Recent advances in targeting IP$_3$R/Bcl-2-channel complexes by peptide molecules: novel therapeutic opportunities for Bcl-2-dependent cancers?

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This publication provides an overview of the recent scientific work of Geert Bultynck, highlighting some key findings and results by means of re-publication of some of the original data and overview figures. For these figures, copyright statements were included.

Abstract

Intracellular Ca$^{2+}$-release channels like the inositol 1,4,5-trisphosphate receptor (IP$_3$R) play a pivotal role in a plethora of cell biological and physiological processes. IP$_3$R-mediated Ca$^{2+}$ flux from the endoplasmic reticulum, the main intracellular Ca$^{2+}$ store, ought to be tightly regulated. This is achieved by a complex network of associated proteins. Recently, IP$_3$Rs have been identified as targets for the anti-apoptotic Bcl-2-family members, Bcl-2 and Bcl-Xl, which control the Ca$^{2+}$-flux properties of the channel. In collaboration with the Distelhorst lab, our lab has focused on the identification of the molecular determinants responsible for IP$_3$R/Bcl-2-complex formation. These studies revealed a novel role for Bcl-2 as an endogenous inhibitor of IP$_3$R-mediated Ca$^{2+}$ flux, thereby suppressing pro-apoptotic Ca$^{2+}$ signals. In addition, novel peptide tools were developed that disturb IP$_3$R/Bcl-2 complexes. These tools may become particularly useful in Bcl-2-dependent cancers, like chronic lymphocytic leukemia to alleviate Bcl-2’s inhibitory role on IP$_3$Rs and to provoke pro-apoptotic Ca$^{2+}$ signals. Furthermore, molecular studies revealed important differences between Bcl-2 and Bcl-Xl at the level of the BH4 domain, underlying differences in the regulation of IP$_3$R-channel activity between Bcl-2 and Bcl-Xl. This also opens the avenue to selectively target Bcl-2 in cancer cells, while maintaining essential Bcl-Xl pro-survival functions in healthy cells.

Keywords: Inositol 1,4,5-trisphosphate receptor, Calcium signaling, Apoptosis, Anti-cancer strategies, Protein complexes.
Abbreviations

Bcl-2: B-cell lymphoma 2
Bcl-XL: B-cell lymphoma-extra large
BH: Bcl-2 homology
CytC: cytochrome c
Cx: connexin
ER: endoplasmic reticulum
FHIT: fragile histidine triad
IAP: inhibitor of apoptosis
IP3R: inositol 1,4,5-trisphosphate receptor
MCU1: mitochondrial Ca\(^{2+}\) uniporter 1
PLC: phospholipase C
PML: promyelocytic leukemia
PTEN: phosphatase and tensin homolog; phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)) phosphatase
SERCA: sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase
Smac: second mitochondria-derived activator of caspase
TMBIM: transmembrane Bax Inhibitor containing motif
TPC: two-pore channels
VDAC: voltage-dependent anion channel
1. ION CHANNELS: AN APPETIZER

Ion channels play a pivotal role in human health by coordinating physiological processes at the cellular level. Their properties require a tight regulation for physiological signaling. For instance, $K_{\text{ATP}}$ channels determine the resting membrane potential in the $\beta$ cell of the isle of Langerhans (1, 2). When blood glucose levels rise, cellular ATP levels rise and close the $K_{\text{ATP}}$ channels, thereby increasing the resting membrane potential. This results in the opening of voltage-dependent $Ca^{2+}$ channels and $Ca^{2+}$-induced release of insulin, whose actions result in the lowering of blood glucose levels. Inhibition or hyperactivation of $K_{\text{ATP}}$ channels are associated with pathophysiological conditions, like diabetes or hyperinsulinemic hypoglycemia, respectively.

Over the last decade, the study of ion channels and their molecular and biophysical properties gained important insights in regulating essential physiologically processes. Malfunctioning or dysregulation of channels often underlie severe pathophysiological conditions. The importance of channelopathies underlying these conditions is underscored by a recent Special Issue in Pflugers Archiv (3). Our lab has been focusing on the role of inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) and connexin (Cx) hemichannels. Both channel types have been implicated in a variety of pathophysiological conditions, including neurodegenerative diseases, pancreatic dysfunction, cardiac arrhythmias and cardiac hypertrophy (4-15). Moreover, by analyzing mutations of these channels associated with pathological conditions, it has become increasingly clear that altered channel functions underpin a plethora of diseases (4, 16, 17). As a result, strategies to selectively target these channelopathies emerged. An example of this is the development of novel pharmacophores based on “RXP”-based motifs known to bind Cx43 to preserve action potential propagation in arrhythmic hearts (18, 19). Here we describe our work in collaboration with Dr. Distelhorst (Case Western Reserve University, Cleveland, OH) that resulted in novel peptide-based strategies to target IP$_3$R channels in specific pathophysiological conditions by analyzing their regulation by protein-protein interactions at the molecular level using a plethora of biophysical and cellular approaches. These peptides prove to be promising lead compounds for peptide-based compounds or peptidomimetics.

2. INTRACELLULAR Ca$^{2+}$ SIGNALING: A CENTRAL ROLE FOR IP$_3$RS

Intracellular Ca$^{2+}$ signaling controls a variety of cellular and physiological processes that ultimately control cell plasticity, homeostasis, survival and death (20, 21). Moreover, intercellular signaling, including Ca$^{2+}$-mediated signaling, coordinates these processes at the tissue or organ level (22).

In somatic cells, intracellular Ca$^{2+}$-release channels, like the IP$_3$R, plays a central role in Ca$^{2+}$ signaling in response to extracellular signals, like hormones, growth factors and antibodies (23, 24). These biomolecules lead to the activation of phospholipase C (PLC) $\beta/\gamma$ and the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). Elevated IP$_3$ levels cause opening of IP$_3$Rs, which are present at the endoplasmic reticulum (ER) membranes. The ER functions as the main intracellular Ca$^{2+}$ store and the opening of ER Ca$^{2+}$-release channels lead an increase in the cytosolic [Ca$^{2+}$] from about 100 nM to about 1 $\mu$M. Nevertheless, in specialized Ca$^{2+}$ microdomains, [Ca$^{2+}$] can rise up to 100 $\mu$M, e.g. near the mouth of the IP$_3$R channel and rise up to 20 $\mu$M in the ER/mitochondrial interspace (25). At distinct places, ER membranes and
mitochondrial membranes are in very close contact, creating Ca\(^{2+}\) hot spots (26, 27) (Figure 1). In these zones, IP\(_3\)Rs indirectly interact with proteins of the outer mitochondrial membrane that have been shown to mediate Ca\(^{2+}\) transport, like the voltage-dependent anion channel (VDAC) (27).

Transport across the inner mitochondrial members is mediated by the recently

![Figure 1. ER Ca\(^{2+}\)-handling components regulate mitochondrial function and bio-energetics.](image)

Figure 1. ER Ca\(^{2+}\)-handling components regulate mitochondrial function and bio-energetics. The ER-Ca\(^{2+}\) content is regulated by channels and pumps (IP\(_3\)Rs, RyRs, SERCAs) and by Ca\(^{2+}\)-binding chaperones (CaBCs). IP\(_3\) stimulates Ca\(^{2+}\) (red dots) release from the ER into cytosol and mitochondria. Mitochondrial Ca\(^{2+}\), transported via VDAC and MCU1, is directly or indirectly controls cellular energy metabolism and the production of Reactive Oxygen Species (ROS). IP\(_3\)Rs are tightly regulated by accessory proteins involved in cell death and survival, like Bcl-2, Bcl-XL, PKB/Akt and the recently identified PML. Different regulatory mechanisms occur at the IP\(_3\)R promoting cell survival (like Bcl-2, Bcl-XL, PKB/Akt) or cell death (like PML). The latter prevents the survival of altered, damaged or oncogenic cells. ROS production may contribute to the survival of cells by efficient detection of damaged/altered mitochondria and their removal by autophagy, while preventing excessive apoptosis. Controlled apoptosis is important to eliminate cells, in which the removal of altered mitochondria by autophagy is not sufficient, thereby avoiding tumor genesis. In this process, PML may play a crucial role, as it promotes IP\(_3\)-mediated Ca\(^{2+}\) transfer from the ER into the mitochondria by dephosphorylating and suppressing PKB/Akt activity through PP2A. While PKB/Akt suppresses IP\(_3\)-channel activity by phosphorylation of the IP\(_3\)R, the recruitment of PP2A via PML at the interorganellar ER/mitochondrial complex dephosphorylates and inactivates PKB/Akt. This suppresses PKB-dependent phosphorylation of IP\(_3\)R and thus promotes Ca\(^{2+}\) release through this channel and Ca\(^{2+}\) transfer into the mitochondria. At the mitochondrial level, the tumor suppressor Fhit increases the affinity for the mitochondrial Ca\(^{2+}\)-uniporter, promoting mitochondrial Ca\(^{2+}\) uptake physiological levels of agonist-induced Ca\(^{2+}\) signals. Green arrows: stimulation; red lines: inhibition; black arrows: Ca\(^{2+}\) flux. This figure was originally published in Journal of Aging Research. Jean-Paul Decuyper, Giovanni Monaco, Ludwig Missiaen, Humbert De Smedt, Jan B. Parys, and Geert Bultynck. IP\(_3\) Receptors, Mitochondria, and Ca\(^{2+}\) Signaling: Implications for Aging. Journal of Aging Research. 2011, 920178. © 2011 Jean-Paul Decuyper et al.
identified and cloned mitochondrial Ca\(^{2+}\) uniporter (MCU1) (28, 29). As a result, IP\(_3\)-Rs tightly influence mitochondrial biology and function (30, 31). A recent report highlighted the importance of basal IP\(_3\)-R function for regulating mitochondrial ATP production (32). Hence, it is not surprising that ER Ca\(^{2+}\) homeostasis ought to be tightly regulated and disturbances in ER Ca\(^{2+}\) homeostasis underlie a plethora of diseases (33). Not only somatic cells depend on IP\(_3\)-R function, oocytes too require IP\(_3\)-Rs for initiating Ca\(^{2+}\) signaling upon fertilization in response to PLC\(\zeta\), the recently identified sperm factor (34, 35). Sperm cells lacking PLC\(\zeta\) fail to induce Ca\(^{2+}\) signaling and embryonal development, while recombinant PLC\(\zeta\) restored these anomalies (34, 35). Besides the ER, other intracellular organelles seem to function as intracellular Ca\(^{2+}\) stores, which display distinct Ca\(^{2+}\)-release and – uptake mechanisms. For instance, lysosomes seem to release Ca\(^{2+}\) in response to NAADP, which targets the recently identified two pore channels (TPCs) (36, 37). Ca\(^{2+}\) signals originating from the TPCs seem to cross-talk to IP\(_3\)-R-mediated Ca\(^{2+}\) signaling (38, 39). Hence, it is important to understand that intracellular organelles are connected and affect each other through Ca\(^{2+}\) signaling. When excessive intracellular Ca\(^{2+}\) signaling is occurring, the ER Ca\(^{2+}\) content is maintained through activation of store-operated Ca\(^{2+}\) channels (SOCs). Recently, the molecular determinants underpinning these channels have been identified in genome wide siRNA studies, leading to the identification of Stim as the ER Ca\(^{2+}\) sensor and Orai as the plasmalemmal Ca\(^{2+}\)-influx channel (40). In normal conditions, IP\(_3\)-R activity is tightly regulated by associated proteins, thereby acting as either direct regulators or scaffolds for other proteins, like kinases and phosphatases (20). An increasing number of reports indicate that proteins controlling cell survival and death interact with the IP\(_3\)-R and tightly control its activity, including the promyelocytic leukemia (PML) tumor suppressor (41). Recent studies indicated that anti-apoptotic Bcl-2-family members, like Bcl-2, Bcl-Xl and Mcl-1, directly interact with IP\(_3\)-Rs, thereby regulating their Ca\(^{2+}\)-flux properties (42-46). Different molecular mechanisms for this regulation have been proposed (Figure 2). Some studies showed that IP\(_3\)-Rs are sensitized towards basal levels of IP\(_3\) when the ratio of anti-apoptotic over pro-apoptotic Bcl-2-family members is elevated. For instance, Bax/Bak-double-knockout cells display enhanced PKA-dependent phosphorylation of the IP\(_3\)-R, thereby increasing the basal Ca\(^{2+}\)-leak rate through this hypersensitive IP\(_3\)-R channel and lowering the steady state ER Ca\(^{2+}\) levels (46). This is consistent with seminal work from Rizzuto and co-workers showing that Bcl-2 overexpression reduces the ER Ca\(^{2+}\)-store content (47, 48). Other studies indicated that overexpression of Bcl-Xl stimulated IP\(_3\)-R-dependent Ca\(^{2+}\) oscillations, thereby enhancing mitochondrial bioenergetics and cell survival (44, 45). In this paradigm, the lowering of the ER Ca\(^{2+}\) levels was not a requisite for IP\(_3\)-R-dependent Bcl-Xl-mediated protection. While pro-survival Ca\(^{2+}\) oscillations seemed to be enhanced by Bcl-Xl, Bcl-2 seems to suppress pro-apoptotic Ca\(^{2+}\) signals associated with mitochondrial outer membrane permeabilization (42, 49). Importantly, these effects of Bcl-2 on Ca\(^{2+}\) signaling were not related to changes in steady state ER Ca\(^{2+}\) levels, but involved a direct inhibitory modulation of the IP\(_3\)-R Ca\(^{2+}\)-flux properties. Recently, other Bcl-2-family members have been shown to interact with the IP\(_3\)-R, including NrZ, the zebrafish homologue of Bcl-2like10 (50, 51). NrZ tightly controls Ca\(^{2+}\) fluxes in the yolk syncytial layer during early development of zebrafish. Importantly, NrZ-imposed inhibition of IP\(_3\)-R-mediated Ca\(^{2+}\) fluxes in the yolk syncytial layer not
only prevents apoptosis but also controls cytoskeletal dynamics and cell movements (50, 52). Zebrafish embryos lacking Nrz display pre-mature acto-myosin contraction, which lead to epiboly arrest (independent of apoptosis) and embryonic mortality (50, 53). Recent studies elucidated that altered IP$_3$R activity underlies pathophysiological conditions (reviewed in (5, 6)). In neurodegenerative diseases, a central role for changed IP$_3$R function has been proposed as very upstream and proximal mechanism in the development of the pathology (54). For instance, presenilins mutations associated with familial Alzheimer’s Disease lead excessive IP$_3$R-mediated Ca$^{2+}$signaling, which results in increased Reactive Oxygen Species (ROS) production (4). Importantly, these features precede the appearance of pathophysiological phenotypes, like the accumulation of amyloid β and/or neuronal cell death. Similar findings were observed for the regulation of IP$_3$Rs by mutant huntingtin protein, associated with Huntington’s disease (6, 55). Besides the IP$_3$R, other ER and mitochondrial Ca$^{2+}$-transporting systems have been implicated in the control of apoptosis. It is fascinating how many tumor suppressor, anti-apoptotic or pro-apoptotic proteins regulate ER and mitochondrial Ca$^{2+}$ homeostasis and dynamics, including the TransMembrane Bax Inhibitor containing Motif (TMBIM)-protein family (56-61), VDAC (62-64) and the fragile histidine triad (FHIT) protein (65).

3. BCL-2 PROTEINS

3.1. BCL-2 IN THE APOPTOTIC PATHWAY

The family of Bcl-2 proteins has been divided in two different classes: i) the anti-apoptotic Bcl-2-family members and ii) the pro-apoptotic Bcl-2-family members (66, 67) (Figure 3). Anti-apoptotic Bcl-2-family members like Bcl-2, Bcl-XL, Bcl-w, Bfl-1 and Mcl-1 inhibit apoptosis by counteracting the activity of pro-apoptotic.

Bcl-2-family members mainly through their hydrophobic cleft which neutralizes the BH3 domain of pro-apoptotic Bcl-2-family members (68) (Figure 4). Anti-apoptotic Bcl-2-family members have been proposed to contain 4 BH domains (BH1-4). The pro-apoptotic can be divided in two different groups: i) the multi-domain pro-apoptotic proteins containing BH1-3 domains responsible for executing mitochondrial outer membrane permeabilization upon activation and oligomerization (like Bax and Bak) and ii) the BH3-only proteins containing only the third BH domain that act either as direct “activators” of Bax and Bak (like Bid and Bim) or as “sensitizers” that interfere with the inhibitory function of Bcl-2 towards Bax/Bak and Bid/Bim (like Bad, Bik, Noxa, BNIP3, Hrk, Puma, Bmf and MULE) (69). However, Bax/Bak may also be directly activated by other proteins, including p53 and some of the sensitizer BH3 domains like Puma, Bmf and Noxa (70-72). Two not mutually exclusive models are proposed: i) an indirect model in which BH3-only proteins replace anti-apoptotic Bcl-2 proteins from Bax/Bak, causing release, activation and oligomerization of Bax/Bak and ii) a direct model, in which activator BH3-only proteins directly bind and activate Bax/Bak by inducing conformational changes and oligomerization (67, 73). Upon cellular stress, damage or derangement, BH3-only proteins will accumulate thereby causing direct Bax/Bak activation and/or inhibition of anti-apoptotic Bcl-2 proteins (74). Further prominent roles have been proposed for BH3-only protein like activated tBid in the recruitment of Bax to outer mitochondrial membranes, leading to Bax oligomerization and activation (74, 75). The activation of Bak by tBid requires the presence of VDAC2, which is responsible for the recruitment of Bak to outer mitochondrial membranes (76, 77). Furthermore, it is important to note that a recent paper from the Green lab indicated an important role for sphingolipids and derivatives in setting the threshold for Bax/Bak activation (78, 79). Sphingomyelinase is present in mitochondria-associated ER membranes that convert sphingomyeline into ceramide, which is further converted to sphingosine, sphingosine-1-phosphate and hexadecanal. As depicted in the model of Holville and Seamus, upon exposure to BH3-only proteins, hexadecanal binds to Bax and promotes its oligomerization and sphingosine-1-phosphate promotes Bak oligomerization (79). Apoptosis will be initiated by the formation of Bax/Bak pores in the outer mitochondrial membrane and the release of pro-apoptosis factors from mitochondrial intermembrane space into the cytosol like second mitochondria-derived activator of caspase (Smac) and CytC (67).
While CytC causes caspase activation, Smac binds and neutralizes Inhibitors of Apoptosis (IAPs) proteins with X-linked IAP (XIAP) as one of the most potent endogenous inhibitors of activated initiator and executioner caspases (cysteine proteases) (80-84). In addition to direct inhibition of caspases, IAPs can terminate caspase activity by ubiquitin-targeted proteasome degradation pathway (85), antagonize Smac release from mitochondria (86) and exert anti-apoptotic effects through TAK1-dependent activation of JNK1 (87). Caspases induce cleavage of different cellular proteins, resulting in morphological characteristics of apoptotic cells, including condensed nuclei, DNA laddering and the flipping of phosphatidylserine from inner to the outer leaflet of the plasma membrane (67). Importantly, also ion channels have been shown to be targets of pro-apoptotic factors, including CytC and caspases like the effector caspase 3. For instance, IP₃Rs are cleaved by caspase 3 leading to the formation of a stable C-terminal domain containing the Ca²⁺-channel pore, which contributes to the observed Ca²⁺ overload during late apoptosis (88, 89). Ca²⁺ release through (truncated) IP₃Rs seems to function as feed-forward mechanism that likely is amplified by CytC release itself, which directly binds to IP₃Rs and prevents their inhibition by high intracellular [Ca²⁺] (90-92). As such, a deadly positive feedback loop from the mitochondria to the ER with the release of CytC and the cleavage of IP₃Rs by caspase 3 and from the ER to the mitochondria with the excessive release of Ca²⁺ through unleashed IP₃R-channel activity or Ca²⁺-channel pore fragments (93). Recently, also Pannexin 1 (Panx1) channels have been proposed as targets for caspase 3 in apoptosis (94, 95). Truncation of Panx1 by caspase 3 removes its C-terminal auto-inhibitory domain from the channel pore, thereby leading to the release of ATP from apoptotic cells (96). Extracellular ATP seems to serve as a potent chemo-attractant responsible for the recruitment of macrophages to the dying cell and their...
removal by the immune system (94, 95).

3.2. APOPTOSIS PATHWAYS IN CANCER CELLS

An important pathway by which cancer cells can arise is the dysregulation of pro-survival versus pro-death mechanisms e.g. by mutations in tumor suppressor genes or up-regulation of pro-survival genes (97). An important tumor suppressor is p53, which is mutated in ~50% of the tumors (98). In normal cells, cell stress including DNA damage or alterations activates p53, leading to cell cycle arrest and the induction of apoptosis. Its pro-apoptotic activity involves i) transcriptional up-regulation of genes encoding pro-apoptotic proteins including PUMA, Bax and Bid and ii) (non-transcriptional) direct inhibition of anti-apoptotic Bcl-2/Bcl-XL or direct activation of Bax (99, 100). Other cancer cells display increased survival signaling for instance by up-regulating anti-apoptotic Bcl-2 proteins like Bcl-2 and Mcl-1 (101) or IAP protein-family members including survivin (102-104) or by activating the phosphatidylinositol-3 kinase (PKB/Akt) pathway (105, 106). Increased PKB/Akt signaling is observed in cancer cells in which the gene encoding phosphatase and tensin homolog (PTEN), which functions as a phostidylinositol 3,4,5-trisphosphate (PIP3) phosphatase, has been inactivated (106).

In any case, while cancer cells experience on-going death signaling by checkpoint violation, genomic instability and oncogene activation, they seem to escape their apoptotic fate (107). This has prompted researchers to map the apoptotic escape pathways in Bcl-2-dependent cancers like B-cell lymphomas (107). This was done using a BH3-profiling approach, which exploits the ability of selective, 20-amino-acid long BH3-domain peptides derived from BH3-only proteins to trigger mitochondrial outer membrane permeabilization in mitochondria isolated from distinct cancer cells (69, 108, 109).

The BH3-profiling approach has been adapted to whole-cell assays allowing analysis of human biopsies and mixed cell populations (110). The approach is based on the fact that each anti-apoptotic Bcl-2-family members has distinct BH3-domain-binding properties (69). For instance, the BH3 domain of Bim and Bid are promiscuous and bind all anti-apoptotic Bcl-2-family members. However, the BH3 domain of Bad selectively binds Bcl-2, Bcl-XL and Bcl-w, while the BH3 domain of Noxa selectively binds Mcl-1. Thus, BH3-Bad will cause CytC release from mitochondria derived from cancer cells that depend on Bcl-2 up-regulation, while BH3-Noxa indicates dependence on Mcl-1 up-regulation (110). Based on a study done in diffuse large-B-cell lymphoma cells, which is a very heterogenous disease, the group of Dr. Letai identified three classes of apoptotic blocks that cancer cells use to escape apoptosis (109). Class A displayed defects in the up-regulation of activator BH3-only proteins, class B displayed mutations in Bax/Bak effector proteins, while class C display up-regulated levels of anti-apoptotic Bcl-2 or Mcl-1 thereby preventing Bax/Bak activation. The latter class of cancer cells has been described as “primed to death”, it means that these cells are addicted to high levels of anti-apoptotic Bcl-2-family members because of their aberrant oncogenic behavior that causes up-regulation of activator BH3-only proteins like Bim or effectors like activated Bax. In these “primed to death” cells, Bcl-2 is loaded with Bim and targeting Bcl-2 with the BH3-mimetic drug ABT-737, which is a functional mimic of the BH3 domain of Bad triggers mitochondrial outer membrane permeabilization and thus apoptotic cell death (111). The sensitivity of cancer cells to ABT-737 was shown to correlate with the amount of Bim bound to Bcl-2.
4. REGULATION OF IP₃RS BY BCL-2

Here, we will discuss the identification of the molecular determinants underlying the regulation of IP₃Rs by anti-apoptotic Bcl-2 proteins and how one may exploit these molecular determinants to modulate IP₃R activity in pathophysiological conditions associated with altered IP₃R function by targeting or using the BH4 domain of Bcl-2.

4.1. IP₃RS ARE MOLECULAR TARGETS FOR BCL-2

Different reports indicated that Bcl-2 not only functioned at the mitochondrial membrane, but also exerted its anti-apoptotic function at the ER membranes by regulating intracellular Ca²⁺ homeostasis and dynamics. However, the report by Rong et al. showed for the first time that Bcl-2 directly interacted with IP₃Rs in intact cells (112) (Figure 5).

Using recombinantly expressed IP₃R fragments as GST-fusion proteins and purified Bcl-2, we obtained biochemical evidence for this interaction via GST-pull-down assays and Surface Plasmon Resonance (SPR) (Figure 6).

Using a peptide-based screening approach, we mapped the Bcl-2-binding site to a 20 amino acid stretch located in the centre of the modulatory domain of the IP₃R (aa 1389-1408), which was a daunting task given the fact that this channel consists of 2749 amino acids. This aa stretch has been used as a synthetic peptide and has been named IDP (IP₃R-derived peptide).

4.2. BCL-2 SUPPRESSES IP₃R ACTIVITY

The binding of Bcl-2 to the IP₃R caused inhibition of IP₃R Ca²⁺-flux activity. This claim is supported by evidence obtained from a variety of biophysical methods, including single IP₃R-channel data in lipid bilayers, unidirectional ⁴⁵Ca²⁺-flux assays in permeabilized cells, fluorescent Ca²⁺ imaging in intact cells triggered with either an agonist or cell-permeable IP₃. Indeed, WEHI7.2 cells, deficient for Bcl-2, displayed enhanced IP₃R-mediated Ca²⁺ release, while overexpression of Bcl-2 in these cells dampened Ca²⁺ release in intact cells in response to either T-cell receptor stimulation using the antibody anti-CD3 or to cell-permeable IP₃.

4.3. BCL-2 PROTECTS AGAINST IP₃R-MEDIATED APOPTOSIS

The immature T-cell line, WEHI7.2, is an excellent model to study IP₃R/Ca²⁺-dependent apoptosis in response to anti-CD3. Indeed, exposing WEHI7.2 cells to high [anti-CD3] (20 µg/ml) leads apoptosis, as shown by nuclear fragmentation and Annexin V-FITC labeling. Overexpressing Bcl-2 suppresses anti-CD3-induced apoptosis.

4.4. IDP REVERSES BCL-2’S SUPPRESSIVE ACTION ON IP₃RS

Importantly, the actions of Bcl-2 on the IP₃R channels could be reversed by the use of IDP. Indeed, the inhibition of IP₃R-mediated Ca²⁺ release by Bcl-2 overexpression was reversed by adding a cell-permeable version of IDP, i.e. IDP coupled to cell-penetrating sequence of the HIV viral protein TAT, thereby promoting anti-CD3-induced Ca²⁺ release. Furthermore, direct assessment of IP₃R activity under uni-directional Ca²⁺-flux conditions in saponin-permeabilized MEF cells, loaded to steady state with ⁴⁵Ca²⁺, displayed an increased responsiveness towards IP₃ in the presence of IDP. The latter technique allows a direct access to the ER Ca²⁺ stores in the absence of mitochondrial and ER Ca²⁺-uptake activity. These findings were confirmed in lipid bilayer experiments using purified Bcl-2.
Figure 5. Schematic overview of IP₃R structure with indication of the molecular determinant (Domain3) containing the hot spot for Bcl-2 binding (IDP). The different domains correspond to the fragments that are naturally formed upon mild trypsin-digestion of IP₃R1 and thus likely retain the proper conformation and higher-order structure.

Figure 6. A typical SPR experiment showing the association of GST-IP₃R Domain3 to the immobilized biotinylated BH4 domain of Bcl-2. Panel A, Increased concentration of the GST-IP₃R Domain3 were applied to biotin-BH4-Bcl-2 and demonstrate a bona fide interaction. Panel B, GST was used as a control and did not display any specific binding towards biotin-BH4-Bcl-2. Panel C, Comparison of the GST-IP₃R Domain3 of IP₁R₁, IP₂R₂ and IP₃R₃, indicating that the binding of BH4-Bcl-2 is conserved among all IP₃R isoforms. GST is shown as a negative control. Panel D, A total protein staining of SDS-PAGE-analyzed, purified GST-fusion proteins applied in the SPR experiments. All curves display the specific binding, obtained after subtracting the background binding to a scrambled version of biotin-BH4-Bcl-2. This figure was originally published in Cell Death and Differentiation. Giovanni Monaco, Elke Decrock, Haidar Akl, Raf Ponsaert, Tim Vervliet, Tomas Luyten, Marc De Maeyer, Ludwig Missiaen, Clark W Distelhorst, Humbert De Smedt, Jan B Parys, Luc Leybaert, Geert Bultynck. Selective regulation of IP₃-receptor-mediated Ca²⁺ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-Xl. Cell Death and Differentiation. 2011, doi: 10.1038/cdd.2011.97. This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. Copyright © 2011, Nature Publishing Group.
4.5. A STABILIZED FORM OF IDP PROMOTES PRO-APOPTOTIC Ca\(^{2+}\) SIGNALS IN BCL-2-DEPENDENT MALIGNANCIES, LIKE CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Next, we wished to elucidate whether Bcl-2-dependent cancers could be targeted by TAT-IDP (113). Therefore, we turned our focus on CLL, which in almost all cases is due to an up-regulation of the anti-apoptotic Bcl-2-protein levels. However, we noticed that IDP displayed reduced affinity upon prolonged application to cells. The reason for this pore stability might be due to the presence of a putative protease cleavage site in the sequence of the peptide. Therefore, we developed a stabilized version of IDP, in which the putative residues targeted by the protease were altered from two Asp residues into two Ala residues. First, we confirmed whether this peptide retained its ability to interact with Bcl-2. We found that a biotinylated version IDP DD/AA was able to pull down Bcl-2 using streptavidin beads. In addition, using unidirectional \(^{45}\)Ca\(^{2+}\) fluxes, we found that IDP DD/AA was able to stimulate IP\(_3\)-induced Ca\(^{2+}\) release in MEF cells. Finally, we also mutated these two Asp residues into Ala residues in the IP\(_3\)R domain responsible for interaction with Bcl-2. Using GST-pull down and SPR approaches, we found that GST-IP\(_3\)R domain DD/AA was equally potent as the wild-type GST-IP\(_3\)R domain to bind Bcl-2. Next, applying TAT-IDP DD/AA to CLL isolated from human patients caused excessive Ca\(^{2+}\) oscillations. These Ca\(^{2+}\) patterns were less pronounced when TAT-IDP was used or when TAT-IDP DD/AA was applied to normal B cells. In addition, these Ca\(^{2+}\) oscillations were dependent on the IP\(_3\)R activity, since blocking IP\(_3\)Rs using xestospongin B abolished TAT-IDP DD/AA-induced Ca\(^{2+}\) oscillations. Importantly, the excessive Ca\(^{2+}\) signals were accompanied with an increase in cell death through activation of the apoptotic program in the CLL cells. Apoptosis was assessed using FACS analysis of PI/Annexin V-FITC-stained cells and using a caspase-3-activation assay. Finally, comparing CLL with normal B cells revealed that TAT-IDP DD/AA was much less potent in provoking apoptosis in the normal B cells versus the CLL (Figure 7). Hence, these results are the first to indicate that targeting Bcl-2 at the level of the IP\(_3\)R holds therapeutic potential for the treatment of malignancies that depend on augmented Bcl-2 levels and provide hope for future strategies to target these currently incurable diseases.

Figure 7. A cell-death assay showing the enhanced sensitivity of CLL towards TAT-IDP DD/AA. Data display viable cells, which were identified by their lack of propidium iodide uptake. This research was originally published in Blood. Fei Zhong, Michael W. Harr, Geert Bultynck, Giovanni Monaco, Jan B. Parys, Humbert De Smedt, Yi-Ping Rong, Jason K. Molitioris, Minh Lam, Christopher Ryder, Shigemi Matsuyama, and Clark W. Distelhorst. Induction of Ca\(^{2+}\)-driven apoptosis in chronic lymphocytic leukemia cells by peptide-mediated disruption of Bcl-2–IP\(_3\) receptor interaction. Blood. 2011, 117:2924-2934. Copyright © 2011, The American Society of Hematology.
4.6. THE BH4 DOMAIN OF BCL-2 IS RESPONSIBLE FOR BINDING AND SUPPRESSING IP₃RS

While we mapped the Bcl-2-binding site on the IP₃R to a 20 amino acid stretch, we too identified the region on Bcl-2 that is responsible for binding and suppressing IP₃Rs (114).

We found that the BH4 domain of Bcl-2 was essential for Bcl-2’s targeting of IP₃Rs. Indeed, a Bcl-2 version lacking the complete BH4 domain (Bcl-2 ΔBH4) did neither bind nor inhibit IP₃Rs. Moreover, we also found that the BH4 domain by itself was sufficient to modulate IP₃R activity. Indeed, a 25-mer peptide (aa 6-30) corresponding the BH4 domain of Bcl-2 was sufficient to pull down the IP₃R and to inhibit IP₃R-mediated Ca²⁺ fluxes in permeabilized ⁴⁵Ca²⁺-loaded MEF cells. Importantly, adding a TAT-version of the BH4 domain of Bcl-2 to WEHI7.2 cells was sufficient to provoke inhibition of anti-CD3-induced Ca²⁺ release and apoptosis. All these effects of the BH4 domain could be suppressed by IDP, indicating that the BH4 domain targets the 20 amino acid stretch in the central, modulatory domain of the IP₃R. These experiments pointed towards an important and novel role for the BH4 domain of Bcl-2. These novel insights also indicate that IDP targets a different region on Bcl-2 than the current BH3-mimetic molecules, like ABT-737 and the orally available ABT-263, targeting the hydrophobic cleft of Bcl-2 formed by the BH1, BH2 and BH3 domain. Based on these findings, we are now combining TAT-IDP with ABT-737 to examine whether these two agents act synergistically to induce cell death in Bcl-2-dependent cancers.

4.7. THE BH4 DOMAIN BIOLOGY OF BCL-2 AND BCL-XL AT THE LEVEL OF IP₃RS IS VERY DIFFERENT

Next, we examined whether the BH4 domain of Bcl-2 and Bcl-Xl displayed similar molecular and functional properties towards IP₃Rs (115) (Figure 8).

Figure 8. Schematic overview of the structure of Bcl-2 and IP₃R1, indicating the molecular determinants involved in IP₃R1/Bcl-2-complex formation and the comparison of Bcl-2 and Bcl-Xl at the level of the BH4 domain. IDP indicates the IP₃R-derived peptide corresponding to the Bcl-2-binding site of the IP₃R. This peptide acts as a sink for Bcl-2, thereby competing for Bcl-2 binding to IP₃Rs. As a result, IDP will disrupt the binding of Bcl-2 to the IP₃R. Figure taken from Giovanni Monaco, Elke Decrock, Haidar Akl, Raf Ponsaerts, Tim Vervliet, Tomas Luyten, Marc De Maeyer, Ludwig Missiaen, Clark W Distelhorst, Humbert De Smedt, Jan B Parys, Luc Leybaert, and Geert Bultynck, Cell Death and Differentiation, 2011. doi:10.1038/cdd.2011.97. This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. Copyright © 2011, Nature Publishing Group.
This characterization is important, since there seems a consensus that Bcl-2 suppresses pro-apoptotic Ca\(^{2+}\) signals, while Bcl-XI enhances pro-survival Ca\(^{2+}\) oscillations. Furthermore, the overall structure of Bcl-2 and Bcl-XI is very similar. Nevertheless, the localization of Bcl-2 seems to differ from that of Bcl-XI. Indeed, Bcl-2 seems to be present at different intracellular compartments, Bcl-XI seem to preferentially accumulate at the mitochondria.

Importantly, our results indicate that although the BH4 domains of Bcl-2 and Bcl-XI are very similar, they differ in their ability to bind and regulate IP\(_3\)Rs. Indeed, while BH4-Bcl-2 inhibits IP\(_3\)-induced Ca\(^{2+}\) release, BH4-Bcl-XI does not (Figure 9).

Moreover, GST-pull down experiments using purified GST-IP\(_3\)R domain3 revealed an interaction of the IP\(_3\)R fragment with BH4-Bcl-2, but not with BH4-Bcl-XI. These results were confirmed by SPR experiments in which we measured the binding of purified GST-IP\(_3\)R Domain3 to biotin-BH4-Bcl-2 or biotin-BH4-Bcl-XI, immobilized on a streptavidin-coated sensor chip. Scrambled peptides were used as controls. While GST-IP\(_3\)R Domain3 specifically associated with immobilized biotin-BH4-Bcl-2, it did not with biotin-BH4-Bcl-XI.

Next, we determined the residues in BH4-Bcl-2 critical for inhibiting IP\(_3\)Rs. We identified 4 key residues that were essential for BH4-Bcl-2’s properties. Strikingly, while most residues were conserved between BH4-Bcl-2 and BH4-Bcl-XI, one of these residues was completely different. Indeed, Lys17 in BH4-Bcl-2 corresponded to Asp11 in BH4-Bcl-XI. Strikingly, changing Lys17 in BH4-Bcl-2 into an Asp completely blocked its ability to bind and inhibit IP\(_3\)Rs. In contrast, changing Asp11 in BH4-Bcl-XI into a Lys turned this peptide into an active component that is able to bind and inhibit IP\(_3\)Rs. These observations were obtained from GST-pull down assays, SPR approaches and unidirectional 45Ca\(^{2+}\)-flux assays in permeabilized MEF cells.

Finally, we assessed the anti-apoptotic properties of both BH4-Bcl-2 and BH4-Bcl-XI. We also applied an innovative electroporation approach to load the BH4-domain peptides into intact cells (116, 117). As apoptotic triggers that act through Ca\(^{2+}\) signaling, we used Cytochrome C

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**Figure 9.** Comparison of BH4-Bcl-2 and BH4-Bcl-XI regarding their ability to inhibit IP\(_3\)-induced Ca\(^{2+}\) release under unidirectional flux conditions from the ER of 45Ca\(^{2+}\)-loaded MEF cells. Figure taken from Giovanni Monaco, Elke Decrock, Haidar Akl, Raf Ponsaerts, Tim Vervliet, Tomas Luyten, Marc De Maeyer, Ludwig Missiaen, Clark W Distelhorst, Humbert De Smedt, Jan B Parys, Luc Leybaert, and Geert Bultynck, Cell Death and Differentiation, 2011. doi:10.1038/cdd.2011.97. This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. Copyright © 2011, Nature Publishing Group.
and staurosporin. We monitored cell death in the electroporated area using a DAPI staining and a caspACE-fluorescent marker. As a control, we applied the scrambled peptides of the BH4 domains. Importantly, while both BH4-Bcl-2 and BH4-Bcl-Xl protected against the apoptotic stimuli, BH4-Bcl-2 was significantly more potent than BH4-Bcl-Xl. Furthermore, the anti-apoptotic potency of BH4-Bcl-2 was reduced by changing Lys17 into an Asp, while the potency of BH4-Bcl-Xl was enhanced by changing Asp11 into a Lys. Moreover, the anti-apoptotic activity of BH4-Bcl-2 was completely alleviated by co-electroporation of IDP, while that of BH4-Bcl-Xl was not affected by the presence of IDP. These data provide strong evidence that the anti-apoptotic activity of BH4-Bcl-2 and BH4-Bcl-Xl underpins different molecular targets. While BH4-Bcl-2 targets the IP$_3$R, thereby suppressing its activity, BH4-Bcl-Xl seems to target other proteins. Given the biological context in which Bcl-Xl operates in an intact cell, we propose that BH4-Bcl-Xl might affect mitochondrial targets, like the voltage-dependent anion channel VDAC, which has been implicated in the initiation of apoptosis.

4.8. LYS17 CONTROLS THE POTENCY OF FULL-LENGTH BCL-2 TO BIND AND INHIBIT IP$_3$R AND TO PROTECT AGAINST IP$_3$R-MEDIATED APOPTOSIS

Finally, we mutated Lys17 in full-length 3xFLAG-tagged Bcl-2 and expressed both the wild-type and mutant Bcl-2 protein in COS cells. First, in GST-pull-down assays using GST-IP$_3$R Domain3, Bcl-2 K/D was much less potent than Bcl-2 in binding to the IP$_3$R fragment. Second, overexpression of Bcl-2 K/D in intact COS cells was much less effective in suppressing IP$_3$R-mediated agonist-induced Ca$^{2+}$ signaling compared to wild-type Bcl-2 (Figure 10).

**Figure 10.** Agonist-induced Ca$^{2+}$ signaling in empty vector (A), 3xFLAG-Bcl-2 (B) or 3xFLAG-Bcl-2 K/D (C)-expressing COS cells. Typical Ca$^{2+}$ traces elicited by 1 uM ATP in the absence of extracellular Ca$^{2+}$ to prevent Ca$^{2+}$ influx (addition of the Ca$^{2+}$ chelator BAPTA in extracellular medium) are depicted. Overexpression of 3xFLAG-Bcl-2 suppresses ATP-induced Ca$^{2+}$ signals, while 3xFLAG-Bcl-2 K/D is much less effective in suppressing these signals than wild-type 3xFLAG-Bcl-2. Importantly, these effects were independent of effects on the ER Ca$^{2+}$-store content, since thapsigargin-induced Ca$^{2+}$ signals were not significantly different among the different conditions. Figure taken from Giovanni Monaco, Elke Decrock, Haidar Akl, Raf Ponsaerts, Tim Vervliet, Tomas Luyten, Marc De Maeyer, Ludwig Missiaen, Clark W Distelhorst, Humbert De Smedt, Jan B Parys, Luc Leybaert, and Geert Bultynck, *Cell Death and Differentiation*, 2011. doi:10.1038/cdd.2011.97. This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. Copyright © 2011, Nature Publishing Group.
Third, Bcl-2 K/D was much less effective in protecting COS cells against staurosporin-induced apoptosis than wild-type Bcl-2. The latter findings were confirmed in WEHI7.2 cells (Figure 11), which contain very low endogenous levels of Bcl-2 using a variety of apoptosis assays [poly-(ADP-ribose)-polymerase (PARP) cleavage, caspase-3 activation and FACS analysis of Annexin V-FITC/propidium iodide-stained cells]. Collectively, these data indicate that Lys17 is a major determinant in the BH4-domain biology of Bcl-2 to bind and suppress IP$_3$Rs, and underlies an important difference in the molecular mechanisms by which Bcl-2 and Bcl-XI affect IP$_3$R-mediated Ca$^{2+}$ signaling and apoptosis.

4.9. THE RELEVANCE OF OUR FINDINGS

4.9.1. Selective regulation of IP$_3$Rs by Bcl-2 versus Bcl-XI

Our data revealed an important difference between Bcl-2 and Bcl-XI at the level of the BH4 domain. One single residue in BH4-Bcl-2 that carries an opposite charge in BH4-Bcl-XI seems to determine its selective action on the IP$_3$R. This finding is highly relevant, given the controversy in the Ca$^{2+}$-signaling field whether Bcl-2-family members prevent pro-apoptotic Ca$^{2+}$ signals, promote pro-survival Ca$^{2+}$ oscillations or lower the ER Ca$^{2+}$ content. Now, our data provide an explanation and a model for the different findings, since different labs have been using different Bcl-2-family members in their studies. Now, we found that Bcl-2 and Bcl-XI act very different at the level of their BH4 domain. Thus, we propose two binding sites for Bcl-2-family members at the IP$_3$R with distinct molecular properties both at the level of the IP$_3$R and the Bcl-2 proteins (Figure 12). Furthermore, these data may also indicate that Bcl-2 and Bcl-XI through their respective BH4 domain not only display different binding properties towards IP$_3$Rs, but also towards other targets. In this respect, a recent study of the Friedler group showed that ASPP2, a pro-apoptotic protein that binds p53, Bcl-2 and NF-KB, binds with about 10-fold higher affinity to the BH4 domain of Bcl-2 than to the BH4 domain of Bcl-XI (118). The difference was attributed to the Lys17 in the BH4 domain of Bcl-2, since mutating this residue into an alanine or aspartate residue (like in the BH4 domain of Bcl-XI) severely diminished the binding of ASPP2.

Figure 11. Staurosporin-induced apoptosis in stable WEHI7.2 cells ectopically expressing empty vector, Bcl-2 and Bcl-2 K/D. Panel A, Expression level of Bcl-2 and Bcl-2 K/D assessed by Western-blotting analysis in the different WEHI7.2 cells. Panel B, Apoptosis assay (caspase-3-mediated PARP cleavage as a measure for apoptosis) in the different WEHI7.2 cells. Overexpression of Bcl-2 prevents PARP cleavage, whereas Bcl-2 K/D is much less effective than wild-type Bcl-2. Figure taken from Giovanni Monaco, Elke Decrock, Haidar Akl, Raf Ponsaerts, Tim Vervliet, Tomas Luyten, Marc De Maeyer, Ludwig Missiaen, Clark W Distelhorst, Humbert De Smedt, Jan B Parys, Luc Leybaert, and Geert Bultynck, Cell Death and Differentiation, 2011. doi:10.1038/cdd.2011.97. This work is licensed under the Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. Copyright © 2011, Nature Publishing Group.
Hence, it is feasible that Bcl-2 and Bcl-XI counteract pro-apoptotic proteins with different potencies or even affect a different set of targets. Our results also suggest that electrostatic differentiation between pro- and anti-apoptotic proteins as observed for the interaction network may be a more common theme for selective recognition of targets that control cell death and cell survival processes (119). In our model, the central, modulatory domain of the IP$_3$R is a target for Bcl-2, but not for Bcl-XI (112, 114). The binding occurs via the BH4 domain of Bcl-2. In contrast, the C-terminal binding site identified by the Foskett group may bind both Bcl-2 and Bcl-XI (44, 45). Recently, Mcl-1 was proposed to target this C-terminal site (43). The binding likely occurs via the hydrophobic cleft of Bcl-2 and Bcl-XI, which is involved in binding, scaffolding and neutralizing the BH3 domain of pro-apoptotic Bcl-2 family members. Since the hydrophobic cleft of Bcl-2 is very similar to the one of Bcl-XI, one does not expect major differences in binding Bcl-2 and Bcl-XI at the C-terminal binding site of the IP$_3$R. In this respect, a conference report by the Foskett group proposed that the C-terminal domain of the IP$_3$R may mimic BH3-domain features of pro-apoptotic Bcl-2-family members (120). Importantly, the C-terminal domain of the IP$_3$R containing the Ca$^{2+}$-channel pore is an important nexus for gating of the channel and has been shown to interact with N-terminal IP$_3$-binding domain (121-123). Thus, one may propose that the binding of Bcl-2 or Bcl-XI at this site may enhance the efficiency of coupling between N-terminal ligand binding and C-terminal channel opening, thereby sensitizing the channel towards lower levels of IP$_3$ and thus promote pro-survival Ca$^{2+}$ oscillations. In fact, we too observed binding of both Bcl-2 and Bcl-XI at this site, confirming these findings (Monaco G et al, unpublished results). However, at the functional level, it seems that the BH4-domain biology of Bcl-2 dominates over its hydrophobic cleft biology and thus leads to inhibition of IP$_3$R-channel opening, which is
particularly beneficial to prevent apoptosis by suppressing massive \(\text{Ca}^{2+}\) fluxes from the ER into the mitochondria upon strong agonist stimulations. In contrast, the BH4-domain biology of Bcl-XI seems not to control IP\(_3\)R activity. Thus, its hydrophobic cleft biology likely will be the dominant factor leading to enhanced IP\(_3\)R activity. The latter will promote survival by energizing mitochondria through subtle \(\text{Ca}^{2+}\) oscillations that drive mitochondrial dehydrogenases and ATP-synthesizing enzymes.

4.9.2. IDP-based molecules

Taken together, results obtained from our integrated biophysical, molecular biology and cell biology approaches indicate that IDP selectively targets the BH4 domain of Bcl-2, while not affecting the anti-apoptotic activity of Bcl-XI. This is an important advantage over the current BH3-mimetic tools, which affect both Bcl-2 and Bcl-XI (124, 125). Given the cellular role of Bcl-XI in the survival of thrombocytes (126), current BH3-mimetic molecules may cause side effects, like thrombocytopenia, apoptosis and dysfunction of platelets (127-129). These might be avoided by developing tools that selectively inhibit Bcl-2’s anti-apoptotic action while maintaining essential functions of Bcl-XI for the survival of normal cells. Hence, IDP or its stabilized form serve as templates for the development of novel peptidomimetic molecules to selectively counteract Bcl-2 in Bcl-2-dependent malignancies, including CLL, diffuse large B-cell lymphoma, small lung cell carcinoma and many other cancers. In addition, IDP-based molecules may act synergistically with BH3 mimetics, thereby sensitizing cancer cells towards these BH3-mimetic molecules. In this respect, it is interesting to note that BH3-mimetic molecules too affect \(\text{Ca}^{2+}\) signaling through Bcl-2-family members by depleting ER \(\text{Ca}^{2+}\) stores (130). Thus combining BH3-mimetic compounds with IDP-based molecules will target the two functional domains of anti-apoptotic Bcl-2 proteins, the hydrophobic cleft and the BH4 domain, which likely exert distinct properties in regulating \(\text{Ca}^{2+}\)-signaling events and have distinct properties in targeting intracellular \(\text{Ca}^{2+}\)-release channels (131).

5. CONCLUSIONS

Here, we discussed the potential of peptides as novel and innovative tools to target ion channels and to modulate their activity, i.e., to enhance or inhibit their opening. We obtained the proof-of-principle for the regulation of IP\(_3\)R-based intracellular \(\text{Ca}^{2+}\)-release channels by the anti-apoptotic Bcl-2-family members. The molecular mechanisms underlying the regulation of IP\(_3\)Rs by Bcl-2 proteins were elucidated and exploited to develop cell-permeable peptides that modulate IP\(_3\)R/Bcl-2 complexes and IP\(_3\)R function in intact cells. Disturbing IP\(_3\)R/Bcl-2 complexes using IP\(_3\)R-derived peptides that target the BH4 domain of Bcl-2 caused pro-apoptotic \(\text{Ca}^{2+}\)-signaling events and resulted in apoptotic cell death in human pathophysiological conditions and malignancies that depend on elevated Bcl-2 levels, like the currently incurable cancer CLL. These insights may provide novel strategies to target Bcl-2 in malignant cells dependent on Bcl-2 up-regulation, while maintaining essential Bcl-XI functions in normal cells.
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