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Bcl-2 inhibitors as anti-cancer therapeutics: the impact of and on calcium signaling

Tamara Vervloessem^{\$1}, Martijn Kerkhofs^{\$1}, Rita La Rovere¹, Flore Sneyers¹, Jan B. Parys^{1,2*},
Geert Bultynck^{1,2*}

*1 KU Leuven, Laboratory of Molecular and Cellular Signaling, Campus Gasthuisberg O/N-I
bus 802, Herestraat 49, B-3000 Leuven*

2 Leuven Kanker Instituut (LKI), Campus Gasthuisberg, Herestraat 49, B-3000 Leuven

\$ these authors share first-authorship

** these authors share last-authorship*

Correspondence to: jan.parys@kuleuven.be; geert.bultynck@kuleuven.be

Graphical abstract

Highlights

- Oncogenesis in several cancer types is associated with upregulation of anti-apoptotic Bcl-2-family members
- Bcl-2 inhibitors that target the hydrophobic cleft or the BH4 domain can kill Bcl-2-dependent cancer cells
- Bcl-2 proteins exert part of their anti-apoptotic function through Ca²⁺-signaling modulation
- Bcl-2 inhibitors can trigger cell death involving Ca²⁺ signaling as an off- or on-target effect

1. ABSTRACT

Bcl-2-protein family members are essential regulators of apoptosis. Anti-apoptotic Bcl-2 proteins ensure cell survival *via* different mechanisms, including *via* binding of pro-apoptotic Bcl-2-family members and the modulation of intracellular Ca²⁺-transport systems. Many cancer cells upregulate these proteins to overcome the consequences of ongoing oncogenic stress. Bcl-2 inhibition leading to cell death, therefore emerged as a novel cancer therapy. Different Bcl-2 inhibitors have already been developed including the hydrophobic

cleft-targeting BH3 mimetics, which antagonize Bcl-2's ability to scaffold and neutralize pro-apoptotic Bcl-2-family members. As such, the BH3 mimetics have progressed into clinical studies as precision medicines. Furthermore, new inhibitors that target Bcl-2's BH4 domain have been developed as promising anti-cancer tools. Given Bcl-2's role in Ca^{2+} signaling, these drugs and tools can impact Ca^{2+} signaling. In addition to this, some Bcl-2 inhibitors may have "off-target" effects that cause Ca^{2+} -signaling dysregulation not only in cancer cells but also in healthy cells, resulting in adverse effects. In this review, we aim to provide an up-to-date overview of the involvement of intracellular Ca^{2+} signaling in the working mechanism and "off-target" effects of the different Bcl-2-antagonizing small molecules and peptides.

2. INTRODUCTION

1. *The Bcl-2-protein family: making acquaintance*

The B-cell lymphoma (Bcl)-2-protein family plays a crucial role in controlling the intrinsic, mitochondrial pathway of programmed cell death. Based on intracellular function and sequence homology, three subclasses can be distinguished within this Bcl-2 family: anti-apoptotic proteins, pro-apoptotic multidomain effectors and pro-apoptotic Bcl-2-homology (BH)3-only proteins [1, 2]. An overview of the different Bcl-2-protein family members and their functional class is provided in Table 1. B-cell lymphoma-2 (Bcl-2), Bcl extra-large (Bcl-XL), myeloid cell leukemia 1 (Mcl-1), Bcl-2-like 2 (Bcl-w), Bcl-2 homolog B (Bcl-B) and Bcl-2-related protein A1 (Bcl-2A1 which is also known as A1 or Bfl-1) belong to the anti-apoptotic subclass that shares four BH domains (BH1-4) [3, 4]. These proteins promote cell survival by sequestering and inhibiting pro-apoptotic Bcl-2-family members [5]. The anti-apoptotic Bcl-2 proteins are anchored primarily to membrane surfaces of intracellular organelles by their C-terminal hydrophobic tail [6]. Bcl-2 and Bcl-XL principally reside on the membrane surfaces of the endoplasmic reticulum (ER), mitochondria and nucleus [6, 7].

The pro-apoptotic Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist killer (Bak) and Bcl-2-related ovarian killer (Bok) are the effectors of apoptosis and although originally they were generally described as having only three BH domains, it is now recognized that they also contain four BH domains (BH1-4) [3, 5, 8]. Apoptosis is initiated when Bak and Bax oligomerize at the outer mitochondrial membrane (OMM), resulting in mitochondrial outer membrane permeabilization (MOMP), which is considered the point-of-no-return in mitochondrial apoptosis [9]. This oligomerization is triggered in a direct or indirect manner *via* the BH3-only activators or sensitizers respectively. After permeabilization of the OMM, apoptogenic factors present in the mitochondrial intermembrane space, including cytochrome c and second mitochondria-derived activator of caspase (Smac; also known as Diablo), are

released into the cytosol [10, 11]. Eventually, this results in caspase activation, leading to protein cleavage, nuclear condensation, cellular blebbing and ultimately, cell death [12]. Multidomain Bak is anchored to both the ER and mitochondrial membrane with its C-terminal hydrophobic tail. On the other hand, Bax is located in the cytosol in healthy cells because its C-terminal transmembrane domain is internalized within a hydrophobic pocket shaped by the BH1-3 domains [6, 13]. However, under apoptotic stimuli, Bax undergoes a conformational change exposing its C-terminal transmembrane domain and allowing its translocation to the mitochondria and ER.

The pro-apoptotic BH3-only proteins only share homology in the amphipathic α -helical BH3 domain that is essential for pro-apoptotic function [3]. Activators, like Bcl-2-interacting mediator of cell death (Bim), Bcl-2-interacting domain death agonist (Bid) and perhaps also p53-upregulated modulator of apoptosis (Puma) are able to activate the executioners of apoptosis, Bak and Bax directly [3, 14]. In contrast, sensitizer BH3-only proteins, such as Bcl-2 antagonist of cell death (Bad), Bcl-2-interacting killer (Bik), Bcl-2 modifying factor (Bmf), Noxa (the Latin word for damage) and Harakiri (Hrk) cannot activate Bak and Bax directly [3, 14]. They promote apoptosis indirectly by binding and sequestering the anti-apoptotic proteins causing the release of BH3-only activators.

Contrary to the anti-apoptotic as well as pro-apoptotic multidomain proteins, BH3-only proteins do not contain a C-terminal transmembrane domain and are therefore localized mainly in the cytosol. Nevertheless, they are able to interact with Bak/Bax and the anti-apoptotic proteins at several intracellular locations [15]. Although Bim and Bid can both activate Bak as well as Bax, Sarosiek *et al.* recently showed that significant activation preferences exist between these BH3-only proteins [16]. Using Bak- and/or Bax-knockout cells, they found that Bid preferentially activates Bak, while Bim preferentially activates Bax. These preferences can have an impact on

chemotherapy response, as some chemotherapeutic agents, like topoisomerase inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), require cleavage of Bid to tBid for maximal induction of apoptosis. Therefore, Bak-deficient cells are protected from cell death induced by these agents [16]. This illustrates that cell fate is determined by the balance between pro-survival and pro-apoptotic Bcl-2-family members in the cell [17].

The interaction between anti-apoptotic and pro-apoptotic proteins occurs through their BH domains. The BH1, BH2 and BH3 domain from the anti-apoptotic proteins form a hydrophobic cleft, which can interact with the BH3 domain of the pro-apoptotic activators, sensitizers and effectors [18]. Interestingly, recently also other domains have been implicated in the binding of Bax and Bak to the anti-apoptotic proteins. For example, the transmembrane domain of both Bcl-2 and Bcl-XL has been shown to interact with the transmembrane domain of Bax and this was shown to be sufficient for an attenuation of apoptosis [19]. Moreover, the BH4 domain of Bcl-2 was shown to confer anti-apoptotic activity through its binding to inactive Bax [20].

In healthy cells, the level of free activator BH3-only proteins is insufficient to initiate programmed cell death by activating Bak/Bax [21]. Under conditions of high stress, however, BH3-only proteins become upregulated, leading to an increase in the concentration of free Bim and Bid in the cell, resulting in the direct activation and oligomerization of Bak and Bax in the OMM. Upregulation of BH3-only proteins in response to elevated stress levels occurs by various mechanisms [6, 22]. Most BH3-only proteins are transcriptionally regulated, like Puma and Noxa whose transcription is enhanced by tumor suppressor p53. Post-translational modifications like phosphorylation of Bad and Bik, proteolytic cleavage such as the formation of truncated Bid by caspase-8 and dissociation of BH3-only proteins from sequestering proteins, e.g. release of Bim from dynein light chain 1 and release of dephosphorylated Bad from 14-3-3 proteins are other examples of upregulation of BH3-only protein activity.

Many types of cancer exploit the pro-survival function of the anti-apoptotic Bcl-2-family members, including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL) [23], acute myeloid leukemia (AML) [24] or acute lymphoblastic leukemia (ALL) [25], which are addicted to high levels of Bcl-2 [26]. Due to the ongoing oncogenic stress, these cancer cells contain high levels of activator BH3-only proteins Bim and Bid [27]. To ensure cell survival, the levels of anti-apoptotic proteins, like Bcl-2, are upregulated to sequester and neutralize the BH3-only activators. Hence, in these cells, Bcl-2 is not “free”, but rather loaded with BH3-only proteins like Bim [27, 28]. As a result, these types of cancer have a very low reserve of unbound anti-apoptotic proteins, pushing them closer to the threshold of apoptosis. For this reason, these cancers are said to be “primed-to-death” [9, 28]. The priming of these cancer cells is their Achilles’ heel upon which can be acted to selectively kill them, as will be discussed further.

II. The Bcl-2-protein family and Ca^{2+} signaling: portrait of an intimate relationship

Apart from regulating cell fate through interactions with other Bcl-2-protein family members, the Bcl-2-protein family is able to influence cell survival and death by modulating the cell’s Ca^{2+} signaling machinery [29, 30]. Intracellular Ca^{2+} signals generally originate from the ER, the main intracellular Ca^{2+} store [31]. *Via* the inositol 1,4,5-trisphosphate receptor (IP_3R), the main intracellular Ca^{2+} -release channel located on the ER [32-34], Ca^{2+} is not only released in the cytosol, but also reaches the mitochondria. Ca^{2+} permeation across the OMM occurs *via* the voltage-dependent anion channel (VDAC) isoform 1 [35], while the mitochondrial Ca^{2+} uniporter (MCU) complex is responsible for Ca^{2+} permeation across the inner mitochondrial membrane (IMM) [36]. This MCU complex is a multi-protein complex consisting of the MCU, which makes up the channel pore, and several regulators of MCU activity (i.e. essential MCU regulator (EMRE), EF-hand-containing calcium-binding proteins mitochondrial calcium

uptake 1/2 (MICU1/MICU2), where the latter functions as a gatekeeper and the former is responsible for the cooperative properties of the MCU [37], the mitochondrial calcium uniporter regulator-1 (MCUR1) [38], SLC25A23 [39] and the negative regulator MCUB [40]). Recently, EMRE has emerged as a critical gatekeeper of MCU governing its tight regulation by both cytosolic Ca^{2+} , *via* MICU1/MICU2, and mitochondrial matrix Ca^{2+} [41, 42]. We like to refer to other reviews for an in-depth discussion on the MCU macro-molecular complex, its function and its physiological implications [43-46]. Although MCU displays a low Ca^{2+} affinity, Ca^{2+} released from the ER reaches the mitochondrial matrix due to i) the strong negative mitochondrial membrane potential produced by the electron transport chain, which provides a high driving force for Ca^{2+} flux into the mitochondrial matrix and ii) the close proximity of the ER and the mitochondria which are physically linked, forming mitochondria-associated ER membranes (MAMs) enabling the cell to reach a high, local Ca^{2+} concentration (about 15 μM) in this microdomain [36, 47, 48]. These contact sites do not only contain tethering proteins, like Mitofusin-2 and PERK, to maintain the close apposition of the membranes, but also Ca^{2+} -transport systems, like the IP₃R, the sarco/endoplasmic reticulum ATPase (SERCA) 2b and VDACs [47, 49-51].

Ca^{2+} signals are able to steer cell fate in a pro-survival as well as a pro-apoptotic direction [30]. Basal Ca^{2+} oscillations mediated by IP₃Rs promote mitochondrial bioenergetics and ATP production by activating different Ca^{2+} -dependent enzymes, catalyzing steps preceding or within the tricarboxylic acid (TCA) cycle, like pyruvate-, isocitrate- and α -ketoglutarate dehydrogenases [52]. Mitochondrial Ca^{2+} can also stimulate the mitochondrial metabolism downstream of these TCA enzymes, by stimulating the respiratory chain complex III, the ATP synthase complex V and adenine nucleotide translocase [53]. Moreover, these Ca^{2+} signals also drive the expression of the MCU via the Ca^{2+} -regulated transcription factor CREB, supporting mitochondrial Ca^{2+} uptake and mitochondrial metabolism [54]. Interestingly, redox

nanodomains at the ER-mitochondria interface further boosts ER-derived Ca^{2+} oscillations via H_2O_2 squeezed from compressed mitochondrial cristae [55]. When IP_3R activity is suppressed, ATP production declines, leading to the activation of AMP-activated kinase (AMPK), an inducer of autophagic flux [56, 57]. Ca^{2+} signals do not only drive mitochondrial energy production but can also trigger MOMP and thus apoptosis [58, 59]. This can occur directly through mitochondrial Ca^{2+} overload, which results in a decline in the mitochondrial membrane potential, the production of mitochondrial reactive oxygen species (ROS) (through dissociation of complex II of the electron transport chain [60] and the opening of the mitochondrial permeability transition pore (mPTP), located at the IMM and recently identified as dimers of the c subunit of the F_0/F_1 ATPase [61-63]. mPTP opening leads to complete collapse of the mitochondrial membrane potential and severe mitochondrial swelling, eventually resulting in rupture of the OMM and release of cytochrome c and other pro-apoptotic factors [58, 59].

Excessive Ca^{2+} signals can also lead to MOMP and apoptosis indirectly [30]. For instance, high levels of cytosolic Ca^{2+} will activate calcineurin, the Ca^{2+} /calmodulin-dependent phosphatase PP2B, thereby dephosphorylating and activating sensitizer BH3-only proteins like Bad [64]. In healthy cells, Bad, present in its phosphorylated form, is bound and neutralized by 14-3-3 scaffold proteins. This leads to the release of pro-apoptotic activators, sequestered by anti-apoptotic Bcl-2-family members, eliciting apoptosis [64]. In summary, intracellular Ca^{2+} can both promote cell survival as well as apoptosis. Since Ca^{2+} signaling can have such a profound impact on the survival of cells, it is no surprise that a myriad of regulators, including a growing list of oncogenes and tumor suppressors, fine-tunes ER Ca^{2+} -release events. Among these regulators, several members of the Bcl-2-protein family modulate Ca^{2+} signaling at both the ER, the mitochondria and the ER-mitochondrial interface, thereby impacting cell survival and apoptosis sensitivity [29, 30, 65]. Ca^{2+} -signaling regulation by Bcl-2 proteins at the level of ER

and mitochondria takes place at least at two distinct levels: Ca^{2+} release by the IP_3R and mitochondrial Ca^{2+} uptake through the OMM by VDAC1.

a. IP_3R

IP_3Rs contain multiple sites responsible for their interaction with various Bcl-2-family members [66]. Bcl-2, Bcl-XL and Mcl-1 are all three reported to bind to the IP_3R thereby exerting their anti-apoptotic activity [29, 67, 68]. For instance, Bcl-2 as well as Mcl-1 were found to increase the Ca^{2+} leak from the ER in an IP_3R -mediated manner, leading to a decreased ER Ca^{2+} store content [68-71]. This results in less Ca^{2+} being released from the ER and being able to contribute to mitochondrial Ca^{2+} overload, thus conferring protection against apoptosis. However, it should be noted that other groups have found that ER Ca^{2+} content was not changed upon Bcl-2 overexpression [72-76]. However, high expression levels of Bcl-2 can lead to a disruption of the ER structure [77], which may result in a reduced ER Ca^{2+} -store content. Furthermore, Bcl-2 is able to promote pro-survival Ca^{2+} signals, through a mechanism involving the sensitization of the IP_3R to IP_3 [68].

Conversely, Bcl-2 can also inhibit IP_3R -mediated Ca^{2+} release [78-81]. Different mechanisms can account for this, including a direct inhibition of the IP_3R by Bcl-2 and the recruitment of other inhibitory proteins to the IP_3Rs through Bcl-2 serving as a linker protein. Evidence for a direct role of Bcl-2 is based on different interaction studies whereby Bcl-2's BH4 domain facilitates the direct binding to the regulatory and coupling domain of the IP_3R in purified cell systems [79, 80]. Moreover, the BH4 domain of Bcl-2 by itself was sufficient to suppress IP_3 -dependent Ca^{2+} release from the ER in both permeabilized and intact cells and prevented Ca^{2+} -mediated apoptosis. The low-affinity modulation of the IP_3R by the BH4 domain of Bcl-2 is overcome by a second binding site, involving Bcl-2's C-terminal transmembrane domain and IP_3R 's 6th transmembrane domain [75]. It is anticipated that this interaction causes an increase

in the “local concentration” of Bcl-2’s BH4 domain in the proximity of the IP₃R channel. On the contrary, an indirect role for Bcl-2 was found by Chang *et al.* [82]. They showed that the BH4 domain of Bcl-2 serves as a docking place for the phosphatase calcineurin [82]. In turn, calcineurin interacts with DARPP-32, a negative regulator of protein phosphatase 1 (PP1), thereby inhibiting its function. Upon IP₃R-mediated Ca²⁺ release calcineurin gets activated, leading to the dephosphorylation and inhibition of DARPP-32. This alleviates PP1 inhibition, leading to dephosphorylation of the IP₃R, resulting in a decreased Ca²⁺ release from the ER and protection against apoptosis [82]. Moreover, the formation of a macromolecular complex containing IP₃R1, PP1 and Bcl-2 has also been confirmed in breast cancer cells [81]. Hereby, Bcl-2 serves as a linker protein restricting IP₃R activity via the sequestering of PP1. However, another explanation might be that the interaction of Bcl-2 and calcineurin is of greater importance because dephosphorylation of Bcl-2 promotes its anti-apoptotic effect and thus may enhance its IP₃R-inhibitory properties [83]. Furthermore, the zebrafish Bcl-2 homolog Nrz interacts with the IP₃R, inhibiting the channel. In this way, Nrz regulates Ca²⁺ trafficking and cytoskeletal dynamics, enabling normal zebrafish epiboly progression and development [84]. In further work, Nrz was shown to bind via the BH4-BH3-BH1 region to the ligand-binding domain of the IP₃R, thereby preventing the agonist-induced Ca²⁺ release and consequently blocking early vertebrate development [85]. The IP₃R/Nrz complex was dynamically regulated by Nrz phosphorylation, which alleviates its IP₃R-binding and –inhibitory properties. As such, Nrz phosphorylation triggers the onset of Ca²⁺ waves that are critical for proper epiboly progression and actin ring formation. Recently, Bcl-2like10, the human ortholog of the zebrafish Nrz, has been identified to bind the IP₃-binding domain [86]. Together with IRBIT, when present in a phosphorylated state, Bcl-2-like10 suppresses ER-mitochondrial Ca²⁺ transfer and thus apoptosis. Upon apoptotic stimulation, IRBIT becomes dephosphorylated and acts as an inhibitor of Bcl-2like10 and a facilitator of ER-mitochondrial contact site formation,

enhancing pro-apoptotic Ca^{2+} transfer from ER to mitochondria. Very recently, our lab found that human Bcl-2 can interact with the ligand-binding domain of the IP_3R (Ivanova H et al, unpublished results).

In a similar way to Bcl-2, Bcl-XL is able to stimulate pro-survival Ca^{2+} signals as well as inhibit pro-apoptotic Ca^{2+} signals from the IP_3R [87, 88]. Although Bcl-2 and Bcl-XL share considerable sequence homology and are part of the anti-apoptotic group of Bcl-2-protein family members, surprisingly, their interaction with the IP_3R differs significantly at the level of binding determinants. Whereas Bcl-2 *via* its BH4 domain binds a region in the central, regulatory domain of the IP_3R [76, 79] Bcl-XL primarily interacts with the IP_3R 's C-terminal BH3-like motives through the hydrophobic groove consisting of the BH1-3 domains [88]. Indeed, a critical single amino-acid difference between Bcl-2 and Bcl-XL at the center of their BH4 domain accounts for a strong interaction between the BH4 domain of Bcl-2 and the central IP_3R domain, while the interaction of the BH4 domain of Bcl-XL with this central IP_3R domain is much weaker [76]. In the case of Bcl-XL [88], binding to the BH3-like motives of the IP_3R promotes pro-survival Ca^{2+} signaling at low Bcl-XL concentrations. Interestingly, however, high levels of Bcl-XL inhibit the IP_3R through a specific binding at the same interaction site used by Bcl-2's BH4 domain, i.e. the central, modulatory domain. However, the determinants for Bcl-XL binding to this central domain were not determined, but may possibly involve Bcl-XL's BH4 domain [88]. Another role for the Bcl-XL- IP_3R interaction, in particular the interaction between Bcl-XL and IP_3R , is the stimulation of mitochondrial metabolism [89]. Both the BH4 domain and the transmembrane domain of Bcl-XL were identified as important for its localization to the MAMs and for its interaction with the IP_3R . Bcl-XL overexpression increased transient mitochondrial Ca^{2+} levels and improved Ca^{2+} -dependent bioenergetics. Interestingly, under conditions of cellular stress (e.g. in the presence of thapsigargin or

tunicamycin) Bcl-XL seems to translocate to the MAMs and its interaction to IP₃R3 is tightened [89].

Regarding the interaction of Bcl-2-protein family members with the IP₃R, not only the anti-apoptotic proteins play a role. Recently, the pro-apoptotic Bok has been shown to bind to the IP₃R, although evidence suggests that this binding does not alter the IP₃R's Ca²⁺-release function directly. The interaction occurs through binding of Bok's putative BH4 domain to a stretch of amino acids in the regulatory domain of the IP₃R. This interaction between Bok and the IP₃R protects the latter against caspase-mediated cleavage *in vivo* and Bok thus regulates IP₃R proteolysis [90]. Conversely, IP₃R binding is necessary for Bok stability. Schulman *et al.* showed that essentially all cellular Bok is bound to the IP₃R, while the unbound Bok in the cytosol was readily ubiquitinated and degraded via the proteasomal pathway, possibly indicating that free Bok threatens cell survival [91].

b. VDAC

Another way for the Bcl-2-protein family to influence Ca²⁺ signaling is through binding the gateway of Ca²⁺ to the mitochondria: VDAC1. VDAC1 is able to switch between an open and a closed state [92]. The open state allows for exchange of large molecules like ATP or Krebs cycle substrates, but also of ROS and Ca²⁺ between the cytosol and the intermembrane space. On the other hand, VDAC1's closed state inhibits the flow of large molecules but has a higher conductance for Ca²⁺ [92, 93]. Again, Bcl-2, Bcl-XL and Mcl-1 have been reported to bind and affect VDAC1's function [94, 95].

Not much is known about the molecular determinants allowing for interaction between VDAC1 and Bcl-2. A study by Arbel & Shoshan-Barmatz showed that VDAC1 binds to Bcl-2 *via* its N-terminal domain [95]. However, the region of Bcl-2 that is responsible for this interaction is not known, although likely Bcl-2's BH4 domain is not involved [96]. The functional implication

of the VDAC1-Bcl-2 interaction is that it protects against cell death [95]. However, it was not addressed how this protection was conferred, e.g. through an effect on VDAC1's Ca^{2+} conduction capacity [95]. Alternatively, Bcl-2 could prevent cytochrome c release mediated by VDAC1 [97, 98].

For Bcl-XL, similarly to what was proposed for the IP_3R , both an inhibitory and a stimulatory effect on VDAC1-mediated Ca^{2+} uptake were observed. The inhibitory effect of Bcl-XL on VDAC1 could be pinpointed to its BH4 domain, which interacts with VDAC1's N-terminus [96]. This interaction protects against apoptosis by reducing mitochondrial Ca^{2+} uptake and subsequent mitochondrial Ca^{2+} overload [96]. Conversely, Bcl-XL is able to promote Ca^{2+} uptake by VDAC1, again purportedly through its interaction with the N-terminal domain of VDAC1, as peptides based on this sequence were able to abolish Bcl-XL's effect on mitochondrial Ca^{2+} uptake [94]. The dichotomous impact of Bcl-XL on VDAC1 could stem from the use of different experimental conditions in different studies, but could also reflect a dual cellular function, where Bcl-XL may promote the transfer of basal pro-survival Ca^{2+} signals into the mitochondria, but suppresses the transfer of excessive pro-apoptotic Ca^{2+} signals into the mitochondria. However, the molecular mechanisms underlying these opposing effects of Bcl-XL on VDAC1 remain unclear [30].

It is clear that Ca^{2+} signaling is of paramount importance in cell fate decisions. Therefore, Ca^{2+} -signaling remodeling appears to be an important aspect underlying tumorigenesis, thereby impacting several cancer hallmarks like apoptosis resistance, metabolic alterations and metastasis [99-101]. Furthermore, given the intimate relationship between the anti-apoptotic Bcl-2 proteins and the cell's Ca^{2+} signaling machinery, notably the IP_3R and VDAC1, upregulation of these proteins can be used by cancer cells to exert their rewiring [27, 30]. For

example, certain diffuse large B-cell lymphoma (DLBCL) cells display an increased expression of IP₃R2, thereby rendering them vulnerable to “toxic” Ca²⁺ signaling, eliciting cell death [102, 103]. While the reason for this upregulation is not known, these cells also display a concomitant upregulation of anti-apoptotic Bcl-2. Via its inhibitory interaction with the IP₃R, Bcl-2 prevents Ca²⁺-mediated apoptosis. Hence, these cells find themselves at the edge of apoptosis, being addicted to high levels of Bcl-2 to dampen the upregulated IP₃R2 channels. Thus, disrupting this interaction can push these cells over the apoptotic edge, as will be discussed below [27, 79, 102]. In concert with the concept of “priming-to-death” at the mitochondria [28], one could state that these cells are “primed-to-death” at the ER.

Not only IP₃R expression/function is modified in cancer, the same holds true for VDAC1. Various studies found a link between VDAC1 expression and tumor growth, survival and migration in different types of cancer [104-106] and as mentioned before, it seems that Bcl-2-protein family members exert at least part of their anti-apoptotic function through VDAC1 [94, 95]. Besides apoptosis resistance, Bcl-2-protein family members can also enhance other VDAC-mediated cancer hallmarks *via* their interaction with VDAC [107]. Mcl-1 was found to do this by promoting VDAC-mediated mitochondrial Ca²⁺ uptake, causing a subsequent increase in mitochondrial ROS production, which is responsible for an increased migration of non-small cell lung cancer cells [107].

In summary, the anti-apoptotic Bcl-2-protein family members enable cancer cell survival in two ways: i) through the fulfilment of their canonical function i.e. counteracting the pro-apoptotic Bcl-2-protein family members; and ii) *via* their interference with Ca²⁺ signaling proteins like the IP₃R or VDAC1. However, cancer cells survive despite being exposed to pro-apoptotic signaling, rendering them uniquely addicted to anti-apoptotic mediators like Bcl-2, a property that can be exploited to kill cancer cells while maintaining survival of normal cells. Below we will discuss some anti-cancer drugs that were developed using concepts based on insights in

Bcl-2-protein family function/interactions and the knowledge of the determinants for these molecular interactions. The focus will lie on the consequences of these anti-cancer drugs on the cellular Ca^{2+} -signaling profile.

3. TARGETING ANTI-APOPTOTIC BCL-2 AND THE IMPACT ON Ca^{2+} SIGNALING

In this section, we will mainly focus on the chemical and peptide-based inhibitors of anti-apoptotic Bcl-2 proteins. Of note, earlier approaches also focused on Bcl-2 mRNA degradation via an antisense oligonucleotide called oblimersen (also known as Genasense or G3139) (Table 2) [108]. It was used in the clinic to inhibit the expression of Bcl-2 in various indications: CLL [109], multiple myeloma [110] and melanoma [111]. In addition to Bcl-2 downregulation, oblimersen can also inhibit VDAC (Table 2) [112], thereby promoting Ca^{2+} -induced intramitochondrial oxidative stress *via* the accumulation of superoxide anions and triggering mPTP opening [113]. Hence, in melanoma cell lines, oblimersen-induced apoptosis was independent of Bcl-2 down-regulation and thus may be due to VDAC inhibition [112]. Yet, due to disappointing results in clinical trials, oblimersen was abandoned and focus shifted towards targeting Bcl-2 at its protein level. We also want to highlight that very recently selective Bcl-XL inhibitors like WEHI-539 [114], A-1155463 [115] and A-1331852 [116] and Mcl-1 inhibitors like UMI-77 [117] and S63845 [118] have been developed, but their impact on Ca^{2+} signaling and the contribution of Ca^{2+} signaling to their mechanisms of action have yet to be determined.

I. BH3I-2'

BH3I-2' is a cell-permeable compound that disrupts the interaction between pro-apoptotic and anti-apoptotic Bcl-2-family members (Fig. 1). Exposure of cells with experimentally induced pancreatitis [119] or leukemic cells [120] to BH3I-2' induced cytochrome c release and MOMP. This observed cytotoxic effect was found to be Bax-dependent [120].

After physiological agonist stimulation a Ca^{2+} wave propagates from the apical towards the basolateral pole of acinar cells [121]. This Ca^{2+} wave has a predominant role in regulating enzyme secretion and function of the pancreatic acinar cell. A major component of the Ca^{2+} propagation is due to Ca^{2+} -induced Ca^{2+} release (CICR), whereby a small rise in cytosolic Ca^{2+} promotes a further increase in Ca^{2+} from intracellular stores. In pathological conditions, this oscillatory pattern is changed to a sustained increase in cytosolic Ca^{2+} [122]. Antagonizing Bcl-2 *via* BH3I-2' causes in the pancreatic acinar cells a slow and transient IP_3R /ryanodine receptor (RyR)-dependent Ca^{2+} increase in the cytosol followed by a plateau that does not decline to baseline levels (Table 2) [123]. This elicits apoptosis in the acinar cells. Both blocking the RyR and the IP_3R partially or overexpression of Bcl-2 in these cells markedly decreased the response towards BH3I-2'. Combining these findings underpins the critical role for the upregulation of pro-survival Bcl-2 proteins to impede cell death in pancreatitis caused by the cytosolic Ca^{2+} increase observed during pathological conditions [119]. On the contrary, BH3I-2' antagonizes the anti-apoptotic effects of Bcl-2 resulting in IP_3R /RyR-dependent cytosolic Ca^{2+} increase (Fig. 2) and cell death.

II. Molecules designed to target the hydrophobic cleft of anti-apoptotic Bcl-2-family members

a. BH3 mimetics

A very promising approach to treat Bcl-2-dependent cancer cells is the use of BH3-mimetic drugs, such as ABT-737 and its orally bioavailable derivative, ABT-236 (navitoclax), which

display a high affinity for Bcl-2 as well as for Bcl-XL and Bcl-w [124]. These small molecules function in a similar way to sensitizer BH3-only proteins as they bind the hydrophobic cleft of the anti-apoptotic proteins (Fig. 1), displacing activator BH3-only proteins from Bcl-2 without directly activating pro-apoptotic Bax or Bak [28, 125]. BH3 mimetics initiate MOMP and induce apoptotic cell death only in “primed-to-death” cancer cells, but not in normal cells as here the anti-apoptotic Bcl-2/Bcl-XL proteins are not loaded with BH3-only activators [27]. However, due to Bcl-XL’s critical role in platelet survival, concomitant thrombocytopenia caused by Bcl-XL inhibition limits the efficacy achievable with these agents (Table 2) [126, 127].

Indeed, exposure of platelets of both healthy volunteers or CLL patients to ABT-737 or navitoclax resulted in a rapid concentration-dependent cytosolic Ca^{2+} rise [128] leading to the depletion of the intracellular Ca^{2+} stores [129], mitochondrial depolarization [130] concomitant with diminished platelet activation [128] and molecular characteristics of apoptosis (cytochrome c release and caspase-9 and -3 activation). However, in some of these studies [128, 129], the effect of ABT-737 on platelets Ca^{2+} homeostasis was only assessed after prolonged incubation, which thus may likely represent a consequence of platelet death rather than the cause. This is supported by a study that demonstrated that ABT-737 treatment did neither affect the ER SERCA pumps nor the IP_3R Ca^{2+} -release channels [131]. Hence, the altered Ca^{2+} signaling in platelets observed during prolonged treatment with ABT-737 likely is not a proximal event but rather a consequence of ABT-737-induced apoptosis in platelets.

The adverse effect on platelets of the first generation BH3 mimetics led to the development of the selective Bcl-2 inhibitor ABT-199 (venetoclax), which has been recently approved for the treatment of 17p-deleted CLL patients [132]. Venetoclax is a potent cancer cell killer in several tumors, including multiple myeloma [133, 134], follicular lymphoma [134], DLBCL [135, 136] and CLL [137] while sparing platelets [135, 137].

However, a major function of the anti-apoptotic Bcl-2 lies in the control of the ER-mitochondrial Ca^{2+} transfer *via* the inhibition of the IP_3R (see above, part IIa). Additionally, the recent identification of BH3 domain-like helices in the C-terminal tail of the IP_3R [138] and their role in the recruitment of Bcl-XL to the IP_3R may underlie a role for Ca^{2+} signaling in these cell death properties of BH3 mimetics in particular compounds targeting Bcl-XL.

Indeed ABT-737, which binds to Bcl-XL's hydrophobic cleft, completely blocked inhibition of the IP_3R by high concentrations of Bcl-XL [138]. In contrast, venetoclax did neither interfere with the inhibition of IP_3Rs by Bcl-2 nor affected ER Ca^{2+} store content (Table 2) [75]. Follow-up work revealed that venetoclax did not adversely affect Ca^{2+} signaling, since acute application of venetoclax did not alter intracellular Ca^{2+} homeostasis nor ER-mitochondrial Ca^{2+} trafficking in Bcl-2-dependent and non-dependent cell line models [136]. Moreover, intracellular Ca^{2+} buffering (BAPTA-AM) did not suppress venetoclax-induced cell death, indicating that venetoclax-induced cell death does not act through intracellular Ca^{2+} overload. Yet, BAPTA-AM appeared to enhance venetoclax-induced cell death. The molecular mechanisms underlying this phenomenon require further study. It is possible that BAPTA-AM affects the expression of Bcl-2-family members. As such, BAPTA-AM may enhance the pro-apoptotic effect of Bcl-2 inhibitors or create a synthetic lethality between intracellular Ca^{2+} buffering and Bcl-2 inhibitors. In ovarian cancer cells, BAPTA-AM resulted in a downregulation of the Mcl-1 protein levels via a mechanism potentially involving mTORC1 inhibition [139]. As a consequence, treatment with BAPTA-AM or ABT-737 separately did not result in cell death in ovarian cancer cells, while the combination of BAPTA-AM with ABT-737 was able to trigger apoptosis. This correlates with the dependence of ovarian cancer cells on both Bcl-XL and Mcl-1 proteins for their survival [140]. Also, long-term treatment of venetoclax may result in remodeling of the ER-mitochondrial contact sites, underlying a Ca^{2+} -dependent modulation of venetoclax-induced cell death. Interestingly, prolonged treatment of ovarian cancer cells with

ABT-737 has been proposed to alter ER-mitochondrial Ca^{2+} transduction, leading to an increased cisplatin-mediated cytosolic and mitochondrial Ca^{2+} response [141]. As such, ABT-737 supported and synergized with the cisplatin-mediated apoptosis.

Finally, it is important to note that the impact of BH3 mimetics on Ca^{2+} signaling and the contribution of Ca^{2+} signaling to cell death induced by BH3 mimetics might be (cancer) cell type dependent. In melanoma cells, ABT-737 has been shown to trigger a slow rise in cytosolic Ca^{2+} levels, which correlated with the induction of ER stress [142]. However, the mechanism underlying this ABT-737-induced Ca^{2+} rise was not further investigated. Also, in this work, BAPTA-AM treatment did neither enhance nor suppress apoptosis induced by ABT-737 in melanoma cells [142], suggesting that the contribution of Ca^{2+} signaling to the apoptotic effects of BH3 mimetics are cell type and context dependent, including the varying dependence of these cells on distinct Bcl-2-family members for cell survival and the ability of these Bcl-2-family members to regulate Ca^{2+} signaling and/or Ca^{2+} -transport systems, like IP_3Rs .

b. The gossypol family

Gossypol is a natural polyphenol compound derived from cottonseed and was initially used as an anti-fertility agent for men [143]. Previously, gossypol was considered to function as a pan-Bcl-2 antagonist [144] and demonstrated anti-tumor efficacy against a wide range of human carcinoma cell lines [145, 146]. Despite the use of gossypol as a BH3 mimetic in preclinical studies, proof for the anti-apoptotic Bcl-2 inhibition as a mechanism for the induction of cell death is limited. Indeed, while gossypol treatment elicited the characteristics of apoptosis both at the morphological (mitochondrial damage) and the biochemical level (release of cytochrome c and caspase cleavage), it has currently been shown that cell death was mainly induced in a non-specific way that required neither Bax/Bak nor caspase-9 and was independent of Bcl-2 proteins (Table 2) [147].

Recently, Albershardt *et al.* [148] stated that gossypol activated the unfolded protein response (UPR) (Fig. 2, Table 2), rather than inhibiting the anti-apoptotic Bcl-2-family members in leukemia cells. In this case, the UPR involves phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) followed by the induction of activating transcription factor (ATF) 4, ATF3, and ultimately the BH3-only protein Noxa, which in turn can inhibit the anti-apoptotic Bcl-2-family member Mcl-1. Further investigation of the underlying mechanism by which gossypol induced the UPR and Noxa led to the finding that leukemia cell lines, upon gossypol treatment, displayed a rapid increase in the activity of phospholipase A2 (PLA2), which subsequently caused a rise in cytoplasmic Ca²⁺ (Fig. 2, Table 2) [149]. Subsequently the ER-stress pathway is activated and Noxa is transcriptionally induced. This gossypol-mediated cytosolic Ca²⁺ increase is a common phenomenon, which is also seen in kidney [150] and hepatoma cells [151]. In leukemia cells, the rapid increase in cytosolic Ca²⁺ preceded the induction of the ER-stress response, whereas buffering intracellular Ca²⁺ decreased the levels of ATF4, ATF3, and abolished the effect on Noxa [149]. In addition, pretreatment with aristolochic acid, an inhibitor of PLA2, suppressed the gossypol-induced PLA2 activity and reduced the subsequent cytoplasmic Ca²⁺ rise and Noxa induction.

Furthermore, this pathway was also induced by apogossypol, a gossypol derivative without the two reactive aldehyde groups, and by AT-101, the D-enantiomer, which is even more potent than gossypol itself for tumor growth inhibition [149].

When determining the morphological characteristics of cell death induced by the gossypol family, Varadarajan *et al.* [152] observed distinct ultra-structural changes in CLL cells. While gossypol induced mitochondrial swelling, loss of cristae, phospholipid inclusions and subsequently apoptosis in CLL cells, exposure of these cells to apogossypol induced no significant mitochondrial damage but caused a malformed ER network (Fig. 2, Table 1).

Similar changes were observed in a variety of non-tumor and tumor cell lines like Jurkat T-

lymphocytes, HeLa cells, mouse embryonic fibroblasts, Chinese hamster ovary cells and even in the fission yeast *Schizosaccharomyces pombe*, meaning that this phenomenon is evolutionary conserved. Interestingly, although exposure to apogossypol resulted in phosphorylation of eIF2 α , CHOP accumulation and XBP1 splicing, these signs occurred at much later time points than the redistribution of the ER membranes. In addition, the malformation of the ER membranes was still induced in cells lacking core UPR proteins e.g. PERK, phosphorylable eIF2 α , IRE1, XBP1, ATF6 or CHOP. Therefore, the authors concluded that this reorganization of ER membranes occurred independently of the UPR [152]. These results seem to conflict with the observations made by Albershardt *et al.* [148]. Nonetheless, while the latter linked the effect of apogossypol to the occurrence of ER stress and the canonical UPR, they did not establish whether these effects were still observed in the absence of the main UPR-related proteins. Using hierarchical clustering of transcription profiles, apogossypol, and the related TW37, were identified as inducers of ER membrane redistribution, via a mechanism dependent on Na⁺ influx in the cell (Fig. 2, Table 2) [153].

These findings suggest the possibility that the gossypol family not only compromises Bcl-2 function, but also modulates Ca²⁺ and Na⁺ homeostasis (Table 2).

c. HA14-1

Ethyl [2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)]-4*H*-chromene-3-carboxylate (HA14-1) is a synthetic, cell-permeable, small molecule that was developed based on the predicted structure of Bcl-2. HA14-1 targets the BH3-domain binding site on the Bcl-2-surface pocket (Fig.1) to induce apoptosis of tumor cells [154].

HA14-1-induced apoptosis has been proposed to be Bax/Bak-dependent (Table 2) [155, 156], although additional mechanism may contribute, like dysregulation of intracellular Ca²⁺ homeostasis through SERCA inhibition [131]. HA14-1 exposure induced the typical molecular

events of apoptosis like loss of mitochondrial membrane potential (e.g. in isolated cardiac mitochondria, pancreatic acinar cells [119] and HL-60 leukemia cells [155]), followed by ROS formation, cytochrome c release, caspase activation and DNA fragmentation. These molecular events were prevented upon the chelation of intracellular Ca^{2+} [155]. This observation together with the increase in cytosolic Ca^{2+} due to a depletion of the ER Ca^{2+} store [123, 155] suggests an essential role for Ca^{2+} in the initiation of apoptosis after HA14-1 exposure. Nevertheless, when applying higher concentrations of HA14-1 ($> 30 \mu\text{M}$), a pan-caspase inhibitor, Z-VAD-fmk, was unable to block the HA14-1-induced cell death completely [155], meaning that both a caspase-dependent and -independent pathway exist. However, HA14-1 is highly unstable with a half-life of 15 minutes when incubated in standard culture conditions [157]. Moreover, decomposition of HA14-1 generates ROS, which has a potent pro-apoptotic activity due to its ability to induce oxidative damage to lipids, proteins and DNA [158]. Hence, it is possible that not only the direct consequences of HA14-1 but also the effect of ROS, formed due to HA14-1 decomposition, on cell viability were measured. Consequently, a more stable analog of HA14-1 (sHA14-1) was designed, which did not generate ROS and still compromised Bcl-2 function in drug-resistant cancer cells [159]. sHA14-1 exposure caused a cytosolic Ca^{2+} increase and was cytotoxic in a wide range of acute lymphoblastic leukemia (ALL) cell lines. Interestingly, the kinetic profile of the intracellular Ca^{2+} increase after challenging with sHA14-1 was very similar to that induced by thapsigargin, a SERCA inhibitor [160]. Moreover, pretreatment with sHA14-1 depleted the ER Ca^{2+} store [159]. These results imply that sHA14-1 targets and antagonizes SERCA like thapsigargin. Indeed, the activity of SERCA 2b and SERCA 1a were both directly inhibited by sHA14-1, without affecting the IP_3R (Fig. 2, Table 2).

Furthermore, the SERCA2b-inhibitory properties were recently confirmed in HeLa cells and described as one of the mechanisms responsible for the deregulated intracellular Ca^{2+} homeostasis in platelets of healthy human volunteers [131]. Additionally, HA14-1 reduced

thrombin-induced IP₃R-mediated Ca²⁺ release in platelets (Table 2), likely related to the partial ER Ca²⁺-store depletion caused by HA14-1-mediated SERCA inhibition. Therefore, the impaired Ca²⁺ signaling in platelets can be referred to as an off-target effect of HA14-1 on SERCA activity.

Recently, the effect of HA14-1 on experimental pancreatitis has also been examined [119]. Hyperstimulation of pancreatic acinar cells with 100 nM cholecystokinin (CCK)-8 (an experimental *in vitro* model of pancreatitis) resulted in both upregulated Bcl-2 and Bcl-XL levels and consequently protects pancreatic cells from cell death at the level of the mitochondria. In contrast, exposure of these cells towards HA14-1 resulted in mitochondrial depolarization, cytochrome c release and eventually cell death. However, although stimulation of pancreatic acinar cells with 5 nM CCK-8 induced a rapid and transient increase in cytosolic Ca²⁺, HA14-1 treatment of these cells only caused a slow and transient increase in cytosolic Ca²⁺ [123]. The latter could not be linked to the effect on mitochondrial energetics because inhibition of the mitochondrial ATP production (with rotenone or oligomycin) did not provoke the release of Ca²⁺ from intracellular stores. Hence, the HA14-1-induced increase in cytosolic Ca²⁺ was rather a consequence of the depletion of the ER stores. Indeed, blocking both the RyR and IP₃R partially reduced the amplitude of the HA14-1-induced Ca²⁺ release and the induction of apoptosis.

These collective data from independent studies propose a dual targeting mechanism of HA14-1 in the apoptotic machinery that includes both the mitochondria and the ER.

d. Obatoclax

Obatoclax is a small-molecule inhibitor of the Bcl-2 family used in the treatment of different types of cancer [142, 161-163]. By inhibiting Bcl-2, Bcl-XL, and Mcl-1, obatoclax has been proposed to function as a BH3 mimetic (Fig. 1). However, it does not cause prominent

thrombocytopenia, which is normally related to Bcl-XL inhibition. Additionally, many different mechanisms of action have been proposed, some of which require Bax/Bak, [164, 165] while others do not [147] or point to autophagy [142]. Therefore, the cell death-mediating mechanism of obatoclax as a BH3 mimetic remains obscure (Table 2).

A recent study indicated that a wide range of melanoma cells were sensitive towards obatoclax treatment, but the kinetics of obatoclax-induced apoptosis was relatively slow [142]. This is due to the fact that obatoclax also led to ER stress and the activation of the UPR. The UPR pathway enabled apoptosis resistance by transcriptionally upregulating Mcl-1 (Fig. 2, Table 2), a previously identified factor critical for the survival of melanoma cells in response to ER stress [166, 167]. As such, knockdown of Mcl-1 or knockdown of UPR components (like ATF6, IRE1 or XBP-1), which abrogated Mcl-1 upregulation, sensitized cells towards obatoclax-induced cell death. This is consistent with previous work showing that the IRE1/XBP-1 arm of the UPR is important for ER stress-induced upregulation of Mcl-1 through activation of the transcription factor Ets-1 [168]. In addition, melanoma cells can be sensitized towards acute ER-stress induced apoptosis by the application of obatoclax, which further increased Noxa expression levels [165]. It was also shown that obatoclax-induced cell death could be suppressed by BAPTA-AM, indicating an important role for cytosolic Ca^{2+} in this process. However, it was not addressed how obatoclax impacted Ca^{2+} signaling and how cytosolic Ca^{2+} buffering could prevent obatoclax-induced cell death, for instance by preventing mitochondrial Ca^{2+} overload or by enhancing cytoprotective UPR signaling and subsequent Mcl-1 upregulation. This may also suggest that the previously reported increased apoptotic efficacy of obatoclax in the presence of thapsigargin could be due to an increased accumulation of Ca^{2+} in the cytosol.

e. Disarib

Disarib is a recently described selective Bcl-2 inhibitor that does not cross-react with Bcl-XL, Mcl-1 or Bcl-w [169]. Additionally, unlike the BH3 mimetics, disarib predominantly targets the BH1 domain of Bcl-2 and not the BH3 domain [169], thereby selectively disturbing the Bcl-2/Bak interaction (Fig. 1) [170]. By targeting anti-apoptotic Bcl-2, disarib has a potent action on Bcl-2-dependent B cell leukemia cancer cells [169, 171], breast adenocarcinoma and ovarian carcinoma [171] while it spares platelets (Table 2). The cytotoxic effects were accompanied both *in vitro* and *in vivo* by loss of the mitochondrial membrane potential, translocation of phosphatidylserine to the plasma membrane concomitantly with the activation of Bak/Bax, caspase-9 and -3 followed by PARP1 cleavage, which are all important features of the intrinsic apoptotic pathway [171]. Moreover, a systematic comparison between disarib and venetoclax indicated that disarib exhibits a better tumor growth inhibition than venetoclax.

III. Molecules designed to target the BH4 domain of anti-apoptotic Bcl-2-family members

a. BIRD-2

An important part of Bcl-2's anti-apoptotic property is to target the IP₃R directly, thereby inhibiting aberrant IP₃-mediated Ca²⁺ release [78, 79]. The BH4 domain of Bcl-2 is the main determinant responsible for the IP₃R inhibition by Bcl-2 [80]. The BH4 domain of Bcl-2 targets a region of 20 amino acids, located in the central, modulatory domain of the IP₃R. These insights in the molecular determinants underlying the BH4-domain binding to the central IP₃R domain led to the development of a stabilized cell-permeable peptide tool comprising 20 amino acids corresponding to the Bcl-2-binding site on the IP₃R. In this peptide, two Asp residues, dispensable for Bcl-2 binding, were replaced by two Ala residues [172]. It is proposed that this peptide is more stable due to mutation of a putative protease-cleavage site. However, the exact mechanism responsible for the increased activity of this peptide (TAT-IDP_{DD/AA}) compared to the native peptide sequence (TAT-IDP) is not yet fully elucidated. This peptide was

subsequently named Bcl-2-IP₃R disrupter-2 (BIRD-2) [134, 173]. BIRD-2 disrupts the Bcl-2/IP₃R complexes and so abolishes Bcl-2's inhibitory effect on the IP₃R (Fig. 1, Table 2) but does not interfere with Bcl-2/Bim complexes [79]. Furthermore, BIRD-2 selectively targets Bcl-2 but not Bcl-XL due to a critical amino-acid difference between Bcl-2 and Bcl-XL at the level of their BH4 domain [76]. Consequently, BIRD-2 triggers Ca²⁺-mediated apoptosis in Bcl-2-dependent lymphoma cancers like CLL [172] and DLBCL [102], lung cancer cells [174] and multiple myeloma [134] without displaying much cytotoxic effect in cells that are not “primed-to-death” at the ER [102]. A crucial role in the BIRD-2 working mechanism is assigned to intracellular Ca²⁺ since (i) the BIRD-2-induced apoptosis is reduced upon intracellular Ca²⁺ chelation or IP₃R inhibition [102] and (ii) the susceptibility to BIRD-2-induced Ca²⁺ release and cell death correlated with the expression levels of IP₃R2 [102], the isoform being most sensitive to its endogenous ligand, IP₃ [175]. Additionally, BIRD-2 provoked a marked decrease in *in vivo* tumor growth in xenografted mouse models [134]. Remarkably, BIRD-2-induced cell death is in multiple myeloma accompanied by Bax translocation and caspase-3 activation [134], while in lung cancer cells a primary role in BIRD-2-induced cell death has been attributed to calpain activation [174]. Additionally, an important finding is that combining BIRD-2 with a Bcl-2 inhibitor (HA14-1 [173] or BH3 mimetics [134, 174]) enhanced cell death compared to cell death induced by the single agent [134, 173, 174]. Moreover, prolonged exposure to BIRD-2 enhanced Bim expression at both the protein and mRNA level and this in a Ca²⁺-mediated manner in multiple myeloma [134]. However, it remains unclear whether Bim upregulation is a common feature of BIRD-2-sensitive cancer cells and thus synergistic actions between BIRD-2 and BH3 mimetics are found in other cancer cells besides multiple myeloma.

b. BDA-366

BDA-366 is a recently described, novel, small molecule that targets the BH4 domain of Bcl-2 with very high affinity and selectivity [176]. In lung cancer [176] and multiple myeloma [177] cells, this interaction converted anti-apoptotic Bcl-2 into a pro-death signaling molecule by the exposure of its BH3 domain (Fig. 1, Table 2). Moreover, the conformational change of Bcl-2 coincided with a reduced phosphorylation of Bcl-2 at Ser70 [177]. Consequently, apoptotic cell death [176], which was associated with cytochrome c release, caspase-3 activation and PARP cleavage, was induced *via* a Bcl-2- and Bax-dependent mechanism and this both *in vitro* as well as *in vivo* xenograft mouse models [176, 177]. Indeed, cell death was diminished or even absent when knocking down Bcl-2 or Bax [176]. Additionally, BDA-366 appeared to disrupt IP₃R/Bcl-2 complexes in these cancer cells, resulting in an increase in basal Ca²⁺ levels. However, further work is needed to validate these findings in other Bcl-2-dependent and -independent cancer cells. BDA-366 also induced cell death in lung cancer cells that do not express Bcl-2, suggesting a Bcl-2-independent action of BDA-366.

b) VDAC-derived peptides

As stated in the introduction, a crucial role in the survival of many cancer cells is ascribed to the interplay between VDAC1 and multiple anti-apoptotic proteins [178, 179], which are often upregulated in cancer. Hence, VDAC1-based cell-penetrating peptides that can disrupt this complex formation demonstrate potential as an innovative approach to anti-cancer therapy. VDAC1-based peptides opposed the interaction between VDAC1 and the anti-apoptotic proteins Bcl-2 [179] and Bcl-XL [180] and hexokinase I [181], a mitochondrially bound protein important for the glycolytic rate. This abrogated their anti-apoptotic effects and stimulated VDAC1 oligomerization [182]. Moreover, it has been observed previously that VDAC1 oligomerization is coupled to apoptosis induction [183, 184]. Furthermore, unlike the BH4 domain of Bcl-2, the BH4 domain of Bcl-XL can target VDAC1, thereby suppressing the

VDAC1-mediated mitochondrial Ca^{2+} uptake [185]. One promising VDAC1-targeting peptide is the cell permeable Antp-LP4 that effectively and selectively induces concentration-dependent, apoptotic cell death in peripheral mononuclear cells (PBMCs) isolated from CLL patients while sparing PBMCs from healthy individuals [182]. Counteracting the VDAC1/Bcl-XL interaction Antp-LP4, favored ER-mitochondrial Ca^{2+} transfer and abolished Bcl-XL apoptotic protection (Table 2) [182]. Additionally, Antp-LP4 adversely affected the cellular bio-energetics (Table 2). The mechanism of action implied a detachment of hexokinase I, followed by the dissipation of the mitochondrial membrane potential and consequently a reduction in the ATP production (Table 2). On the contrary, application of N-terminal VDAC1-based peptides deteriorates the promoting effect of Mcl-1 and VDAC1 on lung cancer cell migration by restricting the mitochondrial Ca^{2+} transfer, thereby limiting the Ca^{2+} -dependent ROS production (Table 2) [186].

4. CONCLUSION

Targeting anti-apoptotic Bcl-2 proteins is an attractive strategy for novel anti-cancer therapies as many cancer cells have upregulated levels of anti-apoptotic proteins to combat pro-death signalling as a consequence of oncogenic stress. Bcl-2 exerts its pro-survival effect both at the level of the mitochondria and of the ER. Hence, major efforts have been undertaken to antagonize the interaction between pro- and anti-apoptotic Bcl-2-family members. A promising class of molecules represent the BH3 mimetics, including venetoclax, which is already applied in the clinic to treat CLL patients. Alternatively, another new and appealing strategy to target anti-apoptotic Bcl-2-protein family members is to antagonize their functional impact on Ca^{2+} -transport systems at the ER and mitochondria. Consequently, Bcl-2 inhibitors have booked successful results in inducing cancer cell death not only by directly antagonizing Bcl-2-family members but also by interfering with their Ca^{2+} -regulatory properties. Furthermore, it also seems that both pathways can impact each other, whereby tools that elicit pro-apoptotic Ca^{2+}

release can result in upregulation of pro-apoptotic Bcl-2-family members or tools that disrupt the complex between anti- and pro-apoptotic Bcl-2 proteins can impact ER-mitochondrial Ca^{2+} transfers. This may form the basis for unexpected synergistic actions between so-called “mitochondrial functions” of Bcl-2 in neutralizing pro-apoptotic Bcl-2 proteins and “ER functions” of Bcl-2 in suppressing pro-apoptotic Ca^{2+} -release events.

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Figure legends

Fig. 1. Antagonizing anti-apoptotic Bcl-2, Bim, Bax and the IP₃R. The anti-apoptotic properties of Bcl-2 enclose both the mitochondria as well as the inositol-1,4,5-trisphosphate receptor (IP₃R). At the mitochondria, Bcl-2 inhibits different pro-apoptotic proteins through its hydrophobic cleft (formed by the BH1-3 domains), which binds the BH3 domain of the pro-apoptotic molecules. In addition to this, Bcl-2 also prevents the activation of Bax by TM/TM interactions and/or *via* its BH4 domain. At the endoplasmic reticulum (ER), Bcl-2 via its BH4 domain and TM can also inhibit aberrant IP₃R-mediated Ca²⁺ release, thereby protecting against Ca²⁺-mediated apoptosis. Different anti-cancer therapies targeting Bcl-2's dual anti-apoptotic role have emerged. Hence, several small molecules induce mitochondrial outer membrane permeabilization by targeting the hydrophobic cleft (BH3I-2'/BH3 mimetics/obatoclax) or specifically the BH3 domain (HA-14-1) or BH1 domain (in the case of disarib) of Bcl-2, thereby releasing pro-apoptotic proteins. Also, the BH4 domain emerged as a promising target for anti-cancer strategies. For instance, BDA-366 converts Bcl-2 in a pro-death signaling molecule and consequently activates Bax directly. At the level of the IP₃R, Bcl-2/IP₃R disrupter (BIRD-2) reverses Bcl-2's anti-apoptotic effect by disturbing the interaction between Bcl-2's BH4 domain and the regulatory and coupling domain of the IP₃R, thereby alleviating IP₃R inhibition by Bcl-2.

Fig. 2: Off-target effects of Bcl-2-targeting compounds on the Ca^{2+} -signaling toolkit. In physiological circumstances the IP_3R releases Ca^{2+} from the ER. At the MAMs, Ca^{2+} is transferred to the mitochondrial intermembrane space by VDAC1. Ca^{2+} is then transported further towards the mitochondrial matrix *via* the MCU complex. Several potential anti-cancer therapeutics that target Bcl-2, display off-target effects at the level of the Ca^{2+} -signaling machinery. BH3I-2' targeting Bcl-2's hydrophobic cleft, e.g. causes $\text{IP}_3\text{R}/\text{RyR}$ -dependent Ca^{2+} release. HA-14-1, another molecule binding the hydrophobic cleft of Bcl-2, inhibits SERCA2b/1a. This causes a depletion of the ER. Furthermore, oblimersen, a molecule affecting Bcl-2's mRNA, is known to inhibit VDAC1, thereby giving rise to increased superoxide production and opening of the mitochondrial permeability transition pore. In addition, gossypol, obatoclox and ABT-737 induces ER stress and subsequent activation of the unfolded protein response (UPR), resulting in an upregulation of Mcl-1. Apogossypol and the related compound TW37, specifically, cause a Na^+ influx and a subsequent reorganization of the ER membrane.

Table 1. Overview of the Bcl-2-protein family members.

Protein	Functional class	Structure
Bcl-2	Anti-apoptotic proteins	
Bcl-2A1/A1/Bfl-1		
Bcl-B		
Bcl-w		
Bcl-XL		
Mcl-1		
Bak	Pro-apoptotic effectors	
Bax		
Bok		
Bid	Pro-apoptotic BH3-only <u>activators</u>	
Bim		
Puma		
Bad	Pro-apoptotic BH3-only <u>sensitizers</u>	
Bik		
Bmf		
Hrk		
Noxa		

Table 2. The on- and off-target effects of pro-survival Bcl-2 inhibitors

Compound	Structural Bcl-2 family	target in the	Main apoptotic-induced process	Off-target effects	Reference off-target effect
ABT-199 (venetoclax)	Selective for Bcl-2's hydrophobic cleft		Bax/Bak-dependent	No direct effect on Ca^{2+} homeostasis or on Ca^{2+} transporters (SERCA, IP_3R)	[136]
ABT-737, ABT-263 (navitoclax)	Bcl-2/Bcl-XL's hydrophobic cleft		Bax/Bak-dependent	Thrombocytopenia due to Bcl-XL inhibition, potentially contributing to Ca^{2+} -store depletion Activation of UPR and Mcl-1 upregulation in melanoma, resulting in apoptosis resistance	[128, 129, 131] [142]
Antp-LP4	Not determined		Revokes anti-apoptotic Bcl-XL protection $\rightarrow$ ER-mitochondrial Ca^{2+} transfer	Interacts with hexokinase I $\rightarrow$ loss of mitochondrial potential $\rightarrow$ ATP production $\downarrow$	[182]
Apogossypol, TW37	Not determined Specific for hydrophobic cleft of Bcl-2-family members?		Bax/Bak- and caspase-9-independent	Redistribution of the ER membranes via Na^+ influx	[149]
BDA-366	BH4 domain of Bcl-2		Converts Bcl-2 into a pro-apoptotic molecule $\rightarrow$ Bax-dependent	Not determined	
BH3I-2'	Hydrophobic cleft		Bax/Bak-dependent	$\text{IP}_3\text{R}/\text{RyR}$ -dependent Ca^{2+} release in pancreatic acinar cells	[123]

BIRD-2	BH4 domain of Bcl-2	Disrupts the Bcl-2/IP ₃ R interaction → IP ₃ R-mediated Ca ²⁺ release	Not determined	
Disarib	Preferentially BH1 domain of Bcl-2	Bax/Bak-dependent	No effect on platelets	[171]
Gossypol family	Not determined Specific for hydrophobic cleft of Bcl-2-family members?	Bax/Bak- and caspase-9-independent	PLA2 activity → in ↑[Ca ²⁺] _{cyt} → activating UPR → No → inhibition of Mcl-1 → apoptosis	[148]
HA-14-1	Hydrophobic cleft of Bcl-2	Bax/Bak-dependent?	ROS? ↑ Inhibits SERCA 2b/1a → depletion of the ER Ca ²⁺ store Impaired IICR activity in platelets	[157] [159] [131]
N-terminal VDAC1-based peptide	Not determined	Revokes anti-apoptotic Mcl-1 and Bcl-2 interaction → cytochrome C release and apoptosis	Ca ²⁺ -dependent ROS production and cell migration	[186]
Obatoclax	Hydrophobic cleft of Bcl-2/Bcl-XL/Mcl-1?	Bax/Bak-dependent?	Induces ER stress and UPR signaling in melanoma → Mcl-1 upregulation	[142]
Oblimersen	Bcl-2's mRNA	Not determined	Inhibits VDAC → O ₂ [*] → opening of mPTP ↑	[112]