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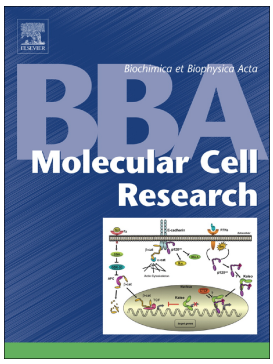


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# Cancer cell death strategies by targeting Bcl-2's BH4 domain

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## Abstract

The Bcl-2-family proteins have long been known for their role as key regulators of apoptosis. Evidence has shown that overexpression of various members of the family is associated with oncogenesis and cancer therapy resistance. Its founding member, anti-apoptotic Bcl-2 regulates cell death at different levels, whereby Bcl-2 emerged as a major drug target to eradicate cancers through cell death. This resulted in the development of venetoclax, a Bcl-2 antagonist that acts as a BH3 mimetic. Venetoclax already entered the clinic to treat relapse chronic lymphocytic leukemia patients. Here, we discuss the role of Bcl-2 as a decision-maker in cell death with focus on the recent advances in anti-cancer therapeutics that target the BH4 domain of Bcl-2, thereby interfering with non-canonical functions of Bcl-2 in $Ca^{2+}$-signaling modulation. In particular, we critically discuss previously developed tools, including the peptide BIRD-2 (Bcl-2/ $IP_3R$-disrupter-2) and the small molecule BDA-366. In addition, we present a preliminary analysis of two recently identified molecules that emerged from a molecular modelling approach to target Bcl-2's BH4 domain, which however failed to induce cell death in two Bcl-2-dependent diffuse large B-cell lymphoma cell models. Overall, antagonizing the non-canonical functions of Bcl-2 by interfering with its BH4-domain biology holds promise to elicit cell death in cancer, though improved tools and on-target antagonizing small molecules remain necessary and to be designed.

**Key words:** anti-apoptotic Bcl-2; calcium signaling; apoptosis; BIRD-2; small molecules; lymphoma

## 1. *Canonical functions of Bcl-2 in control of apoptotic cell death: BH3 profiling and BH3 mimetics*

The Bcl-2-protein family, consisting of both anti- and pro-apoptotic members, critically controls the survival of cells by enabling or preventing the onset of apoptosis [1,2] (**Figure 1 A**). This is a non-inflammatory, coordinated cell death process involving cell shrinkage, DNA fragmentation, membrane blebbing without plasma membrane permeabilization and the clearance of apoptotic bodies by phagocytic cells [3]. These processes are all mediated by the cleavage and activation of cysteinyl-aspartate proteases (caspases). Intrinsic stimuli such as DNA damage, endoplasmic reticulum (ER) stress, and extensive starvation influence the balance, localization and activity of Bcl-2-family proteins, which control apoptosis through regulation of mitochondrial outer membrane permeabilization (MOMP). MOMP is mediated by BAX and BAK, Bcl-2 family proteins, containing multiple Bcl-2-homology (BH)-domains, that form proteinaceous pores in the mitochondrial outer membrane [2,4]. The formation of such pores enables the release of the mitochondrial intermembrane space proteins (cytochrome c and Smac/DIABLO) that, in their turn, induce apoptotic caspase activity in the cytosol [5,6].

The activity of these pro-apoptotic BAX and BAK is controlled by a complex network of protein interactions that take place at the mitochondria outer membrane [1,7]. Activator BH3-only proteins such as Bim and tBid directly trigger BAX/BAK-pore formation. However, anti-apoptotic Bcl-2-family members such as Bcl-2, Bcl-xL, and Mcl-1 counteract this process by binding and neutralization of pro-apoptotic Bcl-2-family members [8]. This inhibitory process of anti-apoptotic Bcl-2 proteins depends on their hydrophobic cleft, formed by the BH3-BH1-BH2 domains, that binds the hydrophobic face of the amphipathic alpha-helical BH3 domain (**Figure 2**) [9]. The aforementioned interaction enables anti-apoptotic Bcl-2 proteins to inhibit BAX/BAK pore formation both through inhibition of activator BH3-only proteins as well as inhibition of the homodimerization of BAX/BAK [10]. Yet, the inhibitory activity of anti-apoptotic Bcl-2 can be counteracted by sensitizer BH3-only proteins, such as Bad and Bik, which are not able to directly activate Bax/Bak. It is clear that not only the BH3 domain-hydrophobic cleft interaction, but also alternative molecular interactions elicit complex formation between pro- and anti-apoptotic Bcl-2-family members [11]. The resulting complexity of interaction establishes an important feature of the Bcl-2-family-interaction network, namely the variable selectivity of each member for each interaction partner [1]. Hence, while Bim and tBid can inhibit all anti-apoptotic Bcl-2-family members, Bad mainly antagonizes Bcl-2, Bcl-xl and Bcl-w, and Noxa mainly Mcl-1 and Bfl-1 [12]. These features are likely to impact the efficacy of chemotherapeutic drugs designed to interfere with the Bcl-2 family network [13,14].

The cellular functions of Bcl-2 proteins have been linked to cancer ever since its founding member, Bcl-2, was identified as an inhibitor of apoptosis. Its discovery was linked to the t(14;18) chromosomal translocation that was observed in human follicular lymphoma cancer models, which enabled its Bcl-2 upregulation [15]. Throughout the years, additional mechanisms by which Bcl-2 is upregulated in cancer have emerged, such as the downregulation of microRNAs that target Bcl-2 mRNA's in chronic lymphocytic leukemia (CLL) [16]. Its upregulation helps cancer cells to circumvent the pro-apoptotic challenges related with oncogenesis such as increased metabolic demands and genomic aberration [17]. Nowadays, upregulation of distinct anti-apoptotic Bcl-2-family members has been observed in a broad variety of cancer types with numerous reports of Bcl-2-dependent cancers [2,18,19].

Seminal work by Letai and co-workers has revealed that cancer cells are primed to death and as such addicted to anti-apoptotic Bcl-2-family members [18]. Indeed, due to ongoing oncogenic stress, cancer cells display augmented levels of pro-apoptotic BH3-only proteins, which would elicit

apoptosis if they were not neutralized by anti-apoptotic Bcl-2 proteins. BH3 profiling is a functional approach that uses distinct sets of BH3-peptides, derived from various sensitizer BH3-only proteins (e.g. Bad, Hrk and Noxa), that antagonize distinct anti-apoptotic Bcl-2-family members (e.g. Bcl-2, Bcl-xl and Mcl-1) to determine the unique dependency of a cancer cell type towards a certain anti-apoptotic Bcl-2-family member [10,20]. Furthermore, BH3 profiling has been taken to the next level via "high throughput dynamic BH3 profiling", which enables a quick, accurate and early identification of effective chemotherapeutic treatments in patients [21,22]. The technique opens the avenue for combinatorial regiments various BH3 mimetic drugs as well as an early identification of successful therapeutic intervention.

Another outcome of the Bcl-2-family network has been the development of small molecule drugs that mimic the BH3 domain of pro-apoptotic sensitizer BH3-only proteins, generally referred to as BH3-mimetics [10]. The first drugs developed were ABT-737 and the orally available compound ABT-263 (navitoclax), which mimicked Bad's BH3 domain and thus inhibited both Bcl-2 and Bcl-xL [23,24]. These drugs proved effective in killing several Bcl-2-dependent cancers in preclinical models but their application in patients was limited as a consequence of thrombocytopenia due to the inhibition of Bcl-xL, which is essential for platelet survival [25]. This prompted the development of the selective Bcl-2 inhibitor ABT-199/venetoclax, which was shown to effectively and selectively kill CLL cells while sparing platelets [26,27]. Nowadays, venetoclax has entered the clinic to treat relapsed CLL patients and it is in advanced stage for the treatment of other cancer types, often in combination with other chemotherapeutic regiments. Interestingly, complementary dynamic BH3 profiles can be used to predict the cooperativity of BH3 mimetic drugs like venetoclax and other chemotherapeutics as was done for the multi-kinase inhibitor TG02 in acute myeloid leukemia [28]. Meanwhile, BH3 mimetic antagonists of other anti-apoptotic proteins have been developed such as A-1155463 and A-1331852 for Bcl-XL or S63845, AMG 176, and AZD5991 for Mcl-1 [29]. Finally, it is important to note that these drugs, despite being high affinity and selective inhibitors of Bcl-2 proteins, can also elicit "off-target" responses that affect cell survival. For instance, venetoclax has been shown to impair the mitochondrial metabolism and respiration by activating an integrated stress response and the ATF4 transcription factor, independently of any Bcl-2-family member [30].

### *2. Non-canonical functions of Bcl-2 in cell fate decisions: $Ca^{2+}$ signaling modulation*

It has become increasingly clear that, beyond the interactions within the Bcl-2-family, Bcl-2 proteins also partner with non-Bcl-2-family members, thereby regulating cell fate in non-canonical manners [31]. A key feature of the non-canonical regulation of cell fate is the impact of Bcl-2-family proteins on $Ca^{2+}$ homeostasis and dynamics [32] (**Figure 1 B**). Here, the ER plays a central role as the main intracellular $Ca^{2+}$-storage organelle. The intimate link between $Ca^{2+}$ signaling and cell death, primarily switched on at the level of the mitochondria, is not surprising. Indeed, the ER and mitochondria are in fact intimately connected through contact sites that are established by mitochondria-associated ER membranes (MAMs) [33]. At these sites, $Ca^{2+}$ fluxes originating from the ER can be transmitted to the mitochondria quasi-synaptically via contact sites that contain inositol 1,4,5-trisphosphate receptors ($IP_3Rs$) [34]. These are tetrameric intracellular $Ca^{2+}$-release channels located at the ER that enable the flux of $Ca^{2+}$ from the ER lumen upon binding of $IP_3$ [35]. The particular channels exist in three isoforms and are composed of several structural domains [36]. $IP_3$ binding occurs at the N-terminal region of the channel resulting in opening of the $Ca^{2+}$-channel pore located at the C-terminal part of the channel. The activation of $IP_3Rs$ leads to increased local $Ca^{2+}$ concentrations in the vicinity of the channels. At the level of the MAMs, these high [$Ca^{2+}$] microdomains are able to overcome the inherent low affinity of $Ca^{2+}$ transport across the mitochondrial inner membrane through the

mitochondrial $Ca^{2+}$ uniporter complex [37]. Transport of $Ca^{2+}$ over the mitochondrial outer membrane at the MAMs occurs via voltage-dependent anion channels type 1 (VDAC1), which are non-selective, high-conductance channels [38,39]. Evidently, $IP_3Rs$ and VDAC1 reside in a multi-protein complex that involves the chaperone GRP75 and spans the ER-mitochondrial membranes, thereby directly enhancing $Ca^{2+}$ flux into mitochondria [40]. $IP_3R3$ isoform was proposed to be the main deliverer of $Ca^{2+}$ to the mitochondria by its preferred location at the MAMs, though recent work has challenged this, and demonstrate that all three $IP_3R$ isoforms are involved in $Ca^{2+}$ flux to the mitochondria with $IP_3R2$ being most efficient [41].

Through these ER-mitochondrial $Ca^{2+}$ fluxes, cell fate processes at the mitochondria are turned on or off. Basal and low-level $Ca^{2+}$ oscillations from the ER into the mitochondria are known to stimulate mitochondrial bioenergetics, thereby promoting survival and suppressing autophagy [42]. Alternatively, excessive $Ca^{2+}$-release events can trigger mitochondrial $Ca^{2+}$ overload, thereby driving the opening of the mitochondrial permeability transition pore (mPTP) followed by mitochondrial swelling and consequently, cell death [43]. Excessive mitochondrial $Ca^{2+}$ can promote mPTP opening both directly by targeting the mPTP and indirectly as a result of $Ca^{2+}$ binding to cardiolipin followed by its dissociation from respiratory complex II of the electron transport chain, resulting in its disassembly and unrestricted reactive oxygen species production [44,45]. Generally, mechanisms that prevent mitochondrial $Ca^{2+}$ overload have been associated with apoptosis resistance, a feature that favors neoplastic behavior and oncogenesis. Yet, basal $Ca^{2+}$ transfers are essential for the survival of cancer cells in order for them to sustain the production of metabolites, enabling proper survival of new cells produced during the cell cycle. B cells lacking $IP_3Rs$ consistently display two features with regards to apoptosis: first, they are more resistant to cell death stimuli; second, they display augmented basal autophagic flux due to reduced mitochondrial bioenergetics [46]. A detailed discussion on the role of ER-mitochondrial $Ca^{2+}$ fluxes underlying cancer hallmarks and oncogenesis has been provided in recent reviews [33,47,48]. Here, we will focus on the role of Bcl-2 in cell fate decisions through $Ca^{2+}$-signaling modulation as well as the targeting methods that have emerged from it.

In the last two decades, $IP_3R$ channels have emerged as key targets of several anti-apoptotic Bcl-2 family members, thereby controlling ER $Ca^{2+}$ homeostasis and dynamics. First hints came from a study on T cell models in which Bcl-2 overexpression enabled cells to retain the ER $Ca^{2+}$ pool when challenged with $H_2O_2$ [49] or thapsigargin, an irreversible blocker of (sarco-/endoplasmic reticulum $Ca^{2+}$-ATPases) SERCA [50]. Bcl-2 was demonstrated to mediate reductions in ER $Ca^{2+}$-loading and reduced pro-apoptotic $Ca^{2+}$ flux from the ER [51]. Evaluation of ER $Ca^{2+}$ concentrations upon Bcl-2-overexpression revealed increased $Ca^{2+}$ leakage from the organelle leading to a reduced ER $Ca^{2+}$ filling state, thereby protecting the cells against cell death induced by ceramide. Different mechanisms elucidating this Bcl-2 controlled ER $Ca^{2+}$ leakage have been put forward. For one, increased $IP_3$-sensitization of $IP_3R$, caused by protein kinase A (PKA)-dependent phosphorylation of Ser1755, which is known to increase basal ER $Ca^{2+}$ leak, is decreased upon Bcl-2 knockdown [52]. Another ER $Ca^{2+}$ leak-promoting target of Bcl-2 is Bax inhibitor-1. The $Ca^{2+}$-permeable channel Bax inhibitor-1, acts as a $H^+/Ca^{2+}$ exchanger that was proposed to be essential for Bcl-2-mediated ER $Ca^{2+}$ leak [53–55]. Bcl-2's BH4 domain was found to play a central role in this regulation, considering that BH4-truncated Bcl-2 was unable to bind Bax inhibitor-1 [56]. Moreover the BH4 domain of Bcl-2, on its own, is capable of promoting Bax inhibitor-1 oligomerization and $Ca^{2+}$ leak activity [54]. Nutrient-deprivation autophagy factor-1 (Naf-1, also referred to as Cisd2) was also proposed to be required for the decrease in ER $Ca^{2+}$-store content provoked by Bcl-2 [57,58]. Of interest, Naf-1 resides in a protein complex with $IP_3Rs$ and Bcl-2, whereby Naf-1 binding to Bcl-2 in part occurs at the level Bcl-2's BH4 domain [59]. Conversely, there have been several reports indicating that Bcl-2 suppresses

$Ca^{2+}$ flux from the ER without affecting the steady-state ER $Ca^{2+}$ levels [60–64]. Altogether, these observations implied that Bcl-2 could directly affect ER $Ca^{2+}$-release channels, such as $IP_3Rs$ and ryanodine receptors (RyRs). Subsequent work revealed that Bcl-2 could functionally interact with $IP_3Rs$ and directly suppress $Ca^{2+}$-flux through the channel without lowering steady-state ER $Ca^{2+}$ levels. A very convincing piece of evidence was provided through direct single-channel measurements, in which purified Bcl-2 proteins could reduce the open probability of purified $IP_3Rs$ proteins that had been reconstituted into lipid bilayers [60].

Further research identified the molecular determinants underlying $IP_3R$/Bcl-2 complex formation. At the level of $IP_3Rs$, three Bcl-2-binding sites were identified: i. a stretch of 20 amino acids located in the central, modulatory region of $IP_3Rs$ (aa 1389-1408 in $IP_3R1$), ii. the ligand-binding domain of $IP_3R1$ (aa 226-604 in $IP_3R1$, i.e. the minimal region responsible for $IP_3$ binding), and iii. the 6$^{th}$ transmembrane domain of $IP_3R1$. At the level of Bcl-2, the N-terminal BH4 domain and the C-terminal transmembrane domain were identified as being decisive for inhibiting $IP_3Rs$ *in cellulo*. Ivanova et al (2016) demonstrated that venetoclax, a selective BH3 mimetic Bcl-2 antagonist occupying Bcl-2's hydrophobic cleft, or mutating Bcl-2's hydrophobic cleft did not interfere with the interaction of Bcl-2 with $IP_3Rs$. Moreover, Bcl-2 loaded with venetoclax or Bcl-2 with mutations in its hydrophobic cleft remained capable of inhibiting $IP_3R$-mediated $Ca^{2+}$ release [65]. Consistent with this, venetoclax did neither provoke $Ca^{2+}$ release by itself in a variety of cell models nor provoked deranged $Ca^{2+}$ signaling that was elicited by physiological agonists [66,67]. Instead, the inhibition of $IP_3R$-mediated $Ca^{2+}$ flux as well as the protection against staurosporine-induced apoptosis by Bcl-2 was severely impaired upon deletion of Bcl-2's transmembrane domain. They also established that the transmembrane domain of Bcl-2 is sufficient for $IP_3R$ binding and inhibition. Bcl-2's transmembrane domain likely assists in inhibiting $IP_3R$ channels by concentrating Bcl-2 in the proximity of the channel. This increased Bcl-2 concentration near the $Ca^{2+}$-channel thereby would allow the BH4 domain to reach its binding site effectively inhibiting $IP_3R$-mediated $Ca^{2+}$ flux despite its low affinity binding. The BH4 domain by itself can target two sites in $IP_3Rs$, namely the central, modulatory region and the ligand-binding domain [68]. Delivery of only the BH4 domain, as a synthetic peptide, is sufficient to suppress $Ca^{2+}$ flux through $IP_3Rs$ and to prevent $IP_3R/Ca^{2+}$-driven apoptosis, e.g. in T cell models exposed to strong T-cell receptor stimulation or in adherent cells exposed to staurosporine [64]. Moreover, Bcl-2 binding to the ligand-binding domain also antagonized its ability to bind $IP_3$ [69]. Since four $IP_3$ molecules are required to bind to one $IP_3R$ tetramer to open the channel, one Bcl-2 protein occupying the ligand-binding core of one $IP_3R$ monomer could be sufficient to inhibit the opening of that particular channel. Furthermore, the α-helical structure of the BH4 domain was shown to be essential in its $IP_3R$ inhibitory functioning with residue Ile14 and Val15 playing a predominant role in its formation [70]. Another residue, Lys17, also plays a key role in the BH4 domain of Bcl-2 as an essential determinant for the Bcl-2/ $IP_3R$ interaction. The Lys17 residue distinguishes Bcl-2's BH4 domain from that of other anti-apoptotic Bcl-2 proteins like Bcl-xL and it is most likely responsible for the divergence in their functioning [64]. By comparison, the Bcl-2-binding site in $IP_3Rs$ is conserved among all three isoforms, although it remains to be established whether all $IP_3R$ isoforms are equally modulated by Bcl-2. In addition, the sequence of the Bcl-2-binding site that is centrally located in the $IP_3R$ is also present in RyRs, another class of tetrameric intracellular $Ca^{2+}$-release channels mainly present in excitable cells. As such, Bcl-2 also binds to RyRs via its BH4 domain, thereby suppressing RyR-mediated $Ca^{2+}$ release [71].

Bcl-2's impact on $IP_3R$ channel functioning is even further complicated by the influence of other proteins. For example, in T-cells, Bcl-2 has been found to dock calcineurin (CaN) and DARPP-32, an inhibitor of protein phosphatase 1 that is regulated by PKA and CaN, onto $IP_3Rs$ [72,73]. The resulting multiprotein complex is thought to create a negative feedback loop by decreasing the PKA-mediated

phosphorylation of $IP_3R$ channels, resulting in an inhibition of $IP_3R$-mediated $Ca^{2+}$ flux. It is crucial that we get a grasp on the complexity of the non-canonical functions of Bcl-2 as the research may be decisive for any Bcl-2 targeting endeavor in search of novel cancer treatments.

### *3. Targeting Bcl-2 via its BH4 domain: controlled cancer cell death*

#### *I. BIRD-2*

Currently, there are no effective anti-cancer drugs designed to target Bcl-2's BH4 domain or its interaction with the $IP_3Rs$. However, the concept of BH4 domain targeting tools is on the rise with promising results from several compounds that were designed to inhibit the Bcl-2/ $IP_3Rs$ interaction. The concept that Bcl-2 binding to $IP_3Rs$ exerted anti-apoptotic effects by suppressing $Ca^{2+}$ release was further grounded by elegant studies using $IP_3R$-derived peptides (IDPs) [74]. These $IP_3R$-derived peptides represent the Bcl-2-binding site in the central, modulatory region of the $IP_3Rs$ and were developed by fusing amino acid 1389-1408 of $IP_3R1$ to a TAT sequence. By targeting Bcl-2's BH4 domain, the peptides are able to disrupt Bcl-2 binding to $IP_3Rs$. In T-cell models, application of cell-permeable versions of such peptides could revert the inhibition on $IP_3R$-mediated $Ca^{2+}$ release by Bcl-2 and subsequent apoptosis in response to strong T-cell receptor stimulation [75]. In following research, stabilized forms of cell permeable IDPs, called BIRD-2 (Bcl-2 / $IP_3$ Receptor Disrupter-2) were developed and assessed in Bcl-2-dependent cancer models [76]. The stability of the IDP was increased by a small modification of the originally discovered IDP, replacing two amino acids that may form a protease cleavage site. BIRD-2 was able to kill a wide variety of Bcl-2-dependent cancer types without the need for additional cell stimulus both *in vitro* and *in vivo*. This includes primary CLL cells from patients [75], established diffuse large B-cell lymphoma (DLBCL) cell lines [74], small cell lung cancer (SCLC) [77], multiple myeloma, follicular lymphoma [76] and ovarian cancer [78]. In these cells, BIRD-2 triggered spontaneous $IP_3R$-mediated $Ca^{2+}$ signaling and excessive $Ca^{2+}$ oscillations, which in their turn provoked cell demise through apoptosis (**Figure 2**). In primary human CLL cells, BIRD-2 treatment was found to increase phosphorylation of residue Ser1755 in $IP_3R1$ [73]. This indicates that BIRD-2 may alleviate the negative feedback regulation of Bcl-2, CaN, and DARPP-32 on $IP_3R1$, thereby augmenting the sensitivity of $IP_3R1$ towards $IP_3$ and thus potentially underlying the increased basal ER $Ca^{2+}$ leak provoked by BIRD-2 treatment [52]. Compelling results also report that local BIRD-2 treatment markedly reduces tumor growth of mouse xenografts [76]. Non-cancerous lymphocytes, alternatively, did not display such a $Ca^{2+}$ signaling-induced apoptosis upon treatment with BIRD-2.

At first, store-operated $Ca^{2+}$ entry (SOCE) was thought to be an important process in BIRD-2-induced cell death since it is known to promote ER $Ca^{2+}$ release. By contrast, it was later revealed that BIRD-2-induced cell death does not require SOCE but rather ER $Ca^{2+}$ store filling in general [79]. This conclusion was supported by a knock-down experiment of STIM1, a protein highly involved in SOCE, showing no significant effect of STIM1 on BIRD-2-induced cell death. However, using the extracellular $Ca^{2+}$ chelator EGTA, extracellular $Ca^{2+}$ was found to play a crucial role in BIRD-2-induced apoptosis. The molecular mechanism mediating this interplay remains elusive.

The mechanisms that render DLBCL cells sensitive to BIRD-2 and thus potentially primed to death at the level of the ER appear to depend on two factors: i. the high expression levels of type 2 $IP_3R$ isoforms [74]; and ii. the constitutively ongoing $IP_3$ signaling downstream of the B-cell receptor [80]. The fact that BIRD-2 sensitivity correlates with $IP_3R2$ expression is fascinating, since $IP_3R2$ is not only the $IP_3R$ isoform with the highest sensitivity to $IP_3$ but it is also the one that may be most efficient in

propelling $Ca^{2+}$ towards the mitochondria [81]. Hence, similarly to the concept of "primed to death" and Bcl-2 addiction at the level of the mitochondria, we hypothesize that cancer cells may also be "primed to death" at the level of the ER and thus addicted to ER-related functions of Bcl-2, in particular in cancer cells with elevated basal $IP_3$ signaling and high levels of $IP_3R2$ [17]. Evidently, the BIRD-2-induced caspase-dependent cell death in DLBCL and CLL was very recently shown to be caused by BIRD-2-induced mitochondrial $Ca^{2+}$ overload [82]. Both cyclosporine A, which desensitizes mPTP towards $Ca^{2+}$, and ruthenium265, a novel inhibitor of mitochondrial $Ca^{2+}$ uptake [83], effectively prevented BIRD-2-induced apoptosis, indicating the involvement of $Ca^{2+}$ overload-dependent activation of mPTP. Instead, BIRD-2-induced cell death did not depend on the presence of Bax/Bak and Bim proteins. Alternatively, in some cancers such as SCLC and multiple myeloma cells, the BIRD-2 induced cell death was demonstrated to be independent from caspase activity but rather involved the activation of $Ca^{2+}$-sensitive proteases (calpains) [76,77]. Moreover, there appears to be a negative correlation between the level of mito-priming/mito-addiction to Bcl-2 that can be targeted by BH3 mimetics such as venetoclax *versus* ER-priming/ER-addiction to Bcl-2 that can be exploited by BH4 antagonists such as BIRD-2. Indeed, cancer cells displayed a reciprocal sensitivity towards venetoclax versus BIRD-2. Hence, cancer cells that were less sensitive to venetoclax were more sensitive to BIRD-2 and *vice versa* [76,84]. Although BH3 mimetics and BIRD-2 target Bcl-2 via distinct mechanisms, there seems a synergistic interplay between BIRD-2 and BH3 mimetics when co-incubated in cancer cells at low concentrations. The observed synergistic apoptotic effect between venetoclax and BIRD-2 might be explained by a convergence in their mode of action, whereby low concentrations of BIRD-2 provoke an increase of Bim-protein levels [76,84]. This Bim upregulation by low [BIRD-2] appeared to be dependent on $Ca^{2+}$ signaling, as cell-permeable $Ca^{2+}$-buffering agents such as BAPTA-AM could prevent Bim upregulation by BIRD-2. These findings suggest that the integral role of Bcl-2 in cancer cell survival, either at the mitochondria via its hydrophobic cleft or at the ER through its BH4 domain, can be exploited in future cancer therapies. However, the reasons why certain DLBCL cancer cells upregulate $IP_3R2$ remain elusive, but it may be linked to mitochondrial metabolism and metabolic demands.

Still, cancer cells with acquired resistance to venetoclax, e.g. by culturing venetoclax-sensitive cancer cells for prolonged times with increasing venetoclax concentrations, do not become sensitized to BIRD-2 [85]. This correlated with lack of changes in $IP_3R$-expression levels, including $IP_3R2$, the key channel for BIRD-2 sensitivity. Furthermore, cells with acquired venetoclax resistance displayed upregulation of Bcl-XL and the cells could become resensitized to venetoclax through antagonizing Bcl-XL using BH3 mimetic compounds that selectively targeted Bcl-XL. Other studies report additional compensatory mechanisms linked to acquired venetoclax resistance being the upregulation of Mcl-1 as well as substantial AKT activation along with the upregulation of Bcl-XL [86,87]. Interestingly HA14-1, another BH3 mimetic drug that also targets sarco/endoplasmic reticulum $Ca^{2+}$-ATPases (SERCA), was demonstrated to potentiate BIRD-2-induced ER $Ca^{2+}$ release as well as the resulting cell death in both primary patient-derived B-CLL cells and in BIRD-2-sensitive DLBCL cells when administered at sub-lethal levels [88,89]. As of yet, HA14-1 is not used to treat cancer patients, due to its adverse effects on platelet survival and cellular homeostasis as a result of the inhibition of SERCA. Furthermore, BIRD-2 can also be used for chemotherapeutic treatment of cancers other than DLBCL or CLL. For example, SCLC cells overexpress Bcl-2 but they are also known to rapidly develop chemotherapeutic resistance, resulting in less effective long-term therapies. However, by targeting multiple functional domains of Bcl-2, BIRD-2 induced cell death in SCLC was demonstrated to work synergistically with the BH3 mimetic drug navitoclax (ABT-263) [77]. These data indicate the great potential of combining Bcl-2-targeting drugs with different properties to enhance BIRD-2-induced cell

death [90]. Altogether, these are compelling results for strong potential of BH4-domain-targeting therapeutic in anti-cancer strategies, alongside the already established BH3-mimetic therapies.

Additionally, BH4 domain-targeting therapeutics could also prove advantageous when incorporated in chemotherapeutic strategies that target proteins other than Bcl-2 family members. A good example of this is the Bruton's tyrosine kinase inhibitor, Ibrutinib, which is effective in B-cell malignancies and has been approved for use in both CLL and mantle cell lymphoma [91]. However, in recent years several cancers with resistance to the highly effective drug have turned up [92]. Interestingly, ibrutinib resistance is likely linked to increased $IP_3$ generation as a result of the concurrent increase in phospholipase C (PLCγ) signaling. Accordingly, BIRD-2 was able to induce cell death in such ibrutinib-resistant cells and ibrutinib was shown to enhance BIRD-2-induced cell death in a series of human myeloma cell lines upon cotreatment [76]. Moreover, BIRD-2 was demonstrated to enhance the effectiveness of cisplatin treatment on ovarian cancer [78]. Their synergy is not surprising considering Bcl-2 family proteins and their role in the intrinsic apoptosis pathway heavily influence the mode of action of the well-established chemotherapeutic drug. Patients that are undergoing long-term cisplatin treatment often display resistance to the drug over time. While the mechanism of cisplatin resistance in ovarian cancer is not fully understood, cisplatin-resistance is known to often involve dysregulated Bcl-2 expression levels in multiple types of cancer [93,94]. In ovarian cancer cells, cisplatin is known to induce pro-apoptotic $Ca^{2+}$ flux [95]. These apoptotic $Ca^{2+}$ fluxes from the ER as well as mitochondrial $Ca^{2+}$ overload induced by cisplatin were reportedly enhanced upon BIRD-2 treatment [78]. A synergistic effect was also observed with the BH3-mimetic drugs ABT-263 (navitoclax) and ABT-737, enhancing cisplatin sensitivity in multiple carcinomas [96,97]. These results demonstrate the possibilities of BH4-antagonizing tools and their potential in combinatorial anti-cancer treatments with other Bcl-2 antagonist such as BH3 mimetics or with other anti-cancer therapeutics such as ibrutinib and cisplatin.

*II. BDA-366*

Of course, peptides have limited therapeutic applicability, certainly, if they ought to be added at several micromolar concentrations in order to elicit a cell death response. Therefore, small molecule BH4-domain antagonists of Bcl-2 with increased potency would have enormous potential to target Bcl-2's functions at the ER and target "ER-primed" cancer cells. About 5 years ago, BDA-366 was identified in a screening as a BH4-domain antagonist that could kill Bcl-2-dependent lung cancer cells as well as multiple myeloma cells [98,99]. It was believed that by binding to the BH4 domain, BDA-366 triggered a conformational change in Bcl-2, thereby exposing its BH3 domain and turning it into a pro-apoptotic protein [100]. As such, BDA-366 sensitivity was proposed to correlate with Bcl-2-expression levels, whereby cancer cells with the highest Bcl-2-protein levels displayed the highest sensitivity to BDA-366. There were also claims that BDA-366 could disrupt $IP_3R$/Bcl-2 complexes, thereby augmenting basal cytosolic $Ca^{2+}$ concentrations [98]. However, recent work has challenged BDA-366 as an "on target" inhibitor of Bcl-2. Cell death induced by BDA-366 was. First evidence that BDA-366-induced cell death was not via selective Bcl-2 antagonism was obtained in acute lymphoblastic leukemia cells and in cell models with engineered Bcl-2 dependence, indicating that BDA-366 triggered MOMP-dependent apoptosis independently of Bcl-2 [101]. This has been further scrutinized in panel of DLBCL cell lines and primary CLL cell models, whereby apoptotic sensitivity to BDA-366 did not correlate with Bcl-2-protein levels in the respective cancer cells [102]. In fact, even cells lacking Bcl-2 displayed high sensitivity towards BDA-366. In *in vitro* assays monitoring the Bax-dependent permeabilization of liposomes, BDA-366 could neither directly activate or sensitize Bax-pore formation nor convert anti-apoptotic Bcl-2 into a pro-apoptotic protein that could activate Bax. Moreover, BDA-366 also did not affect intracellular $Ca^{2+}$ homeostasis in B-cell cancer cells. Finally, it

appeared that BDA-366 provoked Mcl-1 downregulation as well as Bcl-2 dephosphorylation without affecting Bcl-2- and Bcl-xL-protein levels. This feature could be related to its chemical structure as an anthracycline. This class of drugs elicits DNA damage and has already been used in the clinic to induce apoptosis in cancers cells. Such compounds are also known to reduce Mcl-1 levels [102]. Interestingly, DLBCL cells that acquired resistance towards venetoclax through prolonged exposure reportedly display cross-resistance towards BDA-366 [85]. Nevertheless, due to the discrepancy surrounding BDA-366's mode of action, any results concerning cross-resistance between BDA-366 and venetoclax may be unrelated to Bcl-2.

*III. Novel approaches*

A. screening

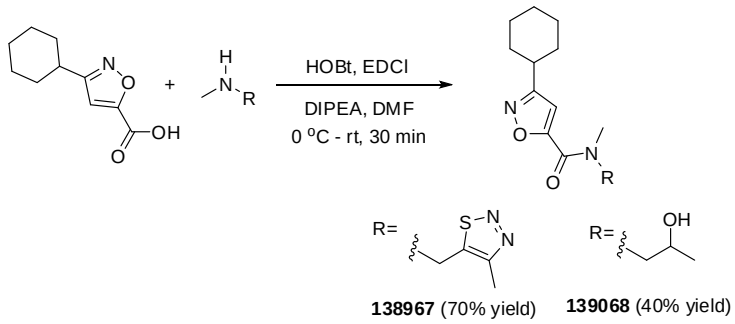
A high throughput cell-death screen, aimed at identifying BIRD-2 mimicking compounds, identified several interesting compounds that induced cell death in Bcl-2 positive multiple myeloma cells was carried out by the Distelhorst lab [103]. This compound screening consisted of a caspase activity assay, an MTS cell viability assay, and cytoplasmic $Ca^{2+}$ measurements. Upon further testing, one lead compound stood out, provoking excessive $Ca^{2+}$ signaling and cell death in both primary CLL and Jurkat cells, while not being toxic towards normal human lymphocytes. Moreover, the lead compound was also shown to induce cell death in small cell lung cancer, histiocytic lymphoma, multiple myeloma, and acute lymphoblastic leukemia. Excitingly, they also quantified the synergy between the lead compound and the BH3-mimetic drug venetoclax was quantified as well, demonstrating synergistic apoptotic effects in combination experiments. These preliminary findings underpin the feasibility of therapeutically targeting Bcl-2's non-canonical function using small molecules, although the structures of these compounds remain elusive. However, further evidence that these molecules are binding to Bcl-2 BH4 domain and disrupt $IP_3R$/Bcl-2 complexes thereby triggering pro-apoptotic $Ca^{2+}$ signaling ought to emerge. Yet, for further pharmaceutical development of clinically relevant compounds, it will be important to identify compounds that are targeting Bcl-2's BH4 domain, display sufficient potency and act in the nM range. For instance, the lead compound identified by the Distelhorst lab needed to be applied at 5 µM to kill about 50% of the multiple myeloma cells, making its use in therapeutically relevant regiments in patients very challenging.

B. Molecular modeling

In an independent work posted on bioRxiv (a preprint server for biology), a virtual screening of the SCUBIDOO database was performed to identify high affinity ligands of the BH4 domain of Bcl-2 [104]. The screening approach used BDA-366 as the standard, since it was the only reported small molecule to selectively bind Bcl-2's BH4 domain at that time, thereby yielding 11 presumed high-affinity ligands. The top three ranked compounds were compound 38831, 139068, and 138967, all with higher Bcl-2-binding affinities compared to BDA-366 in all of the performed virtual analyses (e.g. QM-polarized docking, induced-fit docking with QM/MM optimization, and molecular dynamics simulation using normal model analysis). However, the study did neither include biophysical interaction studies nor cell death studies [104]. Furthermore, we recently demonstrated that while BDA-366 could kill Bcl-2-dependent cancer cells, it acted independently of Bcl-2 [102]. Thus, also compounds based on BDA-366 ought to be critically evaluated whether they are *bona fide* BH4-domain antagonists and can kill cancer cells in a Bcl-2-dependent manner.

Therefore, we synthesized two of the top three ranking compounds (138967 and 139068) by the acid amine coupling reaction between 3-cyclohexylisoxazole-5-carboxylic acid and corresponding N-methylamines in the presence of HOBT, EDCl and DIPEA in DMF as depicted in the scheme presented in **Scheme 1**. The structures of these compounds were characterized by $^{1}H$ NMR, $^{13}C$ NMR analyses

and mass spectrometry (HRMS) (see info presented in Supplementary Material), validating their molecular identity.




**Scheme 1**. Chemical syntheses of 139068 and 138967 with indication of their yield.

Next, we scrutinized the compounds for their ability to elicit cancer cell death in two Bcl-2-dependent cancer cell models, thereby providing a preliminary analysis of their potential use to target Bcl-2-dependent cancers. Hence, we assessed the cell-death properties of compounds 139068 and 138967 in SU-DHL-4 and OCI-LY-1 cells using flow cytometry analysis of annexin V-FITC/7-Amino Actinomycin D (7-AAD)-stained cells, according to previously established methods [74,80]. We have chosen SU-DHL-4 and OCI-LY-1 for the following rationale: (i) SU-DHL-4 cells display the highest $IP_3R2$-expression levels and are highly sensitive to BIRD-2 treatment [74], while (ii) OCI-LY-1 cells display the lowest $IP_3R2$-expression levels and are rather resistant to BIRD-2 treatment. Thus, it is expected that a BH4-domain-targeting molecule would provoke cell death, at least in SU-DHL-4 cells; although if the molecules can trigger a conformational switch turning Bcl-2 into a pro-apoptotic protein, both cell models could be killed by the compounds. Additionally, both cell lines can also be distinguished via their sensitivity towards BH3 mimetic drugs, with higher levels of venetoclax-induced apoptotic cell death in OCI-LY-1 cells when compared to SU-DHL-4 cells. However, the two synthesized BH4-binding compounds, applied for 24 hours at different concentrations (up to 30 µM) did not induce apoptosis in the DLBCL models (**Figure 3**), while venetoclax, the positive control, could provoke cell death in both cancer cell models using exactly the same conditions. Hence, this first analysis argues that these compounds tested in conditions in which venetoclax did trigger cell death could not kill Bcl-2-dependent cancer cells and thus likely do not serve as BH4 inhibitors or at least not as therapeutically relevant BH4-domain inhibitors. Indeed, it remains to be determined whether the compounds can bind to Bcl-2's BH4-domain *in vitro* and whether the molecules could enter the cell models used here. However, it is important to remind that these molecules have emerged from modeling approaches using BDA-366 as a molecule targeting Bcl-2's BH4 domain. Since we and others have shown that BDA-366 is not a bona fide Bcl-2 inhibitor, it may not be surprising that these compounds are not bona fide Bcl-2 inhibitors. Thus, the first bona fide BH4-domain inhibitor of Bcl-2 remains to be developed.

## Conclusions

Anti-apoptotic Bcl-2 exerts important non-canonical anti-apoptotic functions at the level of the ER $Ca^{2+}$ stores. At the molecular level, the BH4 domain of Bcl-2 is a key determinant in this process by binding $IP_3R$ channels and inhibiting their $Ca^{2+}$-flux properties. As such, Bcl-2 protects cells at the level of the ER by suppressing pro-apoptotic $Ca^{2+}$ fluxes that can provoke cell death through mitochondrial $Ca^{2+}$ overload and/or calpain activation. Cancer cells appear to be addicted for their survival to these

ER-related functions of Bcl-2, particularly cancer cells with chronic $IP_3$ signaling and high expression levels of $IP_3R2$. Cell-permeable $IP_3R$-derived peptides corresponding to the Bcl-2-binding site on $IP_3Rs$, including stable variants such as BIRD-2, provide a unique way to target the BH4 domain of Bcl-2 and kill a broad range of cancer cell types by disassembling $IP_3R$/Bcl-2 complexes and triggering intracellular $Ca^{2+}$ overload. Furthermore, BIRD-2 could also potentiate the cell death induced by BH3 mimetics, the Bruton's tyrosine kinase inhibitor, Ibrutinib, and of chemotherapeutic regiments such as cisplatin. While these peptides are promising tools, they may also have limitations for *in vivo* use. Therefore, small molecules targeting the BH4 domain of Bcl-2 ought to be developed. However, we found in previous work that BDA-366, a presumed BH4-domain antagonist, did not interfere with Bcl-2's anti-apoptotic properties and provoked cancer cell death independently of Bcl-2. Here, we also report that two potential BH4 domain inhibitors, identified from a recent virtual screening based on BDA-366, were not effective in killing Bcl-2-dependent DLBCL cancer cell models. Overall, small molecules that target the BH4 domain of Bcl-2, thereby interfering with non-canonical functions of Bcl-2, hold great potential as means for alternative cell death strategies in cancer, including in combinatorial approaches with BH3 mimetics. Yet, the first *bona fide* small molecule BH4-domain inhibitor of Bcl-2 remains to be identified.

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[1] J. Kale, E.J. Osterlund, D.W. Andrews, BCL-2 family proteins: Changing partners in the dance towards death, Cell Death Differ. 25 (2018) 65–80. https://doi.org/10.1038/cdd.2017.186.

[2] R. Singh, A. Letai, K. Sarosiek, Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins, Nat. Rev. Mol. Cell Biol. 20 (2019) 175–193. https://doi.org/10.1038/s41580-018-0089-8.

[3] L. Galluzzi, I. Vitale, S.A. Aaronson, J.M. Abrams, D. Adam, P. Agostinis, E.S. Alnemri, L. Altucci, I. Amelio, D.W. Andrews, M. Annicchiarico-Petruzzelli, A. V. Antonov, E. Arama, E.H. Baehrecke, N.A. Barlev, N.G. Bazan, F. Bernassola, M.J.M. Bertrand, K. Bianchi, M. V. Blagosklonny, K. Blomgren, C. Borner, P. Boya, C. Brenner, M. Campanella, E. Candi, D. Carmona-Gutierrez, F. Cecconi, F.K.M. Chan, N.S. Chandel, E.H. Cheng, J.E. Chipuk, J.A. Cidlowski, A. Ciechanover, G.M. Cohen, M. Conrad, J.R. Cubillos-Ruiz, P.E. Czabotar, V. D'Angiolella, T.M. Dawson, V.L. Dawson, V. De Laurenzi, R. De Maria, K.M. Debatin, R.J. Deberardinis, M. Deshmukh, N. Di Daniele, F. Di Virgilio, V.M. Dixit, S.J. Dixon, C.S. Duckett, B.D. Dynlacht, W.S. El-Deiry, J.W. Elrod, G.M. Fimia, S. Fulda, A.J. García-Sáez, A.D. Garg, C. Garrido, E. Gavathiotis, P. Golstein, E. Gottlieb, D.R. Green, L.A. Greene, H. Gronemeyer, A. Gross, G. Hajnoczky, J.M. Hardwick, I.S. Harris, M.O. Hengartner, C. Hetz, H. Ichijo, M. Jäättelä, B. Joseph, P.J. Jost, P.P. Juin, W.J. Kaiser, M. Karin, T. Kaufmann, O. Kepp, A. Kimchi, R.N. Kitsis, D.J. Klionsky, R.A. Knight, S. Kumar, S.W. Lee, J.J. Lemasters, B. Levine, A. Linkermann, S.A. Lipton, R.A. Lockshin, C. López-Otín, S.W. Lowe, T. Luedde, E. Lugli, M. MacFarlane, F. Madeo, M. Malewicz, W. Malorni, G. Manic, J.C. Marine, S.J. Martin, J.C. Martinou, J.P. Medema, P. Mehlen, P. Meier, S. Melino, E.A. Miao, J.D. Molkentin, U.M. Moll, C. Muñoz-Pinedo, S. Nagata, G. Nuñez, A. Oberst, M. Oren, M. Overholtzer, M. Pagano, T. Panaretakis, M. Pasparakis, J.M. Penninger, D.M. Pereira, S. Pervaiz, M.E. Peter, M. Piacentini, P. Pinton, J.H.M. Prehn, H. Puthalakath, G.A. Rabinovich, M. Rehm, R. Rizzuto, C.M.P. Rodrigues, D.C. Rubinsztein, T. Rudel, K.M. Ryan, E. Sayan, L. Scorrano, F. Shao, Y. Shi, J. Silke, H.U. Simon, A. Sistigu, B.R. Stockwell, A. Strasser, G. Szabadkai, S.W.G. Tait, D. Tang, N. Tavernarakis, A. Thorburn, Y. Tsujimoto, B. Turk, T. Vanden Berghe, P. Vandenabeele, M.G. Vander Heiden, A. Villunger, H.W. Virgin, K.H. Vousden, D. Vucic, E.F. Wagner, H. Walczak, D. Wallach, Y. Wang, J.A. Wells, W. Wood, J. Yuan, Z. Zakeri, B. Zhivotovsky, L. Zitvogel, G. Melino, G. Kroemer, Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018, Cell Death Differ. 25 (2018) 486–541. https://doi.org/10.1038/s41418-017-0012-4.

[4] H. Kalkavan, D.R. Green, MOMP, cell suicide as a BCL-2 family business, Cell Death Differ. 25 (2018) 46–55. https://doi.org/10.1038/cdd.2017.179.

[5] C. Adrain, E.M. Creagh, S.J. Martin, Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2, EMBO J. 20 (2001) 6627–6636. https://doi.org/10.1093/emboj/20.23.6627.

[6] R. Santucci, F. Sinibaldi, P. Cozza, F. Polticelli, L. Fiorucci, Cytochrome c: An extreme multifunctional protein with a key role in cell fate, Int. J. Biol. Macromol. 136 (2019) 1237–1246. https://doi.org/10.1016/j.ijbiomac.2019.06.180.

[7] K.A. Sarosiek, T. Ni Chonghaile, A. Letai, Mitochondria: Gatekeepers of response to chemotherapy, Trends Cell Biol. 23 (2013) 612–619. https://doi.org/10.1016/j.tcb.2013.08.003.

[8] T. Moldoveanu, A.V. Follis, R.W. Kriwacki, D.R. Green, Many players in BCL-2 family affairs, Trends Biochem. Sci. 39 (2014) 101–111. https://doi.org/10.1016/j.tibs.2013.12.006.

[9] M.S. Davids, A. Letai, Targeting the B-cell lymphoma/leukemia 2 family in cancer, J. Clin. Oncol. 30 (2012) 3127–3135. https://doi.org/10.1200/JCO.2011.37.0981.

[10] V. Labi, F. Grespi, F. Baumgartner, A. Villunger, Targeting the Bcl-2-regulated apoptosis pathway by BH3 mimetics: A breakthrough in anticancer therapy?, Cell Death Differ. 15 (2008) 977–987. https://doi.org/10.1038/cdd.2008.37.

[11] M. Kvansakul, M.G. Hinds, The Bcl-2 family: Structures, interactions and targets for drug discovery, Apoptosis. 20 (2015) 136–150. https://doi.org/10.1007/s10495-014-1051-7.

[12] J.M. Adams, S. Cory, The BCL-2 arbiters of apoptosis and their growing role as cancer targets, Cell Death Differ. 25 (2018) 27–36. https://doi.org/10.1038/cdd.2017.161.

[13] J. Pécot, L. Maillet, J. Le Pen, C. Vuillier, S. de C. Trécesson, A. Fétiveau, K.A. Sarosiek, F.J. Bock, F. Braun, A. Letai, S.W.G. Tait, F. Gautier, P.P. Juin, Tight Sequestration of BH3 Proteins by BCL-xL at Subcellular Membranes Contributes to Apoptotic Resistance, Cell Rep. 17 (2016) 3347–3358. https://doi.org/10.1016/j.celrep.2016.11.064.

[14] Q. Liu, E.J. Oesterlund, X. Chi, J. Pogmore, B. Leber, D.W. Andrews, Bim escapes displacement by BH3- mimetic anti-cancer drugs by double-bolt locking both Bcl-XL and Bcl-2, Elife. 8 (2019). https://doi.org/10.7554/eLife.37689.

[15] C. Hua, S. Zorn, J.P. Jensen, R.W. Coupland, Hon Sum Ko, J.J. Wright, A. Bakhshi, Consequences of the t(14;18) chromosomal translocation in follicular lymphoma: Deregulated expression of a chimeric and mutated BCL-2 gene, Oncogene Res. 2 (1988) 263–275. https://europepmc.org/article/MED/3285301 (accessed December 8, 2020).

[16] K.J. Campbell, S.W.G. Tait, Targeting BCL-2 regulated apoptosis in cancer, Open Biol. 8 (2018). https://doi.org/10.1098/rsob.180002.

[17] H. Akl, T. Vervloessem, S. Kiviluoto, M. Bittremieux, J.B. Parys, H. De Smedt, G. Bultynck, A dual role for the anti-apoptotic Bcl-2 protein in cancer: Mitochondria versus endoplasmic reticulum, Biochim. Biophys. Acta - Mol. Cell Res. 1843 (2014) 2240–2252. https://doi.org/10.1016/j.bbamcr.2014.04.017.

[18] M. Certo, V.D.G. Moore, M. Nishino, G. Wei, S. Korsmeyer, S.A. Armstrong, A. Letai, Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members, Cancer Cell. 9 (2006) 351–365. https://doi.org/10.1016/j.ccr.2006.03.027.

[19] V. Del Gaizo Moore, K.D. Schlis, S.E. Sallan, S.A. Armstrong, A. Letai, BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia, Blood. 111 (2008) 2300–2309. https://doi.org/10.1182/blood-2007-06-098012.

[20] M. Butterworth, A. Pettitt, S. Varadarajan, G.M. Cohen, BH3 profiling and a toolkit of BH3-mimetic drugs predict anti-apoptotic dependence of cancer cells, Br. J. Cancer. 114 (2016) 638–641. https://doi.org/10.1038/bjc.2016.49.

[21] J. Montero, A. Letai, Dynamic BH3 profiling-poking cancer cells with a stick, Mol. Cell. Oncol. 3 (2016). https://doi.org/10.1080/23723556.2015.1040144.

[22] P.D. Bhola, E. Ahmed, J.L. Guerriero, E. Sicinska, E. Su, E. Lavrova, J. Ni, O. Chipashvili, T. Hagan, M.S. Pioso, K. McQueeney, K. Ng, A.J. Aguirre, J.M. Cleary, D. Cocozziello, A. Sotayo, J. Ryan, J.J. Zhao, A. Letai, High-throughput dynamic BH3 profiling may quickly and accurately predict effective therapies in solid tumors, Sci. Signal. 13 (2020) 1–12. https://doi.org/10.1126/scisignal.aaz5599.

[23] T. Oltersdorf, S.W. Elmore, A.R. Shoemaker, R.C. Armstrong, D.J. Augeri, B.A. Belli, M. Bruncko, T.L. Deckwerth, J. Dinges, P.J. Hajduk, M.K. Joseph, S. Kitada, S.J. Korsmeyer, A.R. Kunzer, A. Letai, C. Li, M.J. Mitten, D.G. Nettesheim, S.C. Ng, P.M. Nimmer, J.M. O'Connor, A. Oleksijew, A.M. Petros, J.C. Reed, W. Shen, S.K. Tahir, C.B. Thompson, K.J. Tomaselli, B. Wang,

M.D. Wendt, H. Zhang, S.W. Fesik, S.H. Rosenberg, An inhibitor of Bcl-2 family proteins induces regression of solid tumours, Nature. 435 (2005) 677–681. https://doi.org/10.1038/nature03579.

[24] C. Tse, A.R. Shoemaker, J. Adickes, M.G. Anderson, J. Chen, S. Jin, E.F. Johnson, K.C. Marsh, M.J. Mitten, P. Nimmer, L. Roberts, S.K. Tahir, Y. Xiao, X. Yang, H. Zhang, S. Fesik, S.H. Rosenberg, S.W. Elmore, ABT-263: A potent and orally bioavailable Bcl-2 family inhibitor, Cancer Res. 68 (2008) 3421–3428. https://doi.org/10.1158/0008-5472.CAN-07-5836.

[25] M. Vogler, H.A. Hamali, X.M. Sun, E.T.W. Bampton, D. Dinsdale, R.T. Snowden, M.J.S. Dyer, A.H. Goodall, G.M. Cohen, BCL2/BCL-XL inhibition induces apoptosis, disrupts cellular calcium homeostasis, and prevents platelet activation, Blood. 117 (2011) 7145–7154. https://doi.org/10.1182/blood-2011-03-344812.

[26] A.J. Souers, J.D. Leverson, E.R. Boghaert, S.L. Ackler, N.D. Catron, J. Chen, B.D. Dayton, H. Ding, S.H. Enschede, W.J. Fairbrother, D.C.S. Huang, S.G. Hymowitz, S. Jin, S.L. Khaw, P.J. Kovar, L.T. Lam, J. Lee, H.L. Maecker, K.C. Marsh, K.D. Mason, M.J. Mitten, P.M. Nimmer, A. Oleksijew, C.H. Park, C.M. Park, D.C. Phillips, A.W. Roberts, D. Sampath, J.F. Seymour, M.L. Smith, G.M. Sullivan, S.K. Tahir, C. Tse, M.D. Wendt, Y. Xiao, J.C. Xue, H. Zhang, R.A. Humerickhouse, S.H. Rosenberg, S.W. Elmore, ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets, Nat. Med. 19 (2013) 202–208. https://doi.org/10.1038/nm.3048.

[27] M.A. Debrincat, I. Pleines, M. Lebois, R.M. Lane, M.L. Holmes, J. Corbin, C.J. Vandenberg, W.S. Alexander, A.P. Ng, A. Strasser, P. Bouillet, M. Sola-Visner, B.T. Kile, E.C. Josefsson, BCL-2 is dispensable for thrombopoiesis and platelet survival, Cell Death Dis. 6 (2015). https://doi.org/10.1038/cddis.2015.97.

[28] M. Pallis, F. Burrows, J. Ryan, M. Grundy, C. Seedhouse, A. Abdul-Aziz, J. Montero, A. Letai, N. Russell, Complementary dynamic BH3 profiles predict co-operativity between the multi-kinase inhibitor TG02 and the BH3 mimetic ABT-199 in acute myeloid leukaemia cells, Oncotarget. 8 (2017) 16220–16232. https://doi.org/10.18632/oncotarget.8742.

[29] D. Merino, G.L. Kelly, G. Lessene, A.H. Wei, A.W. Roberts, A. Strasser, BH3-Mimetic Drugs: Blazing the Trail for New Cancer Medicines, Cancer Cell. 34 (2018) 879–891. https://doi.org/10.1016/j.ccell.2018.11.004.

[30] A. Roca-Portoles, C. Rodriguez-Blanco, D. Sumpton, C. Cloix, M. Mullin, G.M. Mackay, K. O'Neill, L. Lemgruber, X. Luo, S.W.G. Tait, Venetoclax causes metabolic reprogramming independent of BCL-2 inhibition, Cell Death Dis. 11 (2020). https://doi.org/10.1038/s41419-020-02867-2.

[31] A. Gross, S.G. Katz, Non-apoptotic functions of BCL-2 family proteins, Cell Death Differ. 24 (2017) 1348–1358. https://doi.org/10.1038/cdd.2017.22.

[32] S.J.F. Chong, S. Marchi, G. Petroni, G. Kroemer, L. Galluzzi, S. Pervaiz, Noncanonical Cell Fate Regulation by Bcl-2 Proteins, Trends Cell Biol. 30 (2020) 537–555. https://doi.org/10.1016/j.tcb.2020.03.004.

[33] G. Morciano, S. Marchi, C. Morganti, L. Sbano, M. Bittremieux, M. Kerkhofs, M. Corricelli, A. Danese, A. Karkucinska-Wieckowska, M.R. Wieckowski, G. Bultynck, C. Giorgi, P. Pinton, Role of Mitochondria-Associated ER Membranes in Calcium Regulation in Cancer-Specific Settings, Neoplasia (United States). 20 (2018) 510–523. https://doi.org/10.1016/j.neo.2018.03.005.

[34] G. Csordás, A.P. Thomas, G. Hajnóczky, Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria, EMBO J. 18 (1999) 96–108.

https://doi.org/10.1093/emboj/18.1.96.

[35] J.K. Foskett, C. White, K.H. Cheung, D.O.D. Mak, Inositol trisphosphate receptor $Ca^{2+}$ release channels, Physiol. Rev. 87 (2007) 593–658. https://doi.org/10.1152/physrev.00035.2006.

[36] M.R. Baker, G. Fan, I.I. Serysheva, Structure of $IP_3R$ channel: high-resolution insights from cryo-EM, Curr. Opin. Struct. Biol. 46 (2017) 38–47. https://doi.org/10.1016/j.sbi.2017.05.014.

[37] J. Kevin Foskett, B. Philipson, The mitochondrial $Ca^{2+}$ uniporter complex, J. Mol. Cell. Cardiol. 78 (2015) 3–8. https://doi.org/10.1016/j.yjmcc.2014.11.015.

[38] D. Gincel, H. Zaid, V. Shoshan-Barmatz, Calcium binding and translocation by the voltage-dependent anion channel: A possible regulatory mechanism in mitochondrial function, Biochem. J. 358 (2001) 147–155. https://doi.org/10.1042/0264-6021:3580147.

[39] W. Tan, M. Colombini, VDAC closure increases calcium ion flux, Biochim. Biophys. Acta - Biomembr. 1768 (2007) 2510–2515. https://doi.org/10.1016/j.bbamem.2007.06.002.

[40] G. Szabadkai, K. Bianchi, P. Várnai, D. De Stefani, M.R. Wieckowski, D. Cavagna, A.I. Nagy, T. Balla, R. Rizzuto, Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial $Ca^{2+}$ channels, J. Cell Biol. 175 (2006) 901–911. https://doi.org/10.1083/jcb.200608073.

[41] D.A. Gomes, M. Thompson, N.C. Souto, T.S. Goes, A.M. Goes, M.A. Rodrigues, M. V. Gomez, M.H. Nathanson, M.F. Leite, The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic $Ca^{2+}$ signals into mitochondria, J. Biol. Chem. 280 (2005) 40892–40900. https://doi.org/10.1074/jbc.M506623200.

[42] A. Rossi, P. Pizzo, R. Filadi, Calcium, mitochondria and cell metabolism: A functional triangle in bioenergetics, Biochim. Biophys. Acta - Mol. Cell Res. 1866 (2019) 1068–1078. https://doi.org/10.1016/j.bbamcr.2018.10.016.

[43] H.K. Baumgartner, J. V. Gerasimenko, C. Thorne, P. Ferdek, T. Pozzan, A. V. Tepikin, O.H. Petersen, R. Sutton, A.J.M. Watson, O. V. Gerasimenko, Calcium elevation in mitochondria is the main $Ca^{2+}$ requirement for mitochondrial permeability transition pore (mPTP) opening, J. Biol. Chem. 284 (2009) 20796–20803. https://doi.org/10.1074/jbc.M109.025353.

[44] M.S. Hwang, C.T. Schwall, E. Pazarentzos, C. Datler, N.N. Alder, S. Grimm, Mitochondrial $Ca^{2+}$ influx targets cardiolipin to disintegrate respiratory chain complex II for cell death induction, Cell Death Differ. 21 (2014) 1733–1745. https://doi.org/10.1038/cdd.2014.84.

[45] V. Giorgio, V. Burchell, M. Schiavone, C. Bassot, G. Minervini, V. Petronilli, F. Argenton, M. Forte, S. Tosatto, G. Lippe, P. Bernardi, Ca 2+ binding to F-ATP synthase β subunit triggers the mitochondrial permeability transition , EMBO Rep. 18 (2017) 1065–1076. https://doi.org/10.15252/embr.201643354.

[46] C. Cárdenas, R.A. Miller, I. Smith, T. Bui, J. Molgó, M. Müller, H. Vais, K.H. Cheung, J. Yang, I. Parker, C.B. Thompson, M.J. Birnbaum, K.R. Hallows, J.K. Foskett, Essential Regulation of Cell Bioenergetics by Constitutive InsP3 Receptor $Ca^{2+}$ Transfer to Mitochondria, Cell. 142 (2010) 270–283. https://doi.org/10.1016/j.cell.2010.06.007.

[47] H. Ivanova, M. Kerkhofs, R.M.L. Rovere, G. Bultynck, Endoplasmic reticulum-mitochondrial $Ca^{2+}$ fluxes underlying cancer cell survival, Front. Oncol. 7 (2017). https://doi.org/10.3389/fonc.2017.00070.

[48] M. Kerkhofs, M. Bittremieux, G. Morciano, C. Giorgi, P. Pinton, J.B. Parys, G. Bultynck, Emerging molecular mechanisms in chemotherapy: $Ca^{2+}$ signaling at the mitochondria-associated endoplasmic reticulum membranes, Cell Death Dis. 9 (2018).

https://doi.org/10.1038/s41419-017-0179-0.

[49] C. Distelhorst, M. Lam, T. Mccormick, Bcl-2 inhibits hydrogen peroxide-induced ER $Ca^{2+}$ pool depletion., Undefined. (1996).

[50] H. He, M. Lam, T.S. McCormick, C.W. Distelhorst, Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl- 2, J. Cell Biol. 138 (1997) 1219–1228. https://doi.org/10.1083/jcb.138.6.1219.

[51] P. Pinton, R. Rizzuto, Bcl-2 and $Ca^{2+}$ homeostasis in the endoplasmic reticulum, Cell Death Differ. 13 (2006) 1409–1418. https://doi.org/10.1038/sj.cdd.4401960.

[52] S.A. Oakes, L. Scorrano, J.T. Opferman, M.C. Bassik, M. Nishino, T. Pozzan, S.J. Korsmeyer, Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 105–110. https://doi.org/10.1073/pnas.0408352102.

[53] C. Xu, W. Xu, A.E. Palmer, J.C. Reed, BI-1 regulates endoplasmic reticulum $Ca^{2+}$ homeostasis downstream of Bcl-2 family proteins, J. Biol. Chem. 283 (2008) 11477–11484. https://doi.org/10.1074/jbc.M708385200.

[54] T. Ahn, C.H. Yun, H.R. Kim, H.J. Chae, Cardiolipin, phosphatidylserine, and BH4 domain of Bcl-2 family regulate $Ca^{2+}/H^{+}$ antiporter activity of human Bax inhibitor-1, Cell Calcium. 47 (2010) 387–396. https://doi.org/10.1016/j.ceca.2010.02.005.

[55] G. Bultynck, S. Kiviluoto, N. Henke, H. Ivanova, L. Schneider, V. Rybalchenko, T. Luyten, K. Nuyts, W. De Borggraeve, I. Bezprozvanny, J.B. Parys, H. De Smedt, L. Missiaen, A. Methner, The C terminus of bax inhibitor-1 forms a Ca 2+-permeable channel pore, J. Biol. Chem. 287 (2012) 2544–2557. https://doi.org/10.1074/jbc.M111.275354.

[56] Q. Xu, J.C. Reed, Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast, Mol. Cell. 1 (1998) 337–346. https://doi.org/10.1016/S1097-2765(00)80034-9.

[57] N.C. Chang, M. Nguyen, M. Germain, G.C. Shore, Antagonism of Beclin 1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1, EMBO J. 29 (2010) 606–618. https://doi.org/10.1038/emboj.2009.369.

[58] N.C. Chang, M. Nguyen, J. Bourdon, P.A. Risse, J. Martin, G. Danialou, R. Rizzuto, B.J. Petrof, G.C. Shore, BCL-2-associated autophagy regulator NAF-1 required for maintenance of skeletal muscle, Hum. Mol. Genet. 21 (2012) 2277–2287. https://doi.org/10.1093/hmg/dds048.

[59] S. Tamir, S. Rotem-Bamberger, C. Katz, F. Morcos, K.L. Hailey, J.A. Zuris, C. Wang, A.R. Conlan, C.H. Lipper, M.L. Paddock, R. Mittler, J.N. Onuchicc, P.A. Jennings, A. Friedler, R. Nechushtai, Integrated strategy reveals the protein interface between cancer targets Bcl-2 and NAF-1, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 5177–5182. https://doi.org/10.1073/pnas.1403770111.

[60] R. Chen, I. Valencia, F. Zhong, K.S. McColl, H.L. Roderick, M.D. Bootman, M.J. Berridge, S.J. Conway, A.B. Holmes, G.A. Mignery, P. Velez, C.W. Distelhorst, Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate, J. Cell Biol. 166 (2004) 193–203. https://doi.org/10.1083/jcb.200309146.

[61] F. Zhong, M.C. Davis, K.S. McColl, C.W. Distelhorst, Bcl-2 differentially regulates $Ca^{2+}$ signals according to the strength of T cell receptor activation, J. Cell Biol. 172 (2006) 127–137. https://doi.org/10.1083/jcb.200506189.

[62] C.J. Hanson, M.D. Bootman, C.W. Distelhorst, R.J.H. Wojcikiewicz, H.L. Roderick, Bcl-2 suppresses $Ca^{2+}$ release through inositol 1,4,5-trisphosphate receptors and inhibits $Ca^{2+}$ uptake by mitochondria without affecting ER calcium store content, Cell Calcium. 44 (2008) 324–338. https://doi.org/10.1016/j.ceca.2008.01.003.

[63] Y.P. Rong, A.S. Aromolaran, G. Bultynck, F. Zhong, X. Li, K. McColl, S. Matsuyama, S. Herlitze, H.L. Roderick, M.D. Bootman, G.A. Mignery, J.B. Parys, H. De Smedt, C.W. Distelhorst, Targeting Bcl-2-IP3 Receptor Interaction to Reverse Bcl-2's Inhibition of Apoptotic Calcium Signals, Mol. Cell. 31 (2008) 255–265. https://doi.org/10.1016/j.molcel.2008.06.014.

[64] G. Monaco, E. Decrock, H. Akl, R. Ponsaerts, T. Vervliet, T. Luyten, M. De Maeyer, L. Missiaen, C.W. Distelhorst, H. De Smedt, J.B. Parys, L. Leybaert, G. Bultynck, Selective regulation of IP 3-receptor-mediated Ca 2+ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-Xl, Cell Death Differ. 19 (2012) 295–309. https://doi.org/10.1038/cdd.2011.97.

[65] H. Ivanova, A. Ritane, L. Wagner, T. Luyten, G. Shapovalov, K. Welkenhuyzen, B. Seitaj, G. Monaco, H. De Smedt, N. Prevarskaya, D.I. Yule, J.B. Parys, G. Bultynck, The trans-membrane domain of Bcl-2a, but not its hydrophobic cleft, is a critical determinant for efficient IP3 receptor inhibition, Oncotarget. 7 (2016) 55704–55720. https://doi.org/10.18632/oncotarget.11005.

[66] T. Vervloessem, H. Ivanova, T. Luyten, J.B. Parys, G. Bultynck, The selective Bcl-2 inhibitor venetoclax, a BH3 mimetic, does not dysregulate intracellular $Ca^{2+}$ signaling, Biochim. Biophys. Acta - Mol. Cell Res. 1864 (2017) 968–976. https://doi.org/10.1016/j.bbamcr.2016.11.024.

[67] M.A. Jakubowska, M. Kerkhofs, C. Martines, D.G. Efremov, J. V. Gerasimenko, O. V. Gerasimenko, O.H. Petersen, G. Bultynck, T. Vervliet, P.E. Ferdek, ABT-199 (Venetoclax), a BH3-mimetic Bcl-2 inhibitor, does not cause $Ca^{2+}$-signalling dysregulation or toxicity in pancreatic acinar cells, Br. J. Pharmacol. 176 (2019) 4402–4415. https://doi.org/10.1111/bph.14505.

[68] Y.P. Rong, G. Bultynck, A.S. Aromolaran, F. Zhong, J.B. Parys, H. De Smedt, G.A. Mignery, H.L. Roderick, M.D. Bootman, C.W. Distelhorst, The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP3 receptor, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14397–14402. https://doi.org/10.1073/pnas.0907555106.

[69] H. Ivanova, L.E. Wagner, A. Tanimura, E. Vandermarliere, T. Luyten, K. Welkenhuyzen, K.J. Alzayady, L. Wang, K. Hamada, K. Mikoshiba, H. De Smedt, L. Martens, D.I. Yule, J.B. Parys, G. Bultynck, Bcl-2 and IP3 compete for the ligand-binding domain of $IP_3Rs$ modulating $Ca^{2+}$ signaling output, Cell. Mol. Life Sci. 76 (2019) 3843–3859. https://doi.org/10.1007/s00018-019-03091-8.

[70] G. Monaco, E. Decrock, K. Nuyts, L.E. Wagner, T. Luyten, S. V. Strelkov, L. Missiaen, W.M. De Borggraeve, L. Leybaert, D.I. Yule, H. De Smedt, J.B. Parys, G. Bultynck, Alpha-Helical Destabilization of the Bcl-2-BH4-Domain Peptide Abolishes Its Ability to Inhibit the IP3 Receptor, PLoS One. 8 (2013). https://doi.org/10.1371/journal.pone.0073386.

[71] T. Vervliet, E. Decrock, J. Molgó, V. Sorrentino, L. Missiaen, L. Leybaert, H. De Smedt, N.N. Kasri, J.B. Parys, G. Bultynck, Bcl-2 binds to and inhibits ryanodine receptors, J. Cell Sci. 127 (2014) 2782–2792. https://doi.org/10.1242/jcs.150011.

[72] G. Bultynck, E. Vermassen, K. Szlufcik, P. De Smet, R.A. Fissore, G. Callewaert, L. Missiaen, H. De Smedt, J.B. Parys, Calcineurin and intracellular $Ca^{2+}$-release channels: Regulation or association?, Biochem. Biophys. Res. Commun. 311 (2003) 1181–1193. https://doi.org/10.1016/j.bbrc.2003.08.084.

[73] M.J. Chang, F. Zhong, A.R. Lavik, J.B. Parys, M.J. Berridge, C.W. Distelhorst, Feedback regulation mediated by Bcl-2 and DARPP-32 regulates inositol 1,4,5-trisphosphate receptor phosphorylation and promotes cell survival, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 1186–1191. https://doi.org/10.1073/pnas.1323098111.

[74] H. Akl, G. Monaco, R. La Rovere, K. Welkenhuyzen, S. Kiviluoto, T. Vervliet, J. Molgó, C.W. Distelhorst, L. Missiaen, K. Mikoshiba, J.B. Parys, H. De Smedt, G. Bultynck, $IP_3R2$ levels dictate the apoptotic sensitivity of diffuse large B-cell lymphoma cells to an $IP_3R$-derived peptide targeting the BH4 domain of Bcl-2, Cell Death Dis. 4 (2013) e632. https://doi.org/10.1038/cddis.2013.140.

[75] F. Zhong, M.W. Harr, G. Bultynck, G. Monaco, J.B. Parys, H. De Smedt, Y.P. Rong, J.K. Molitoris, M. Lam, C. Ryder, S. Matsuyama, C.W. Distelhorst, Induction of $Ca^{2+}$-driven apoptosis in chronic lymphocytic leukemia cells by peptide-mediated disruption of Bcl-2-IP3 receptor interaction, Blood. 117 (2011) 2924–2934. https://doi.org/10.1182/blood-2010-09-307405.

[76] A.R. Lavik, F. Zhong, M.J. Chang, E. Greenberg, Y. Choudhary, M.R. Smith, K.S. McColl, J. Pink, F.J. Reu, S. Matsuyama, C.W. Distelhorst, A synthetic peptide targeting the BH4 domain of Bcl-2 induces apoptosis in multiple myeloma and follicular lymphoma cells alone or in combination with agents targeting the BH3-binding pocket of Bcl-2, Oncotarget. 6 (2015) 27388–27402. https://doi.org/10.18632/oncotarget.4482.

[77] E.F. Greenberg, K.S. McColl, F. Zhong, G. Wildey, A. Dowlati, C.W. Distelhorst, Synergistic killing of human small cell lung cancer cells by the Bcl-2-inositol 1,4,5-trisphosphate receptor disruptor BIRD-2 and the BH3-mimetic ABT-263, Cell Death Dis. 6 (2015) e2034. https://doi.org/10.1038/cddis.2015.355.

[78] Q. Xie, Y. Xu, W. Gao, Y. Zhang, J. Su, Y. Liu, Y. Guo, M. Dou, K. Hu, L. Sun, TAT-fused $IP_3R$-derived peptide enhances cisplatin sensitivity of ovarian cancer cells by increasing ER $Ca^{2+}$ release, Int. J. Mol. Med. 41 (2018) 809–817. https://doi.org/10.3892/ijmm.2017.3260.

[79] M. Bittremieux, R.M. La Rovere, M. Schuermans, T. Luyten, K. Mikoshiba, P. Vangheluwe, J.B. Parys, G. Bultynck, Extracellular and ER-stored $Ca^{2+}$ contribute to BIRD-2-induced cell death in diffuse large B-cell lymphoma cells, Cell Death Discov. 4 (2018). https://doi.org/10.1038/s41420-018-0118-6.

[80] M. Bittremieux, R.M. La Rovere, H. Akl, C. Martines, K. Welkenhuyzen, K. Dubron, M. Baes, A. Janssens, P. Vandenberghe, L. Laurenti, K. Rietdorf, G. Morciano, P. Pinton, K. Mikoshiba, M.D. Bootman, D.G. Efremov, H. De Smedt, J.B. Parys, G. Bultynck, Constitutive IP 3 signaling underlies the sensitivity of B-cell cancers to the Bcl-2/IP 3 receptor disruptor BIRD-2, Cell Death Differ. 26 (2019) 531–547. https://doi.org/10.1038/s41418-018-0142-3.

[81] A. Bartok, D. Weaver, T. Golenár, Z. Nichtova, M. Katona, S. Bánsághi, K.J. Alzayady, V.K. Thomas, H. Ando, K. Mikoshiba, S.K. Joseph, D.I. Yule, G. Csordás, G. Hajnóczky, IP3 receptor isoforms differently regulate ER-mitochondrial contacts and local calcium transfer, Nat. Commun. 10 (2019). https://doi.org/10.1038/s41467-019-11646-3.

[82] M. Kerkhofs, R. La Rovere, K. Welkenhuysen, A. Janssens, P. Vandenberghe, M. Madesh, J.B. Parys, G. Bultynck, BIRD-2, a BH4-domain-targeting peptide of Bcl-2, provokes Bax/Bak-independent cell death in B-cell cancers through mitochondrial $Ca^{2+}$-dependent mPTP opening, Cell Calcium. 94 (2021) 102333. https://doi.org/10.1016/j.ceca.2020.102333.

[83] J.J. Woods, N. Nemani, S. Shanmughapriya, A. Kumar, M. Zhang, S.R. Nathan, M. Thomas, E. Carvalho, K. Ramachandran, S. Srikantan, P.B. Stathopulos, J.J. Wilson, M. Madesh, A Selective and Cell-Permeable Mitochondrial Calcium Uniporter (MCU) Inhibitor Preserves Mitochondrial Bioenergetics after Hypoxia/Reoxygenation Injury, ACS Cent. Sci. 5 (2019) 153–166.

https://doi.org/10.1021/acscentsci.8b00773.

[84] T. Vervloessem, H. Akl, T. Tousseyn, H. De Smedt, J.B. Parys, G. Bultynck, Reciprocal sensitivity of diffuse large B-cell lymphoma cells to Bcl-2 inhibitors BIRD-2 versus venetoclax, Oncotarget. 8 (2017) 111656–111671. https://doi.org/10.18632/oncotarget.22898.

[85] M. Kerkhofs, T. Vervloessem, K.B. Stopa, V.M. Smith, M. Vogler, G. Bultynck, Dlbcl cells with acquired resistance to venetoclax are not sensitized to bird-2 but can be resensitized to venetoclax through BCL-XL inhibition, Biomolecules. 10 (2020) 1–13. https://doi.org/10.3390/biom10071081.

[86] G.S. Choudhary, S. Al-Harbi, S. Mazumder, B.T. Hill, M.R. Smith, J. Bodo, E.D. Hsi, A. Almasan, MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies, Cell Death Dis. 6 (2015). https://doi.org/10.1038/cddis.2014.525.

[87] I. Kapoor, J. Bodo, B.T. Hill, E.D. Hsi, A. Almasan, Targeting BCL-2 in B-cell malignancies and overcoming therapeutic resistance, Cell Death Dis. 11 (2020). https://doi.org/10.1038/s41419-020-03144-y.

[88] J.L. Wang, D. Liu, Z.J. Zhang, S. Shan, X. Han, S.M. Srinivasula, C.M. Croce, E.S. Alnemri, Z. Huang, Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7124–7129. https://doi.org/10.1073/pnas.97.13.7124.

[89] H. Akl, R.M.L. La Rovere, A. Janssens, P. Vandenberghe, J.B. Parys, G. Bultynck, HA14-1 potentiates apoptosis in B-cell cancer cells sensitive to a peptide disrupting IP3 receptor / Bcl-2 complexes, Int. J. Dev. Biol. 59 (2015) 391–398. https://doi.org/10.1387/ijdb.150213gb.

[90] H. Akl, I. Vandecaetsbeek, G. Monaco, A. Kauskot, T. Luyten, K. Welkenhuyzen, M. Hoylaerts, H.D. De Smedt, J.B. Parys, G. Bultynck, HA14-1, but not the BH3 mimetic ABT-737, causes $Ca^{2+}$ dysregulation in platelets and human cell lines, Haematologica. 98 (2013) e49. https://doi.org/10.3324/haematol.2012.080598.

[91] Y.T. Tai, K.C. Anderson, Bruton's tyrosine kinase: Oncotarget in myeloma, Oncotarget. 3 (2012) 913–914. https://doi.org/10.18632/oncotarget.655.

[92] J. Ma, P. Lu, A. Guo, S. Cheng, H. Zong, P. Martin, M. Coleman, Y.L. Wang, Characterization of ibrutinib-sensitive and -resistant mantle lymphoma cells, Br. J. Haematol. 166 (2014) 849–861. https://doi.org/10.1111/bjh.12974.

[93] H.J. Cho, J.K. Kim, K.D. Kim, H.K. Yoon, M.Y. Cho, Y.P. Park, J.H. Jeon, E.S. Lee, S.S. Byun, H.M. Lim, E.Y. Song, J.S. Lim, D.Y. Yoon, H.G. Lee, Y.K. Choe, Upregulation of Bcl-2 is associated with cisplatin-resistance via inhibition of Bax translocation in human bladder cancer cells, Cancer Lett. 237 (2006) 56–66. https://doi.org/10.1016/j.canlet.2005.05.039.

[94] G. Leisching, B. Loos, M. Botha, A.M. Engelbrecht, Bcl-2 confers survival in cisplatin treated cervical cancer cells: Circumventing cisplatin dose-dependent toxicity and resistance, J. Transl. Med. 13 (2015) 328. https://doi.org/10.1186/s12967-015-0689-4.

[95] Y. Xu, C. Wang, J. Su, Q. Xie, L. Ma, L. Zeng, Y. Yu, S. Liu, S. Li, Z. Li, L. Sun, Tolerance to endoplasmic reticulum stress mediates cisplatin resistance in human ovarian cancer cells by maintaining endoplasmic reticulum and mitochondrial homeostasis, Oncol. Rep. 34 (2015) 3051–3060. https://doi.org/10.3892/or.2015.4283.

[96] M. Wong, N. Tan, J. Zha, F. V. Peale, P. Yue, W.J. Fairbrother, L.D. Belmont, Navitoclax (ABT-263) reduces Bcl-x L-mediated chemoresistance in ovarian cancer models, Mol. Cancer Ther.

11 (2012) 1026–1035. https://doi.org/10.1158/1535-7163.MCT-11-0693.

[97] Z. Fan, H. Yu, N. Cui, X. Kong, X. Liu, Y. Chang, Y. Wu, L. Sun, G. Wang, ABT737 enhances cholangiocarcinoma sensitivity to cisplatin through regulation of mitochondrial dynamics, Exp. Cell Res. 335 (2015) 68–81. https://doi.org/10.1016/j.yexcr.2015.04.016.

[98] B. Han, D. Park, R. Li, M. Xie, T.K. Owonikoko, G. Zhang, G.L. Sica, C. Ding, J. Zhou, A.T. Magis, Z.G. Chen, D.M. Shin, S.S. Ramalingam, F.R. Khuri, W.J. Curran, X. Deng, Small-Molecule Bcl2 BH4 Antagonist for Lung Cancer Therapy, Cancer Cell. 27 (2015) 852–863. https://doi.org/10.1016/j.ccell.2015.04.010.

[99] J. Deng, D. Park, M. Wang, A. Nooka, Q. Deng, S. Matulis, J. Kaufman, S. Lonial, L.H. Boise, J. Galipeau, X. Deng, BCL2-BH4 antagonist BDA-366 suppresses human myeloma growth, Oncotarget. 7 (2016) 27753–27763. https://doi.org/10.18632/oncotarget.8513.

[100] T. Li, Y. Cui, B. Wu, Molecular dynamics investigations of structural and functional changes in Bcl-2 induced by the novel antagonist BDA-366, J. Biomol. Struct. Dyn. 37 (2019) 2527–2537. https://doi.org/10.1080/07391102.2018.1491424.

[101] M. Villalobos-Ortiz, J. Ryan, T.N. Mashaka, J.T. Opferman, A. Letai, BH3 profiling discriminates on-target small molecule BH3 mimetics from putative mimetics, Cell Death Differ. 27 (2020) 999–1007. https://doi.org/10.1038/s41418-019-0391-9.

[102] T. Vervloessem, B.K. Sasi, E. Xerxa, S. Karamanou, J. Yale, R.M. La Rovere, S. Chakraborty, F. Sneyers, M. Vogler, A. Economou, L. Laurenti, D.W. Andrews, D.G. Efremov, G. Bultynck, BDA-366, a putative Bcl-2 BH4 domain antagonist, induces apoptosis independently of Bcl-2 in a variety of cancer cell models, Cell Death Dis. 11 (2020). https://doi.org/10.1038/s41419-020-02944-6.

[103] C.W. Distelhorst, M.D. Bootman, Creating a new cancer therapeutic agent by targeting the interaction between Bcl-2 and IP3 receptors, Cold Spring Harb. Perspect. Biol. 11 (2019) a035196. https://doi.org/10.1101/cshperspect.a035196.

[104] J. Ireoluwa Yinka, A. Temidayo Olamide, A. Ahmeedah Ololade, B. Olukayode Olusola, O. Ugochukwu Okechukwu, A.-E. Ifelolu Adeseye, S. Lateef Adegboyega, Virtual Screening and Elucidation of Putative Binding Mode for Small Molecule Antagonist of BCL2 BH4 Domain, BioRxiv. (2020).

## Legend

**Figure 1: A schematic overview on the canonical and non-canonical functioning of B-cell lymphoma 2 (Bcl-2) in the regulation of cell death and survival. (A)** The complex molecular network of the Bcl-2 family in the intrinsic apoptosis pathway. Intrinsic death stimuli such as DNA damage and ER stress trigger activator and sensitizer BH3-only family member functioning. In their turn, activator BH3-only proteins (e.g. Bim and tBid) stimulate pore-forming Bcl-2 family members Bax/Bak to form proteinaceous pores in the mitochondrial outer membrane (MOM), while sensitizer BH3-only proteins (e.g. Bim and tBid) inhibit anti-apoptotic Bcl-2 family members. These anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-xL and Mcl-1) via their hydrophobic cleft neutralize both the pro-apoptotic activator BH3-only family members and pore-forming Bax/Bak. Activation and oligomerization of Bax/Bak provokes mitochondrial outer membrane permeabilization (MOMP), thereby releasing of cytochrome C and Smac/DIABLO into the cytosol. The release of cytochrome C enables the formation of the apoptosome and consequently the activation of executioner caspases. The cleavage and activation of these caspases then implements the coordinated cell death process. **(B)** Bcl-2 modulation of $Ca^{2+}$ transfer at the level of the mitochondria-associated ER membranes (MAMs). The aforementioned $Ca^{2+}$ transfers from the ER into the mitochondria are enabled by a multi-protein complex spanning the MAMs that is made up of inositol 1,4,5-trisphosphate receptor ($IP_3R$) channels and voltage-dependent anion channels type 1 (VDAC1) coupled by Grp75 (not shown) along with the mitochondrial calcium uniporter (MCU) complex located on the inner mitochondrial membrane. These ER-mitochondrial $Ca^{2+}$ transfers critically control the outcome of cell death and cell survival decisions. On one hand, a continuous oscillatory influx of $Ca^{2+}$ from the ER into the mitochondria (green arrow) promote ATP-production, mitochondrial bioenergetics and thus cell survival (left). On the other hand, mitochondrial $Ca^{2+}$-overload elicits cell death through opening of the mitochondrial permeability transition pore (mPTP), involving direct actions of $Ca^{2+}$ and indirect actions of $Ca^{2+}$ via ROS produced upon disintegration of respiratory complex II upon dissociation of cardiolipin. This facilitates the flow of ions and $H_2O$ into the mitochondrial matrix resulting in mitochondrial swelling and rupture of the MOM. The rupture of the mitochondria and the resulting release of cytochrome C and Smac/DIABLO prompt a plethora of processes eventually leading to cell death. Created with BioRender.com.

**Figure 2: Schematic representation of the anti-apoptotic Bcl-2 protein, including 4 Bcl-2 homology (BH) domains and a transmembrane domain (TMD) along with its functionality at both the ER (left) and the mitochondria (right).** The different domains of Bcl-2 and the number of their delineating amino acid residues are indicated. Via its BH4 domain, Bcl-2 inhibits the pro-apoptotic release of $Ca^{2+}$ from the ER lumen mediated by the $IP_3$ receptor ($IP_3R$), a function that is inhibited by BIRD-2 peptides. At the mitochondrial outer membrane, Bcl-2 suppresses Bax/Bak-pore formation through its hydrophobic cleft via inhibition of both the Bax/Bak proteins themselves as well as the activator BH3-only proteins that facilitate Bax/Bak pore formation, a function that is inhibited by BH3-mimetic drugs. Consequently, the release of cytochrome C (CytC) and Smac/DIABLO into the cytosol followed by apoptosis resulting from Bax/Bak-pore formation is also inhibited by BH3-mimetic drugs.

**Figure 3: Cell Death analysis of putative BH4-domain inhibitors in DLBCL cell lines.** Cell death was measured using cytometry analysis of Annexin V-FITC/7-AAD-stained cells treated with the different compounds or vehicle for 24 hours (10.000 cells analysed). Cells that stained negative for both Annexin V-FITC and 7-AAD were considered as viable. **(A)** A representative dot plot of SU-DHL-4 cells treated with compound (139068, and 138967), vehicle, BIRD-2 or ABT-199. **(B-E)** The quantitative analysis of % of living cells (Annexin V-FITC negative and 7-AAD negative) on each of the two evaluated cell types (SU-DHL-4 and OCI-LY-1) either for compound 139068 (B and D) or compound

138967 (C and E). The means standard deviation from 3 independent experiments are displayed. BIRD-2 and venetoclax were used as benchmark tools for cell death in these cell models. For a detailed discussion of the materials and methods, we refer to Bittremieux *et al.* (2019) [68]. Flow cytometry data were analyzed using FlowJo™ v10.7.

The authors declare that there is no conflict of interest.

**CREDIT author statement**

Ian de Ridder: writing – original draft and integration of comments; conceptualization

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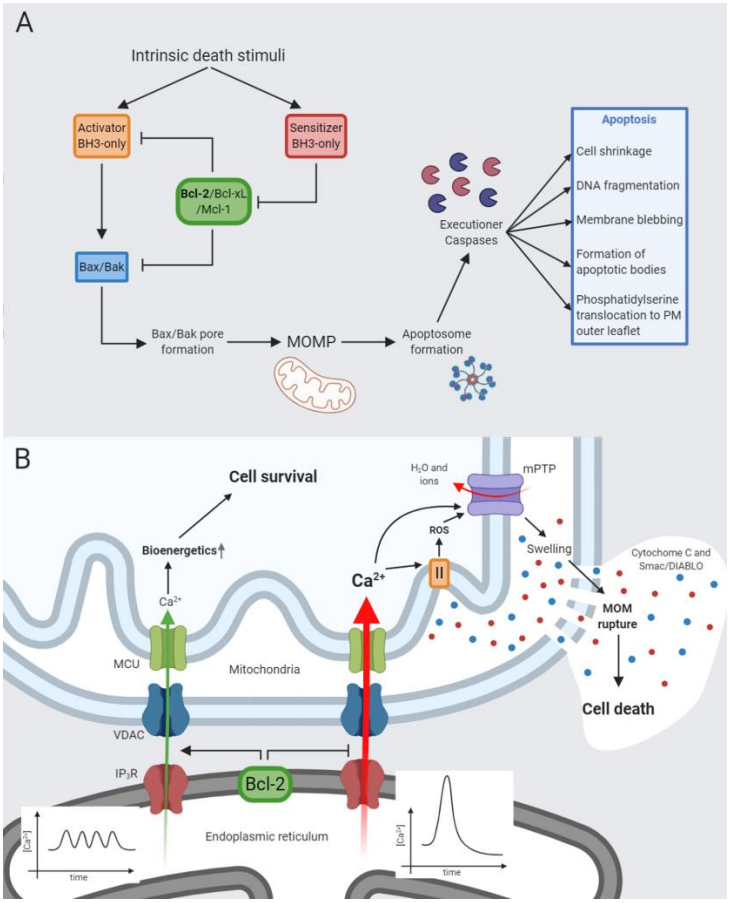
Wim Dehaen: writing – critically revision; supervision; methodology; validation

Geert Bultynck writing – original draft and further revision and editing; original design of work; conceptualization; supervision; funding acquisition

**Highlights**

- Anti-apoptotic Bcl-2 prevents cell death through its mitochondrial and endoplasmic reticulum functions.
- Anti-apoptotic Bcl-2 via its BH4 domain prevents pro-apoptotic, $IP_3$ receptor-driven $Ca^{2+}$ signaling.
- Tools that target Bcl-2's BH4 domain such as BIRD-2 elicit cell death in several cancer cell types.
- Targeting Bcl-2's BH4 domain in anti-cancer strategies is complementary with BH3 mimetics.
- BDA-366 was proposed as the first small molecule BH4-domain antagonist, yet triggers cell death independently of Bcl-2.
- The first bona fide BH4-domain antagonist small molecule still needs to emerge.

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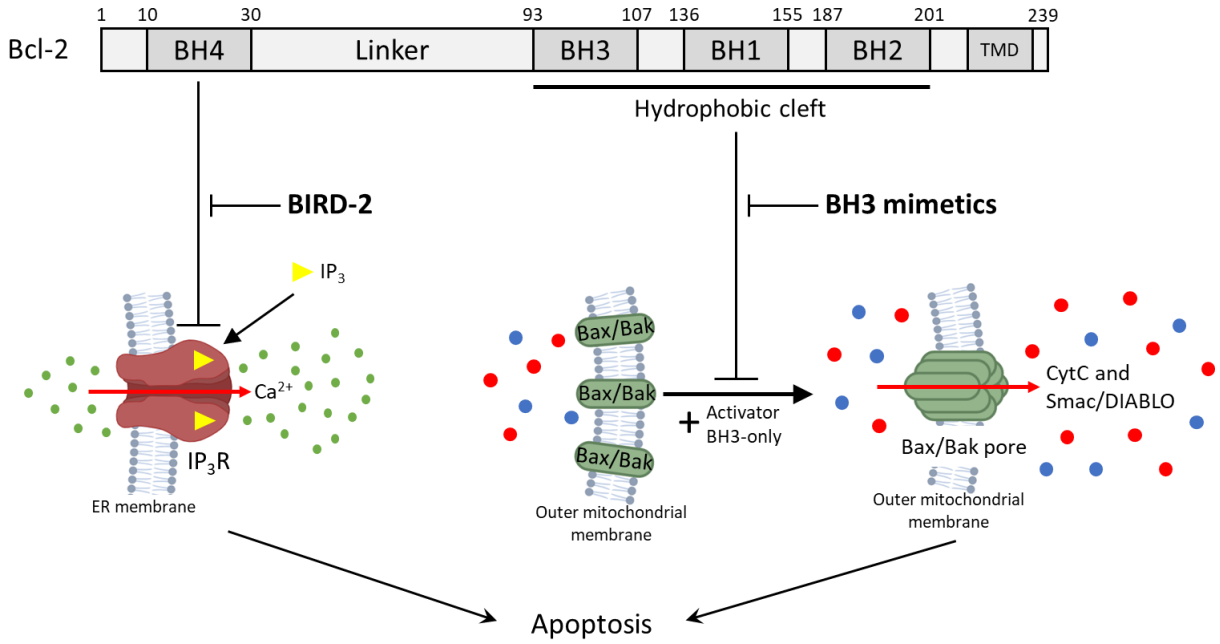


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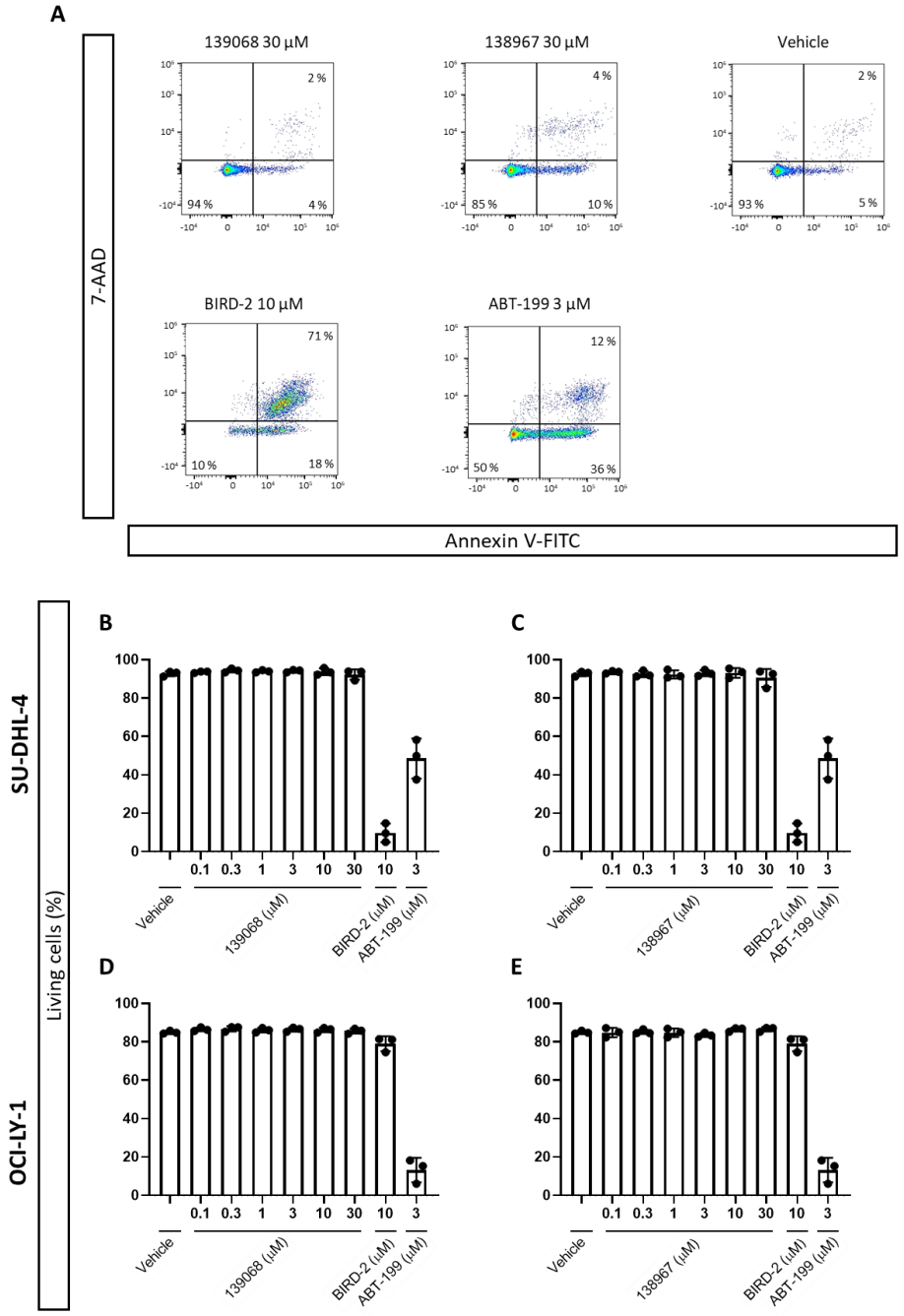


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