elevation in intact cells. Bcl-2's inhibition of T cell-receptor-induced Ca^{2+} elevation and apoptosis are reversed by incubation of cells with TAT-Pep2. These data confirmed that the interaction of Bcl-2 with IP_3 Rs contributes to the regulation of proapoptotic Ca^{2+} signals. Moreover, they highlighted the Bcl-2/ IP_3 R interaction as a potential therapeutic target in diseases associated with Bcl-2's inhibition of cell death.

In continuing studies, we found the interaction of Bcl-2 with the IP_3 R was mediated by the BH4 domain of Bcl-2 (Rong et al. 2009). The presence of a BH4 domain distinguishes the antiapoptotic protein Bcl-2 from its proapoptotic relatives. Deletion of the BH4 domain converts Bcl-2 into a proapoptotic protein, whereas a TAT-BH4 peptide (i.e., a membrane-permeant form of Bcl-2's BH4 domain) inhibits apoptosis and improves survival in models of disease caused by accelerated apoptosis (Hotchkiss et al. 2006). Thus, the BH4 domain of Bcl-2 has antiapoptotic activity independent of full-length protein. It is known that the BH4 domain of Bcl-2 is involved in the interaction with and suppression of Bax (Ding et al. 2010; Barclay et al. 2015), but in addition the BH4 domain of Bcl-2 and domain 3 of IP_3 Rs are responsible for the Bcl-2/ IP_3 R interaction that modulates Ca^{2+} signaling and inhibits apoptosis. TAT-Pep2, which mimics part of the amino acid sequence of domain 3 of IP_3 Rs, prevents the Bcl-2/ IP_3 R interaction by providing alternative docking sites for the BH4 domain of Bcl-2.

The binding of Bcl-2 by domain 3 of IP_3 Rs was confirmed in collaboration with others, and it was also determined that a critical residue in the BH4 domain of Bcl-2 (Lys17), which is not observed in the BH4 domain of Bcl-XL (Asp11), is necessary for binding to the IP_3 R (Monaco et al. 2012). Additional findings indicated the α -helical secondary structure of the BH4 domain of Bcl-2, conferred by the Ile14 and Val15 residues, is essential for inhibiting IP_3 R-mediated Ca^{2+} release (Monaco et al. 2013). Moreover, in contrast to wild-type Bcl-2, a full-length Bcl-2 Ile14Gly/Val15Gly mutant displayed markedly reduced structural stability and a shortened pro-

tein half-life. Also, the mutant protein failed to interact with Bax, and was unable to inhibit IP_3 R-mediated Ca^{2+} release or to protect against Ca^{2+} -mediated apoptosis (Monaco et al. 2018).

The carboxy-terminal portion of Bcl-2, which contains a transmembrane domain that tethers the protein to cellular membranes, also plays a role in regulating of IP_3 R-mediated Ca^{2+} release and apoptosis. Deletion of the transmembrane domain of Bcl-2 yielded a protein with a lesser ability to inhibit both Ca^{2+} signaling and apoptosis (Ivanova et al. 2016). At first sight, this outcome might seem rather predictable. It is obvious that deletion of the transmembrane domain of Bcl-2 would prevent its insertion into ER membranes, and thereby make it less likely to be juxtaposed to IP_3 Rs. However, it appears that the transmembrane domain of Bcl-2 is not only required for locating Bcl-2 on ER membranes, but also increases the association of Bcl-2 with IP_3 Rs. In this case, the Bcl-2/ IP_3 R interaction is not mediated by the BH4 of Bcl-2 and domain 3 of IP_3 Rs, but by the transmembrane carboxy-terminal domains of both proteins. Another striking observation was that the carboxy-terminal domain of Bcl-2 alone had an inhibitory effect on IP_3 R-mediated Ca^{2+} release (Ivanova et al. 2016). The Bcl-2/ IP_3 R interaction therefore appears to have multiple components that promote both protein:protein binding and inhibition of Ca^{2+} release. It is interesting to note that Bcl-2 exists in at least two isoforms, Bcl-2 α and Bcl-2 β , which differ in that Bcl-2 β does not have the carboxy-terminal transmembrane domain and is a cytosolic protein (Ivanova et al. 2016). Bcl-2 β is typically expressed at lower levels than Bcl-2 α , but may have a proapoptotic action that promotes tumor cell death (Warren et al. 2016), consistent with the notion that the carboxy-terminal domain is necessary for interaction with IP_3 Rs and inhibition of apoptosis.

Through minor modification of the TAT-Pep2 sequence, replacing two amino acids encoding a putative protease cleavage site, we improved the stability and thus the potency of this peptide inhibitor of Bcl-2/ IP_3 R interaction (Zhong et al. 2011). This modified peptide was initially referred to as TAT-Pep8, but has been more recently named Bcl-2 IP_3 R Disruptor-2, or

BIRD-2. Using this modified peptide to inhibit Bcl-2/IP₃R interaction in primary human CLL cells, we provided additional evidence of the therapeutic usage of inhibiting Bcl-2/IP₃R interaction, as BIRD-2-treated CLL cells underwent apoptosis, whereas normal lymphocytes did not (Zhong et al. 2011). Additional evidence for the potential therapeutic value of targeting Bcl-2/IP₃R interaction using BIRD-2 has been provided since this initial study. Induction of apoptosis by BIRD-2 in diffuse large B cell lymphoma (DLBCL) cell lines (Akl et al. 2013) and in multiple myeloma cell lines in vitro and in a xenograft mouse model of multiple myeloma (Lavik et al. 2015) has been documented. Importantly, the potential value of targeting Bcl-2/IP₃R interaction using BIRD-2 has been extended in recent studies to nonlymphoid malignancies, including small-cell lung cancer (Greenberg et al. 2015) and ovarian cancer (Xie et al. 2018). Finally, further recent studies indicate that BIRD-2-triggered cytosolic Ca²⁺ signals and cell death are critically dependent on the constitutive IP₃ signaling that occurs downstream from the B cell receptor in B cell malignancies (Bittremieux

et al. 2018a). The cell death induced by BIRD-2 is dependent on Ca²⁺ release from the ER via IP₃Rs, but also requires the presence of extracellular Ca²⁺ to maintain the cytosolic Ca²⁺ signals to drive cells to point of cell death (Bittremieux et al. 2018b). The ER Ca²⁺ store is finite, and when the ER is depleted influx of Ca²⁺ across the cell membrane is required to sustain cytosolic Ca²⁺ signals. It is unclear what mechanism underlies the influx of Ca²⁺ during BIRD-2-induced cell death. In most cells, depletion of ER Ca²⁺ content activates a “store-operated Ca²⁺ entry” (SOCE) mechanism to allow replenishment (Putney 2007; Bodnar et al. 2017; Bootman and Rietdorf 2017; Putney et al. 2017). However, knockdown of the SOCE component STIM1, or addition of pharmacological inhibitors, did not protect against BIRD-2-induced apoptosis (Bittremieux et al. 2018b).

Bcl-2 MEDIATES A NEGATIVE FEEDBACK LOOP THAT MODULATES Ca²⁺ SIGNALING AND CELL DEATH

The discussion above alluded to both direct and indirect actions of Bcl-2 in regulating IP₃R-mediated Ca²⁺ release, and the ability of Bcl-2 to regulate prodeath Ca²⁺ elevations without interfering with physiological IP₃R-mediated Ca²⁺ signals. In addition to its direct bimolecular effect on IP₃Rs, which has been shown, for example, using purified IP₃Rs and recombinant Bcl-2 in simple lipid bilayers (Chen et al. 2004), Bcl-2 can act to dock other proteins with IP₃Rs. For example, Bcl-2 docks both DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of molecular weight 32,000) and calcineurin (a Ca²⁺-sensitive phosphatase) in a complex on the IP₃R (Chang et al. 2014). DARPP-32 is phosphorylated and activated by protein kinase A (PKA), and is dephosphorylated by calcineurin. In its phosphorylated state DARPP-32 can inhibit the activity of protein phosphatase 1 (PP1) (Walaas et al. 2011).

The association of DARPP-32 and calcineurin with IP₃Rs was shown to reduce IP₃R-mediated Ca²⁺ elevation in T cells (Fig. 2; Chang et al. 2014). In brief, type 1 IP₃Rs can be phosphorylated by PKA on Ser1755, thereby sensi-

Figure 2. Bcl-2 regulates IP₃-induced Ca²⁺ release from the endoplasmic reticulum (ER). Bcl-2 docks CaN and DARPP-32 onto the IP₃R, forming a negative feedback loop that regulates IP₃R phosphorylation and prevents Ca²⁺ elevation that induces apoptosis. BIRD-2 inhibits Bcl-2/IP₃R interaction, inducing excessive Ca²⁺ elevation and apoptosis.

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tizing the channels to IP₃ and enhancing cellular Ca²⁺ signaling. The PKA-mediated phosphorylation of type 1 IP₃Rs can be reversed by PP1, which is inhibited by PKA-phosphorylated DARPP-32. The IP₃R/Bcl-2/DARPP-32/calci-neurin complex creates a negative feedback loop that prevents excessive Ca²⁺ release from the ER by regulating IP₃R phosphorylation as follows. Ca²⁺ release arising via IP₃Rs activates calcineurin, which subsequently dephosphorylates DARPP-32 thereby relieving its inhibition of PP1. Active PP1 can dephosphorylate IP₃Rs and consequently attenuate Ca²⁺ release. So, by bringing DARPP-32 and calcineurin into close association with IP₃Rs, Bcl-2 can indirectly contribute to the regulation of IP₃R phosphorylation and Ca²⁺ release from the ER (Chang et al. 2014).

These findings are consistent with evidence from multiple laboratories pointing to a role of Bcl-2 in regulating IP₃R phosphorylation, and thus IP₃R-mediated Ca²⁺ release. We previously reported that Bcl-2 decreases IP₃R phosphorylation (Chen et al. 2004), and other investigators found that PKA mediates phosphorylation of serine 1755 and serine 1589 on type 1 IP₃Rs, thereby increasing IP₃-mediated channel opening and Ca²⁺ release (Volpe and Alderson-Lang 1990; Wagner et al. 2003, 2008). Also, Oakes et al. (2005) found that Bcl-2 regulates IP₃R phosphorylation in the IP₃R regulatory and coupling domain. PKA-mediated protein phosphorylation is typically regulated by PP1 α (Tang et al. 2003), and an IP₃R/PP1 α complex has been implicated in Bcl-2-mediated suppression of ER Ca²⁺ release in breast cancer cells (Xu et al. 2007). Also, it has been established for some time that Bcl-2 binds calcineurin (Shibasaki et al. 1997), and that Bcl-2 increases the association of calcineurin with IP₃Rs (Erin et al. 2003a, b; Erin and Billingsley 2004); this has a neuro-protective effect in primary neuronal cells (Erin et al. 2003b).

Before the work described above, Bultynck and colleagues had predicted that the effects of calcineurin on IP₃R-mediated Ca²⁺ signals were indirect, and may be secondary to PP1 acting with DARPP-32 (Bultynck et al. 2003). Tang et al. (2003) discovered a direct association be-

tween PP1 α and type 1 IP₃R, and established that the association with PP1 α reverses PKA-mediated IP₃R phosphorylation. AKAP9, a multifunctional PKA anchoring protein, docks both PKA and PP1 α to IP₃R (Tu et al. 2004). In experiments with medium spiny neurons from DARPP-32 knockout mice, DARPP-32 was shown to regulate dopamine-induced Ca²⁺ oscillations (Tang and Bezprozvanny 2004). 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 (Svenningsson et al. 2004).

The findings discussed above are relevant to CLL in humans, because we detect DARPP-32 expression in primary human CLL cells. Moreover, treating these cells with BIRD-2 increases phosphorylation of Ser1755 in type 1 IP₃Rs levels followed by enhanced cytosolic Ca²⁺ elevation (Chang et al. 2014). Taken together, our data indicate that BIRD-2-mediated disruption of the Bcl-2/IP₃R interaction (and consequently, also that of DARPP-32 and calcineurin with IP₃Rs) overcomes the Bcl-2-imposed repression of Ser1755 phosphorylation and IP₃R-mediated Ca²⁺ release. These results suggest that CLL cells exploit the IP₃R/Bcl-2/DARPP-32/calci-neurin-mediated negative feedback mechanism to prevent Ca²⁺ elevation and cell death.

ELUCIDATING A SMALL MOLECULE INHIBITOR THAT MIMICS BIRD-2

The actions of the peptide inhibitors, BIRD-2 (TAT-Pep8) and TAT-Pep2, summarized here provide a strong rationale for targeting the Bcl-2/IP₃R interaction as a novel treatment strategy for Bcl-2-positive malignancies. However, peptides rarely become effective therapeutic agents, and a long-term aim is therefore to explore the use of small molecules as a step toward producing effective inhibitors of Bcl-2/IP₃R interactions for clinical use.

To this end, the Distelhorst laboratory has conducted a high-throughput screen with the goal of identifying chemical compounds that mimic the function of BIRD-2 by binding to the BH4 domain of Bcl-2 and disrupting Bcl-

2's interaction with IP₃R. A library of 25,480 compounds was tested using two Bcl-2-positive multiple myeloma cell lines. A caspase activation assay (i.e., stimulation of apoptotic cell death) was performed in the initial screening procedures to detect responses to individual compounds. Of the compounds tested, 38 were positive for caspase activation, and thus advanced to additional testing in our laboratory using both the caspase assay (with myeloma lines), and an MTS cell viability assay (with both myeloma lines and in primary human CLL cells). These studies were followed by cytoplasmic Ca²⁺ measurements using Jurkat cells, and selected primary CLL cells, as summarized in the heat map (Fig. 3). As noted on the heat map, one of the most promising hits, labeled "lead compound," is positive across each of the assays performed. This compound not only induces cell death in the multiple myeloma cell lines, but also induces both cell death and Ca²⁺ elevation in Jurkat cells and CLL cells. Moreover, additional studies show that the lead compound induces cell death in additional hematologic malignancies: acute lymphoblastic leukemia, histiocytic lymphoma, multiple myeloma, and small-cell lung cancer. Moreover, this compound does not induce cell death in normal human lymphocytes (Fig. 4). Interest in this compound is enhanced by evidence it produces synergistic cell death when combined with ABT-199/Venetoclax (Fig. 5). Although this work is still in progress, requiring biophysical studies to document on-target effects and use of mouse models to document in vivo action and monitor potential toxicity, the findings encourage the prospect of developing a novel Bcl-2 inhibitor to supplement the current therapeutic ABT-199/Venetoclax.

ABT-199/Venetoclax induces apoptosis by a different mechanism to BIRD-2. Unlike BIRD-2, ABT-199/Venetoclax neither binds to the BH4 domain of Bcl-2, nor interacts with IP₃Rs, or regulates intracellular Ca²⁺ signaling or homeostasis (Ivanova et al. 2016; Vervoessem et al. 2017b; Jakubowska et al. 2018). Instead, ABT-199/Venetoclax binds within a hydrophobic pocket formed by BH domains 1-3 of Bcl-2. In so doing, it displaces proapoptotic proteins, such as Bim, from Bcl-2, thereby activating Bax

and inducing apoptotic cell death. Because of its fundamental mechanism of action, ABT-199/Venetoclax is an effective therapeutic agent only in malignancies in which levels of both Bcl-2 and Bim are elevated: cells that are said to be addicted to Bcl-2 for their survival (Letai 2008; Davids and Letai 2013).

Thus, ABT-199/Venetoclax responsiveness varies among cancers (Deng et al. 2007). For example, CLL is highly responsive to ABT-199/Venetoclax, although resistance is reported (Davids et al. 2012). On the other hand, ABT-199/Venetoclax response rates are 28% in DLBCL and 31% in FL (Gibson and Davids 2015). Although Bcl-2 is commonly expressed in multiple myeloma at levels comparable to CLL and FL (Pettersson et al. 1992), responses to ABT-199/Venetoclax are limited to a small subset of myeloma lines (Touzeau et al. 2014) and patients with the CCND1/IGH translocation (Touzeau et al. 2014; Gibson and Davids 2015). Also, AML is a Bcl-2-positive malignancy, but ABT-199/Venetoclax is effective in only a fraction of AML patients (Konopleva et al. 2006; Pan et al. 2014). Major reasons for ABT-199/Venetoclax resistance include (1) low expression levels of proapoptotic proteins so the cancer cells are not primed to respond to ABT-199/Venetoclax (Deng et al. 2007); and (2) expression of Mcl-1 or Bcl-xl, which bind and inhibit proapoptotic proteins released from Bcl-2 by ABT-199/Venetoclax (Choudhary et al. 2015).

Interestingly, a reciprocal relationship was observed in the sensitivity of cancer cell lines to ABT-199/Venetoclax and BIRD-2 (Greenberg et al. 2015; Lavik et al. 2015; Vervoessem et al. 2017a). These observations are consistent with Bcl-2 having different prosurvival modes of action: one by sequestering proapoptotic proteins (ABT-199/Venetoclax sensitive) and another by reducing Ca²⁺ release from IP₃Rs (BIRD-2 sensitive). These two modes of action may be linked because incubation of cells with BIRD-2 increases Bim expression in cancer cells that have relatively low Bim levels and thus are insensitive to ABT-199/Venetoclax (Greenberg et al. 2015; Lavik et al. 2015). It is therefore plausible that BIRD-2, or a small molecule with analogous interactions, can trigger cell death either

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Figure 3. Heat map. This diagram reveals the relative significance of 38 drug-like compounds. The compound marked lead compound is the most impressive, in that it induces cell death, as detected by caspase assay and by MTS assay, and also induces Ca²⁺ elevation in both primary human chronic lymphocytic leukemia (CLL) cells and in the Jurkat cell line. In this way this lead compound mimics BIRD-2. Additional experiments not shown here indicate this compound also inhibits Bcl-2's interaction with IP₃Rs, a hallmark of BIRD-2 activity.

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Figure 4. Cell death induction. Whereas the lead compound induces apoptosis in multiple myeloma cells, it does not decrease survival of normal human lymphocytes. This is further indication that this lead compound behaves like BIRD-2.

by promoting substantial elevation of cytosolic Ca^{2+} concentration, or by causing Bim expression and consequent sensitivity to ABT-199/Venetoclax. For these reasons, developing a small molecule that mimics the action of BIRD-2 should be a high priority for both biomedical and pharmaceutical enterprises. The preliminary findings presented here provide strong rationale for this.

CONCLUDING REMARKS

Cancer cells of many types have aberrantly high amounts of Bcl-2 promoting their survival. Although ABT-199/Venetoclax kills some of these cells, specifically those addicted to Bcl-2 for their survival, it is far from being fully effective. A growing number of studies have shown that disruption of the Bcl-2/IP₃R interaction using BIRD-2, or an analogous tool, can promote cancer death. These observations highlight an alternative potential mechanism for killing cancers cells that are supported by high levels of Bcl-2 expression. More work is required to establish exactly how BIRD-2 kills cells and what prevailing conditions are necessary for BIRD-2 to be effective. Current evidence points to the factors such as constitutive IP₃ production, Ca^{2+} influx, type 2 IP₃Rs, mitochondrial Ca^{2+} sequestration and/or Bim expression for effective cancer cell

killing by BIRD-2 (see Kerkhofs et al. 2019 for further discussion). Moreover, it seems that BIRD-2 is unlikely to be always effective as monotherapy, and may work efficaciously in conjunction with other anticancer treatments such as ABT-199/Venetoclax and cisplatin. However, the option of treating cancer with

Figure 5. Synergy between the lead compound and ABT-199. KMS-12-BM myeloma cells were treated for 24 hours with the lead compound and ABT-199. Cell viability was measured by CAT assay and analyzed by Chou and Talaly method in which a combination index (CI) value less than one defines synergy. Symbols represent triplicate determinations in a single experiment.

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agents that counter Bcl-2/IP₃R interactions is now clearly in our grasp based on findings established using BIRD-2 and its analogs, as is the possibility of developing a therapeutic chemical compound that mimics BIRD-2's mode of action.

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