BIOMOLECULES & THERAPEUTICS

Review
Biomol Ther 32(3), 267-280 (2024)

Unlocking the Therapeutic Potential of BCL-2 Associated Protein Family: Exploring BCL-2 Inhibitors in Cancer Therapy

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Abstract

Apoptosis, programmed cell death pathway, is a vital physiological mechanism that ensures cellular homeostasis and overall cellular well-being. In the context of cancer, where evasion of apoptosis is a hallmark, the overexpression of anti-apoptotic proteins like Bcl2, Bcl-xL and Mcl-1 has been documented. Consequently, these proteins have emerged as promising targets for therapeutic interventions. The BCL-2 protein family is central to apoptosis and plays a significant importance in determining cellular fate serving as a critical determinant in this biological process. This review offers a comprehensive exploration of the BCL-2 protein family, emphasizing its dual nature. Specifically, certain members of this family promote cell survival (known as anti-apoptotic proteins), while others are involved in facilitating cell death (referred to as pro-apoptotic and BH3-only proteins). The potential of directly targeting these proteins is examined, particularly due to their involvement in conferring resistance to traditional cancer therapies. The effectiveness of such targeting strategies is also discussed, considering the tumor's propensity for anti-apoptotic pathways. Furthermore, the review highlights emerging research on combination therapies, where BCL-2 inhibitors are used synergistically with other treatments to enhance therapeutic outcomes. By understanding and manipulating the BCL-2 family and its associated pathways, we open doors to innovative and more effective cancer treatments, offering hope for resistant and aggressive cases.

Key Words: Apoptosis, BCL-2 protein, BH3-mimetic drugs, Venetoclax, Nanotechnology, Natural compounds

INTRODUCTION

Apoptosis is a highly coordinated process that occurs in both embryonic as well as adult cells. It carries an important protective role against the development of tumors in adults; hence its absence is detrimental and can lead to cancer. In contrast to the naturally occurring apoptotic process, necrosis is a form of cell injury that occurs due to infections or trauma and is associated with excessive inflammation leading to tissue death (Kulbay *et al*., 2022; Qian *et al*., 2022). Phenotypic characteristics and changes allow for easy differentiation between the two processes. During necrosis, cells swell and their membranes rupture, leaking all their internal organelles and biochemical molecules such as ATP, ADP, heat shock proteins, and nuclear proteins. Moreover, the activation of non-specific nucleases causes the DNA to be cleaved at random sites giving a smear-like structure on an agarose gel. The efflux of various biochemical molecules recruits leukocytes to the site of injury, which initiates an inflammatory response that affects the injured cells and the surrounding tissue (Chan *et al*., 2015) (Fig. 1A). While necrotic cells swell and autolyze, apoptotic cells shrink, and their intact membranes start blebbing. Once the apoptotic cascade is activated, specific nucleases are proteolytically processed and activated to

Open Access **https://doi.org/10.4062/biomolther.2023.149**

Received Aug 25, 2023 **Revised** Nov 5, 2023 **Accepted** Dec 5, 2023
Published Online Apr 9, 2024

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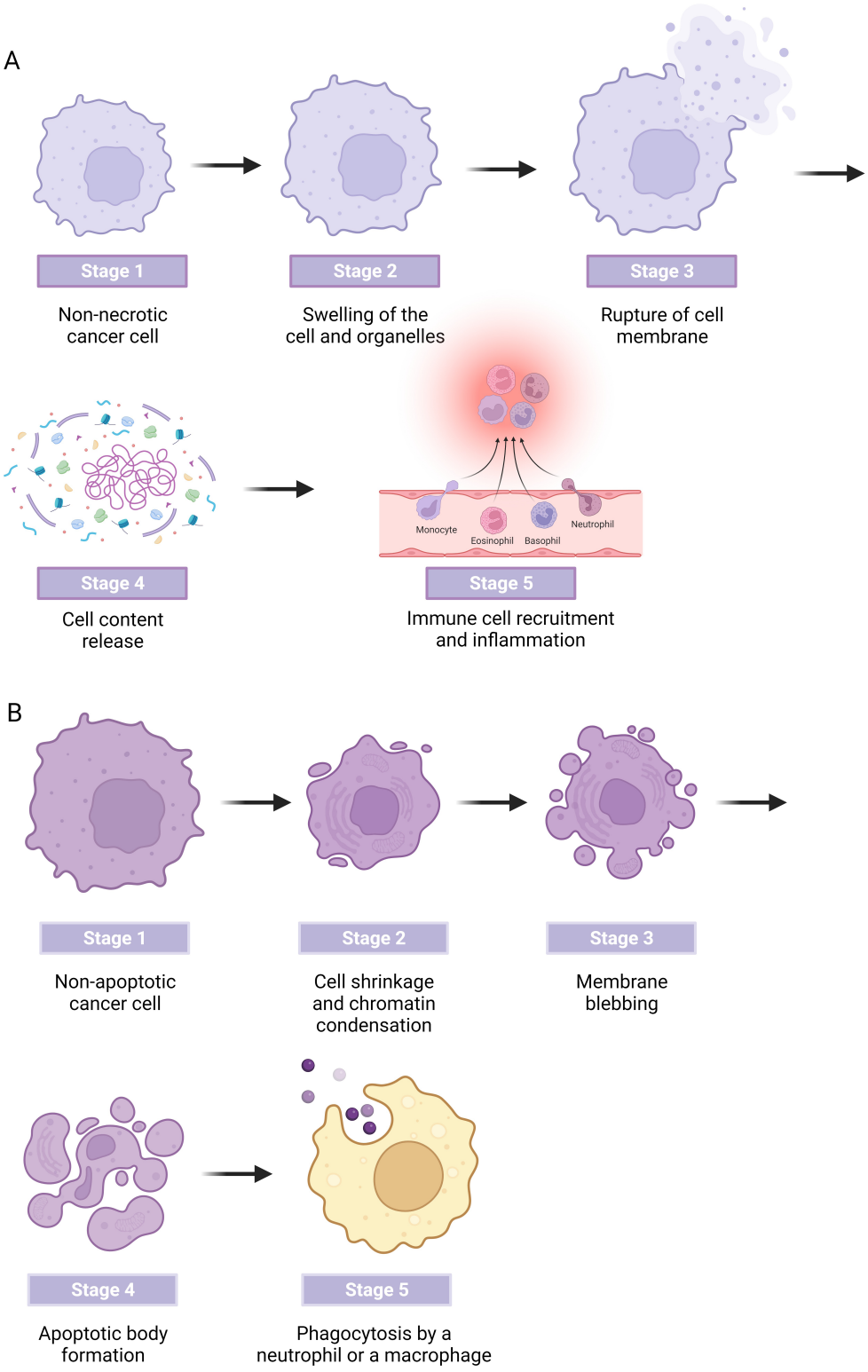

Fig. 1. (A) Illustration of a cell undergoing necrosis. Cells swell and their membranes rupture, leaking all their internal organelles and biochemical molecules such as ATP, ADP, heat shock proteins, and nuclear proteins. Moreover, the activation of non-specific nucleases causes the DNA to be cleaved at random sites giving a smear-like structure on an agarose gel. The efflux of various biochemical molecules recruits leukocytes to the site of injury, which initiates an inflammatory response that not only affects the injured cells but also the surrounding tissue. (B) Apoptotic cells shrink, and their intact membranes start blebbing. Once the apoptotic cascade is activated, specific nucleases are proteolytically processed and activated to fragment the cellular DNA in an orderly manner resulting in a ladder-like structure on an agarose gel. Apoptotic bodies are finally produced which are cleared from the system by phagocytic cells. Created with BioRender.com.

fragment the cellular DNA in an orderly manner resulting in a ladder-like structure on an agarose gel. Apoptotic bodies are finally from the system by phagocytic cells like neutrophils or macrophages without any adverse effects on the surrounding healthy tissue (Saraste and Pulkki, 2000) (Fig. 1B).

APOPTOTIC PATHWAYS

Apoptotic death signals can originate from various stimuli such as DNA damage, cellular stress, exposure to radiation, or nutrient deprivation. These signals can activate two distinct pathways: the extrinsic and the intrinsic pathways (Kerr, 2002) (Fig. 2). The extrinsic pathway (Fig. 2A) is activated through receptor-ligand binding mechanisms where multiple receptors, including the Fas receptor "also called CD95", death receptors (DR4 and DR5), and Tumor necrosis factor receptor (TNF-R1) bind their ligands Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), and Tumor necrosis factor alpha (TNFα), respectively (Xu and Shi, 2007). This binding recruits adaptor proteins and induce the formation of the death-inducing signaling complex (DISC) which will stimulate the caspase cascade rendering them active to proteolytically target various cellular proteins (Kulbay *et al*., 2022; Qian *et al*., 2022). On the other hand, intrinsic activation occurs mainly through the mitochondria, resulting in the formation of what is referred to as an apoptosome (Fig. 2B). BCL-2 protein family is known to be the key player in regulating this pathway (Tsujimoto, 1998). This family can be classified into three major groups based on their function and sequence similarities. The anti-apoptotic (Bcl2, Bcl-xL, Bcl-w, and Mcl-1), Pro-apoptotic (Bax, Bak, and less commonly involved Bok, Bcl-X_5, and Bcl-G_L), and the BH3-only proteins (tBid, Bim, Puma, Bad, Noxa, Bik, Bmf, Hrk, and Bnip3). It is important to note that Bnip3 operates as a constituent of the BH3-only protein family. Nevertheless, its principal function primarily centers on its significant participation in the regulation of the complex process of autophagy. Physiologically, anti-apoptotic proteins promote cell survival mainly by sequestering the pro-apoptotic members, rendering them inactive. When an internal signal is generated, such as DNA damage or increased Reactive Oxygen Species (ROS) formation (Wu and Bratton, 2013), BH3-only members are stimulated to promote cell death (Shamas-Din *et al*., 2011), either indirectly by binding of sensitizer BH3-only proteins to BCL-2 anti-apoptotic proteins, unleashing its inhibitory effect on pro-apoptotic members (Bad, Noxa, Bik, Bmf, Hrk, and Bnip3), or by direct binding of activator BH3-only proteins to BAX and BAK and activating them (tBid, Bim, and Puma). This leads to mitochondrial outer membrane permeabilization (MOMP) causing the efflux of apoptotic mediators like cytochrome c, procaspase-9 which will form the apoptosome and initiate the caspase cascade and cellular proteins destruction. One of the essential apoptotic mediators is the second mitochondria-derived activator (SMAC), called DIABLO, as it acts on the inhibitors of apoptosis proteins (IAPs) and inactivates them (Kulbay *et al*., 2022; Qian *et al*., 2022). As shown earlier, caspases are a group of endoproteases that play a central role in both apoptotic pathways by helping in the break down cellular components for the neat disposal that is characteristic of apoptosis (McIlwain *et al*., 2013).

BCL-2 family proteins

BCL2 is a constituent of the BCL2 protein family, which plays a pivotal role in the modulation of apoptosis. The proteins within this particular family can be classified into two main groups: anti-apoptotic members, such as BCL2 itself, Bcl-xL, MCL1, and pro-apoptotic members, such as BAX, BAK, and BAD (Table 1) (Qian *et al*., 2022). The capacity of these proteins to engage in mutual interactions and assemble into complexes is mostly dictated by their structural domains. At the structural level, BCL2 is comprised of four discrete Bcl-2 homology domains known as BH1, BH2, BH3, and BH4. The BH1, BH2, and BH3 domains congregate to form a hydrophobic groove configuration on the protein's surface, whereas the BH4 domain is situated proximal to the protein's N-terminus (Kulbay *et al*., 2022; Qian *et al*., 2022). A certain group within the BCL2 family, referred to as the 'BH3-only proteins' (such as BAD, BIM, PUMA) (Table 1), exclusively possesses the BH3 domain. This particular domain plays a crucial role as it facilitates the interaction between these proteins and the hydrophobic groove found in anti-apoptotic BCL2 proteins. The binding of a BH3-only protein to BCL2 or its anti-apoptotic counterparts results in the inhibition of their activity, hence triggering the activation of the pro-apoptotic proteins BAX and BAK (Shamas-Din *et al*., 2011). The protein BCL2 can engage in heterodimerization with pro-apoptotic counterparts by utilizing its BH domains. An example of this phenomenon is the interaction between BAX or BAK and BCL2, which results in the inhibition of their pro-apoptotic function. Conversely, the interaction between BH3-only proteins and BCL2 inhibits the sequestration of BAX or BAK by BCL2, therefore facilitating the process of apoptosis (Villalobos-Ortiz *et al*., 2020).

BCL-2 family proteins' mechanism of action and regulation

The major mechanism in which pro-apoptotic proteins function is based on the concept of Mitochondrial Outer Membrane Permeabilization (MOMP), which was explained earlier in this paper. MOMP can be stimulated either direct or indirect method (Shamas-Din *et al*., 2011). The direct method involves the binding of some members of the BH3 pro-apoptotic group-tBid, Bim, Puma, and activation of the Bax and Bak proapoptotic proteins, which will translocate to the outer mitochondrial membrane and create pores, thus allowing the efflux of apoptotic effector proteins to assemble the apoptosome. A key modulator of the intrinsic apoptotic pathway is the caspase-9, as it activates the downstream caspase cascade, including caspase 3, 6, and 7 (Hassig *et al*., 2014).

While pro-apoptotic members prompt the cellular death signals, another group of proteins works on shutting them down using various mechanisms. These are the anti-apoptotic members. Bcl2, Bcl-xL, Bcl-w, and Mcl-1. The significance of understanding these proteins comes from their contribution to many diseases' progression, most commonly cancer, especially after revealing tumor cell hallmarks which include their ability to escape apoptotic signals. (Del Bufalo *et al*., 1997; Tanaka *et al*., 2004).

These proteins contain a site for binding all BH3-only proteins and sequestering them therefore preventing the activation of pro-apoptotic factors such as Bax and Bak. Moreover, they can bind the apoptotic effectors, Bax and Bak, and constraining their activity.

The sensitizer BH3 proteins—Bad, Noxa, Bik, Bmf, Hrk,

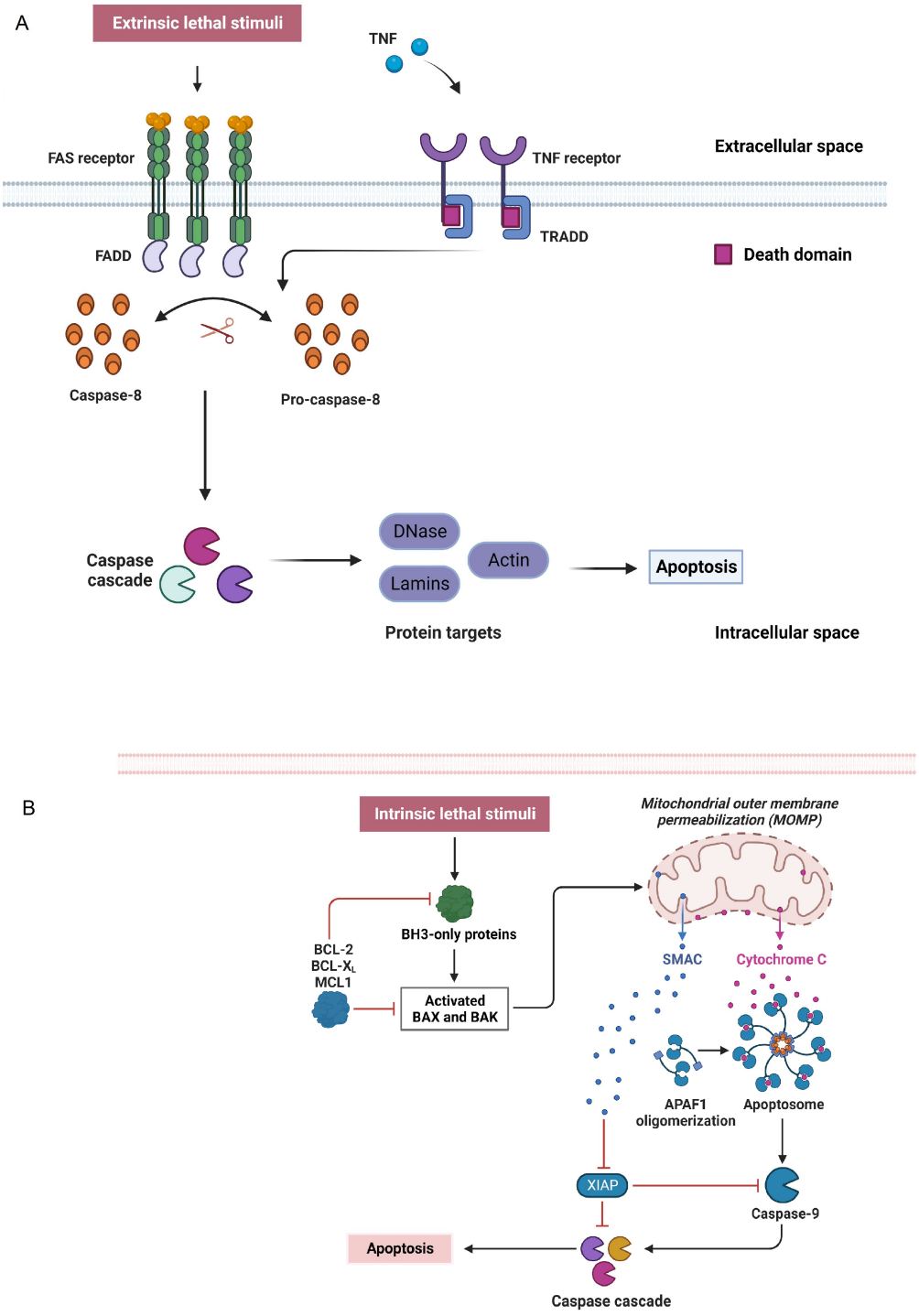

Fig. 2. Apoptotic pathways (A) extrinsic pathway is activated through receptor-ligand binding mechanisms where multiple receptors including the Fas receptor and Tumor necrosis factor receptor (TNF-R1) bind their ligands Fas ligand (FasL), and Tumor necrosis factor alpha (TNFα), respectively. This activates it and unmasks a death domain located in its cytoplasmic tail. Adaptor proteins such as FADD and TRADD bind the death domain and help in recruiting procaspase-8 to the site. Once recruited, procaspase-8 undergoes self-cleavage to transform into the active caspase-8, which will later act on downstream caspases, initiating a caspase cascade that includes caspase-3,6,7. Finally, various cellular protein targets will be proteolytically degraded by caspases and cell death will occur. Together the death receptor, the ligands, adaptor proteins, and the initiator caspase (caspase-8) are referred to as death-inducing signaling complex (DISC). (B) Intrinsic pathway is initiated with the activation of pro-apoptotic proteins such as Bax and Bak, leading to mitochondrial outer membrane permeabilization (MOMP). This permeabilization allows the release of cytochrome c from the mitochondria into the cytoplasm. Subsequently, cytochrome c associates with apoptotic protease activating factor 1 (Apaf-1), forming the apoptosome complex. The apoptosome activates procaspase 9, initiating a cascade of caspase activation. Leading to the cleavage of specific cellular substrates, DNA fragmentation and cellular disassembly. Created with BioRender.com.

Table 1. BCL-2 family protein members

Group	Anti-apoptotic members	Pro-apoptotic members	Pro-apoptotic- BH3-only members
Members	Bcl2, Bcl-xL, Bcl-w, and Mcl-1	Bax/Bak	Bad, Noxa, Bik, Bmf, Hrk, Bnip3, Bid, Bim, and Puma
Role in Apoptosis	Inhibit apoptosis	Enhance apoptosis	Enhance apoptosis

Table 2. BCL-2 family regulation

Mechanisms of regulation	Examples
mRNA surveillance	mRNA stability
Post-transcriptional modifications	Non-coding RNA species (miRNA, lncRNA)
Regulation of gene expression	Transcription factors (STAT, P53, NF-κB)
Post-translational modification	Phosphorylation, acetylation

and Bnip3—bind to the antiapoptotic proteins, thereby liberating activator BH3 proteins to promote MOMP (Letai *et al*., 2002; Kuwana *et al*., 2005; Certo *et al*., 2006). The antiapoptotic proteins bind to both the activator and the sensitizer BH3 proteins but are unable to complex with Bax and Bak. Therefore, antiapoptotic proteins must sequester the BH3 proteins to prevent Bax/Bak activation and cellular evasion of apoptosis (Kim *et al*., 2006a, 2006b).

The balance between the activity of both pro- and anti-apoptotic members of the BCL-2 protein family (Ke *et al*., 2022) must be maintained to ensure proper embryonic development and cellular homeostasis. Although essential for regulating the intrinsic apoptotic pathway, the BCL-2 protein family has a diverse set of functions that makes it unfeasible for certain cell types, such as neuronal cells, to develop and degenerate (Pemberton *et al*., 2021). BCL-2 proteins are majorly involved in cellular metabolism, maintaining mitochondrial homeostasis, cell cycle regulation, energy production, and intracellular calcium secretion and signaling (Gross, 2016). A broad range of mechanisms regulate the expression of this protein family (Table 2). One way is by modulating the mRNA transcription (Kønig *et al*., 2019) or by post-transcriptional methods such as altering the mRNA stability and production of non-coding RNA species like micro-RNAs and long non-coding RNA "lncRNA" (Cui and Placzek, 2018). Posttranslational modifications can also be utilized, including phosphorylation, ubiquitylation, cellular localization, and proteolytic processing (Vucic *et al*., 2011) (Table 2).

Several transcription factors can shuttle back and forth from the cytosol into the nucleus and alter the expression of the BCL-2 protein family. Of these are E2F-1, the nuclear factor-kappa beta (NF-κB) family (Luna-López *et al*., 2013), P53 tumor suppressor (Ozaki and Nakagawara, 2011), and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) (Puthier *et al*., 1999) (Table 2). One of the most complex transcription factors in our systems is the NF-κB as it affects more than 200 target genes that are involved in numerous downstream pathways including inflammation, cell survival, and differentiation, as well as innate immune responses (Liu *et al*., 2017). Inhibitors of apoptosis proteins (IAPs) are targets of the NF-κB pathway. They function by blocking the caspase cascade and preventing both intrinsic and extrinsic pathways (Lee and Collins, 2001).

The "guardian of the genome", the P53 tumor suppressor, is responsible for correcting any DNA damage caused by endogenous reactive oxygen species. It can translocate into the nucleus and regulate the expression of a diverse set of genes related to cellular death, DNA repair, cell cycle arrest, and inhibition of angiogenesis. BCL-2 and Bcl-xL were found to be down-regulated in response to p53 activation in various tumor types, such as breast cancer (Haldar *et al*., 1994).

Non-coding RNA species comprise an alternative regulation mechanism. Micro-RNAs are short RNAs composed of 20-22 nucleotides that resides in the nucleus where it functions as a transcriptional repressor by binding multiple mRNAs and either degrading them by incorporating them in the RNA-induced silencing complex (RISC) or increasing their susceptibility to post-transcriptional modifications that prevent their interaction with the cytoplasmic ribosomes, therefore, preventing translation. It was reported that miR-26a's function as a tumor suppressor as it was found to downregulate the expression of the anti-apoptotic MCL-1 and inhibit breast cancer cell proliferation and migration (Gao *et al*., 2013). Moreover, recently, Mei and his colleagues reported that the knockdown of LncRNA SNHG6 retained cisplatin sensitivity and prevented gastric cancer cell progression through the miR-1297/BCL-2 axis (Mei *et al*., 2021). Modifications are not confined to mRNA but can also occur on the protein level, referred to as post-translational modifications. Various phosphorylation processes by kinases were reported to alter the BCL-2 protein family function. For instance, phosphorylation of BCL-2 at Serine70 (S70) was found to enhance its binding affinity to proapoptotic members like the Bim and Bak, leading to increased cell viability and chemotherapeutic drug resistance (Dai *et al*., 2013). Histone Deacetylase inhibitor (HDACi) was found to induce MCL-1 phosphorylation at Serine159 and Threonine163 sites. These modifications caused an elevated expression in the MCL-1 in colon cancer cells, thus inhibiting apoptosis (Tong *et al*., 2018).

BCL-2 family proteins in diseases

Evidence on the role of BCL2 over-expression in tumorigenesis has been found in a wide range of cancer types. These are B-cell lymphoma, prostate cancer, lung cancer, Acute lymphoblastic leukemia, and breast cancer (Delbridge *et al*., 2016). p53 mutant acute lymphoblastic cells were demonstrated to be resistant to apoptosis by upregulating the Bcl-XL and downregulating the BAX proteins, whereas the wild-type p53+ cells were more sensitive to cell death (Findley *et al*., 1997). Similar outcomes regarding the relation between the TP53 mutation and sensitivity to apoptosis were obtained using erythroleukemic cell lines (Schott *et al*., 1995).The rate of expression of BCL2 was studied in small cell lung carcinoma (Kaiser *et al*., 1996), this study revealed an over-expression of this anti-apoptotic factor in most of the cases and

interestingly, survival rate, although not significant, was found to be higher in BCL2+ patients, which grabs the attention to dig further in the relation between the two. In colorectal cancer (CRC), MCL-1 was found to form a tri-protein complex with alpha-enolase and calmodulin, facilitating its translocation into the nucleus. This subcellular translocation was found to induce resistance to chemotherapeutic agents in CRC (Fu *et al*., 2022). Besides its anti-apoptotic role, MCL-1 regulates fatty acid oxidation (FAO) in certain tumor types, especially those of hematologic origin. FAO is one of the alternative pathways used to generate energy, which aids tumor cells survive and proliferate. Elevated levels of MCL-1 were associated with tumor progression, whereas a genomic deletion in the MCL-1 gene led to energy deprivation in the cells and eventually their death (Prew *et al*., 2022). Dysregulation in BCL-2 protein family is seen in other disease conditions such as autoimmunity, Alzheimer's disease, Down syndrome, Parkinson's, and other Neurodegenerative disorders (Sawa *et al*., 1997; Kitamura *et al*., 1998; Anilkumar and Prehn, 2014). The upregulation of pro-survival proteins as well as the downregulation of pro-apoptotic members are the most encountered alterations. For instance, BCL-2 and Bcl-xL over-expression contributed to the selective neuronal loss found in both Alzheimer's disease (Kitamura *et al*., 1998) and Down syndrome (Sawa *et al*., 1997) patients. Apoptosis is essential for eliminating the autoreactive immune cells, therefore, precluding autoimmunity. Lack of pro-apoptotic BH3-only Bim or the accumulation of BCL-2 has been linked with the enhanced survival and development of T regulatory cells, which with time, predisposes the individual to autoimmune diseases (Tischner *et al*., 2012).

Authentic BH3 mimetics

The fact that BCL-2 protein family intervenes with a diverse set of pathological conditions makes it a druggable target. A new class of anticancer drugs was recently introduced to treat different tumor types. These are the BCL-2 proteins inhibitors, also known as BH3 mimetics. It includes small molecules that antagonize the pro-survival function of anti-apoptotic proteins such as BCL2, Mcl1, and Bcl-xL. BH3 mimetics must have a direct effect on the mitochondria of the anti-apoptotic cell, inhibit the anti-apoptotic factors through direct protein-protein high-affinity interactions, and induce MOMP and apoptosis in a BAX/BAK-dependent manner (Villalobos-Ortiz *et al*., 2020). While many drugs entered the clinical trials stage as potential BCL2 inhibitors, only few were proven to meet the eligibility criteria. Obatoclax, subatoclax, maritoclax, apo gossypol, TW-37, UMI-77, and BDA-366 for example were found to harbor anti-apoptotic functions, but independent of the BAX/BAK axis. These putative mimetics can be distinguished from authentic mimetics by their ability to kill BAX/BAK-deficient cells. (Villalobos-Ortiz *et al*., 2020) have developed a toolkit to distinguish putative BH3 mimetics from the true ones. Their approach was to test 18 different compounds on 5 cell lines over-expressing anti-apoptotic factors like BCL-2, Bcl-xL, and MCL-1. By performing viability tests and measuring the MOMP via the release of cytochrome c using flow cytometry, they could segregate these compounds and better understand their mechanism of action. Nowadays, BH3 mimetics are being used in a variety of diseases, including cancer, cardiovascular disease, and tissue fibrosis with a focus on inducing tumor cell death. Multiple compounds were approved as anticancer drugs for both solid and hematological malignancies. This matter will be further elaborated later in this paper. Their mechanism of action is to interfere with the BH3-only proteins and their interaction with the BCL2 protein, hence activating the BAX/BAK factors and inducing cell death. The major target of BH3 mimetics is a 15-amino acid sequence located in the hydrophobic pocket of the BCL2 anti-apoptotic protein. This sequence is responsible for the binding of BCL2 with the BH3-only sensitizers, eventually leading to the inactivation of BAX/BAK pro-apoptotic proteins. Targeting this sequence allows the BH3 mimetics to block the BCL2 activity, which will help enhance the BAK/BAX activity, leading to cellular death (Bierbrauer *et al*., 2020; Villalobos-Ortiz *et al*., 2020).

BH3 mimetics in disease therapy

Organ fibrosis is characterized by the replacement of the original tissue with fibers. Recently the role of BH3 mimetics in inducing apoptotic signals in myofibroblasts has gained attention as this could lead to reversing fibrosis and restoring organ function (Kuehl and Lagares, 2018). Navitoclax (ABT-263) is a BH3-mimetic drug that targets and inhibits Bcl-xL protein. It significantly reverses fibrosis in patients with scleroderma, a fibrotic connective tissue disorder (Lagares *et al*., 2017). Moncsek *et al*. (2018) examined the effect of A-1331852 BH3 mimetic on senescent cholangiocytes which have been linked with Primary sclerosing cholangitis (PSC). Senescent cholangiocytes consistently secret growth factors which leads to the activation of stromal fibroblasts hence fibrosis. Upon *in vitro* studies, their results showed an almost 80% reduction of fibrosis-inducing growth factors and cytokines (Moncsek *et al*., 2018). Aside from fibrosis, BH3 mimetics were also found to alter cardiomyocyte viability by targeting the MCL-1 anti-apoptotic factor. The inactivation of MCL-1 via the small molecule inhibitor S63845 disrupted the mitochondrial dynamics as well as the cytoskeletal actin filament's organization (Rasmussen *et al*., 2020). ABT-737, an inhibitor originally developed for oncology inhibited T and B lymphocyte proliferation in animal models with Rheumatoid arthritis (RA) and Systemic lupus erythematosus (SLE). BCL-2 proteins were proven to exhibit aberrant expression in various autoimmune disorders such as SLE and RA; therefore, researchers thought of testing the effect of BH3 mimetics used to treat cancer on mouse models with autoreactive lymphocytes. Surprisingly, ABT-737, which targets BCL-2, Bcl-xL, and BCL-w, significantly induced caspase activation and apoptosis in a concentration-dependent manner and helped clear the disease in mice (Bardwell *et al*., 2009). Food and drug administration (FDA) approved the usage of Venetoclax, a BCL-2 inhibitor, to treat relapsed chronic lymphocytic leukemia (CLL). Furthermore, it has been successfully utilized to treat patients with Refractory autoimmune cytopenias like Immune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA) (Gordon *et al*., 2019).

BH3 mimetics and cancer

BH3 mimetic-drug compounds were observed to have an efficient effect on reducing the cell's insensitivity to death signals, a feature that is considered a hallmark of cancer and helps tumor growth and development; therefore, the impact of BH3 mimetics had to be investigated in cancer diseases. The extensive research in this field was accompanied by a better understanding of these compounds, their mechanism of action, and their potential use in cancer therapy. As mentioned earlier, BCL2 family proteins were found to have a

dysregulated expression in various hematological and solid tumor types, such as B/T-cell lymphoma, acute lymphoblastic leukemia, breast, prostate, pancreatic, thyroid, and many cancers. It is essential to think of drugs that can target this family of proteins and halt their activity. Venetoclax (ABT-199) is a BCL2 inhibitor compound approved by the FDA for the treatment of CLL, acute myeloid leukemia (AML), and multiple myeloma (MM). It binds to BCL2 with high affinity and induces the activation of the TP53-independent apoptotic pathway. CLL is of good efficacy in both monotherapy and combination therapy, unlike AML cases which showed good efficacy only in combination therapy rather than monotherapy (Lasica and Anderson, 2021). Sejic and colleagues conducted a series of *in vitro* tests involving cell lines of Epstein-Barr-virus-induced T/NK-lymphoma (Sejic *et al*., 2020). The cell lines were treatment with different BH3 mimetics, including Venetoclax (ABT-199), a selective inhibitor of BCL-2; ABT-737, which targets BCL-2, BCLXL, and BCL-W; A-1331852, a targeted inhibitor of BCL-XL; and S-63845, an inhibitor of MCL-1. The assessment of cellular viability was conducted using flow cytometry at a time point of 48 h after treatment, as described by Sejic *et al*. (2020). The findings revealed a significant decrease in viable cells across the whole range of cell lines when exposed to medicines that inhibit BCL-2, Bcl-xL, and MCL-1. Moreover, by subjecting neuroblastoma cell lines to these inhibitors, it was observed that all treated cells demonstrated decreased viability, thus displaying a favorable response to the administered treatment. This observation implies that BCL-2 does not exert exclusive influence over apoptosis in neuroblastoma, as there are other proteins within this family that also play a role. The significant observation made by Bierbrauer *et al*. (2020) was the increased responsiveness of the cell to A-1331852, which highlights the crucial involvement of Bcl-xL in the pathogenesis of this particular malignancy (Bierbrauer *et al*., 2020). BH3 mimetics were also tested with a combination of other drugs. Navitoclax (ABT-263) antagonizes BCL-2 and MCL-1 function. It was tested in combination with the macrolide antibiotic rapamycin on the follicular lymphoma cell lines, DoHH-2 and SuDHL-4. Results showed a significant increase in the Annexin V staining when both drugs were used compared to the Navitoclax alone. This drug combination was also tested *in vivo* on DoHH-2 and SuDHL-4 animal models, and results showed an approximate increase in tumor growth inhibition of 50% (Ackler *et al*., 2008). A few years later, the effect of Navitoclax was tested against a broader range of lymphomas, including mantel cell lymphoma (MCL), non-Hodgkin's lymphoma (NHL), Diffuse large B-cell lymphoma, in addition to CLL. But this time, it was combined with the nitrogen mustard chemotherapeutic agent bendamustine. Treatment with both navitoclax and bendamustine showed a synergistic response on tumor growth inhibition as it was significantly increased to more than 90%. Moreover, the tumor objective response rate increased, indicating a better overall response to therapy (Ackler *et al*., 2012). Specific inhibitors that target MCL-1 were developed using High throughput screening and structure-based design. AZD5991 is an inhibitor AstraZeneca designed only to target MCL-1. It binds MCL-1 with high affinity and activates the BAK-dependent mitochondrial apoptotic pathway. AZD5991 showed potent antitumor effects both *in vitro* and *in vivo* multiple myeloma and acute myeloid leukemia models (Tron *et al*., 2018). A clinical trial was launched by the developing company (AstraZeneca) "NCT03218683" in 2017 to test the activity of AZD5991 on patients with relapsed acute myeloid leukemia. This study showed promising outcomes for AML and Myelodysplastic syndrome (MDS), Multiple myeloma (MM), and CLL. Amgen (CA, US) has developed another selective MCL-1 inhibitor using high throughput screening; this is AMG-176. Yi *et al*. (2020) hypothesized that AMG-176 effectively kills CLL cells. So, using more than 70 CLL patient samples, they performed cytotoxicity and viability assays using AMG-176 alone and in combination with Venetoclax (BCL-2 inhibitor). Results showed a significant cytotoxic effect of AMG-176 in addition to a synergistic effect when combined with Venetoclax (Yi *et al*., 2020).

The elevated complexity and quantity of biological data, along with the invasion of computational tools, attracted attention towards designing targeted therapies by integrating molecular modeling and artificial intelligence (Balasubramanian, 2022). These therapies are believed to be more efficient, with a lower toxicity and mutagenicity rate (Balasubramanian, 2022). In-silico modeling and docking studies have been run to identify BCL-2 inhibitor compounds (Sahin *et al*., 2021). In-silico compound libraries were used to compute the therapeutic activity value (TAV), followed by hybrid docking and molecular dynamics (MD) (Sahin *et al*., 2021). Several drugs were developed using this approach. Obatoclax mesylate (also known as GX15-070) is a compound initially developed by the University of Montreal, Canada. It targets BCL-2 family members, mostly BCL-2 and MCL-1, and inhibits the sequestration of the pro-apoptotic proteins, BAK and BAX, enhancing apoptotic activation. The mechanisms of this drug have been studied in AML cell line and primary cells (Konopleva *et al*., 2008) and was found to harbor an antiproliferative activity. Moreover, obatoclax has been shown to have anticancer activity in a wide range of *in vitro* cell lines of non-small cell lung cancer, mantle cell lymphoma, melanoma, as well as multiple myeloma (Li *et al*., 2008). Another MCL-1 inhibitor mentioned earlier, S-63845, was also designed using in-silico modeling. As MCL-1 is overexpressed in many cancer types, its inhibition is of valuable anticancer effect. S-63845 was found to bind the BH3-binding groove of MCL-1 and activate the BAX/BAK-dependent mitochondrial apoptotic pathway (Wang *et al*., 2021b). Multiple myeloma patients have an upregulated MCL-1; therefore, studies were conducted to target this protein. One of the studies performed on AML and MM mouse models used S-63845, resulting in a 100% tumor regression (Al-Odat *et al*., 2021). In addition to hematologic malignancies, S-63845 has promising activity against some solid tumor cell lines, such as triple-negative breast cancer (Campbell *et al*., 2018). However, *in vivo* testing has not yet been investigated. GDC-0941, a PI3K inhibitor, enhanced apoptosis and downregulated MCL-1 levels when combined with a BCL-2 inhibitor in AML cells (Jin *et al*., 2013). The drugs mentioned above comprise a small example of using in-silico computational tools and high-throughput screening techniques to develop novel and efficient therapies and solutions.

Natural BCL-2 inhibitors

Natural product extracts (NPEs) represent a tremendous source of novel drugs and chemical compounds. In ancient life, NPEs were used to treat various health conditions ranging from simple headaches to a broader range of complex pathological conditions such as severe wounds, diabetes, cancer, tuberculosis, and many others (Bustanji *et al*., 2006; Moham-

mad *et al*., 2013; Kasabri *et al*., 2014; Motawi *et al*., 2014; Harb *et al*., 2018; Qasem *et al*., 2020; Althaher *et al*., 2022; Eldesouki *et al*., 2022; Bou Malhab *et al*., 2023; Tarawneh *et al*., 2023). In the past few decades, modern medicines have overshadowed these compounds. However, nowadays, and with the emergence of new infectious agents that are drastically evolving, drug discovery organizations are directing their focus toward applying high throughput analysis to generate NPE libraries hoping to come up with potent potential therapeutic agents (Thornburg *et al*., 2018; Althaher *et al*., 2022; Eldesouki *et al*., 2022; Bou Malhab *et al*., 2023; Tarawneh *et al*., 2023)

As discussed earlier, the anti-apoptotic BCL-2 protein family is a validated target for cancer therapy. Few drugs have shown potent effects and were approved by the FDA for disease therapy; therefore, shifting towards natural extracts was the alternative. A large-scale project was initiated in the past two decades by the National Cancer Institute (NCI), where they tested more than 150,000 natural compounds derived mostly from terrestrial plants, marine plants, and invertebrates, and some from microbial sources (Hassig *et al*., 2014). High- Throughput Screening (HTS) techniques were used to screen these products against all antiapoptotic protein family members (Bcl-2, Bcl-W, Bcl-xL, Bcl-B, Mcl-1, and Bfl-1). Results revealed 994 NPEs with confirmed activity against one or more BCL-2 protein members. Perylene quinone mycotoxins like Altertoxins I, II, and alterperylenol isolated from the fungus *Alternaria alternata* and gossypol are examples of these compounds. Moreover, 17% of these NPEs were found to activate Caspase 3/7 pathways in tumor cells, therefore, inducing apoptosis (Table 3) (Ackler *et al*., 2008). Many bacterial endotoxins were found to harbor an anti-cancer effect. Examples are the cytotoxic necrotizing factor (CNF) secreted by Escherichia coli that was found to suppress cellular differentiation and induce apoptosis (Khoshnood *et al*., 2022). *Pseudomonas aeruginosa*, a gram-negative non-fermentative bacillus, secretes several exotoxins, including exotoxin A & T, that induce caspase 3-dependent apoptosis and DNA fragmentation (Wolf and Elsässer-Beile, 2009; Goldufsky *et al*., 2015). Antibiotics produced by bacterial cells are also potent anticancer agents. For instance, Tetrocarcin A secreted by Micromonospora sp. was described to antagonize BCL2 function and inhibit the junctional adhesion molecule A and human epidermal growth factor receptor 2 (HER2) (Vellanki *et al*., 2019). It also showed strong bioactivity against cellular growth as it inhibits both AKT and BCL2 pathways. Several mouse models with various tumor types were treated with Tetrocarcin A, and all exhibited promising tumor suppression, most commonly breast and lung cancer (Nakashima *et al*., 2000; Vellanki *et al*., 2019). This compound antagonizes IAP proteins, which is another mechanism by which it activates apoptosis. Pang X *et al* performed a cell viability assay and tested the effect of Perylenequione derivatives on the gastric and hepatocellular carcinoma cell lines, SGC-7901 and BEL-7402, respectively (Pang *et al*., 2018). A secondary metabolite produced by Streptomyces bacteria is Actimycin A functions in halting the mitochondrial electron transport chain, as well as arresting the cell cycle by increasing the expression of cyclin-dependent kinase inhibitor (CDKI) and decreasing CDK 2/4/6 and cyclin D levels. It was also found that this metabolite causes the loss of mitochondrial membrane potential and elevates the levels of the proapoptotic BAX, making it an enhancer of the apoptotic process (Park *et al*., 2007). *In vitro* studies using lung cancer cells, juxtaglomerular cells and liver cells proved the antiproliferative effect of actinomycin A (Park *et al*., 2007; Han and Park, 2009). Gossypol, a natural polyphenol extracted from cottonseeds that were historically utilized in Chinese herbal medicine, was proven to eliminate many cancer types *in vitro*

Table 3. Different classes of BCL-2 inhibitors

Type of inhibitor	Mechanism of action	Examples
Chemical compounds	Interfere with the BH3-only proteins and their interaction with the BCL2 protein, hence activating the BAX/BAK factors	BH3 mimetics: Venetoclax, Navitoclax, A133852
		In silico generated: Obatoclax mesylate, S63845, GDC-0941
Natural compounds	· Antagonize BCL2 function. · Caspase-3-dependent apoptosis. · Antagonizes IAP proteins	Terrestrial plants, marine plants, and invertebrates, Perylene quinone mycotoxins
Inhibitors in nanoparticles	Tumor-targeted drug delivery Decreases toxicity	· Chemotherapy-BCL2 inhibitor nanoparticle (Doxorubicn-Navitoclax nanoparticle · Metal nanoparticles (Silver nanoparticles) · BCL2 inhibitors combination (Venetoclax-S63845 nanoparticle)
Dual inhibitors	Improve the efficiency of tumor therapy	· AZD4320: targets BCL2 and Bcl-xL · Indole derivative: targets BCL-2 and MCL-1 · BT-1197: targets BCL2 and Bcl-xL
Combination therapies	Bypass potential resistance and modifications in the apoptotic pathway	· BCl2 inhibitors with HDACis · Kinase inhibitors with BCL-2 inhibitors · Immune checkpoint inhibitors with BCL-2 inhibitors · Chemotherapeutic drugs with BCL-2 inhibitors

and *in vivo*. One way it does that is by acting on the Jun N-terminal kinases or JNKs that phosphorylate pro-apoptotic BCL2 members, enhancing cell death (Dhanasekaran and Reddy, 2008). Another way gossypol works is by inhibiting the activity of BCL-xl anti-apoptotic member which allows BAX and BAK proteins to transmit the apoptotic signal. Moreover, gossypol exhibited a synergistic effect when combined with chemotherapy and/or radiation therapy, as it decreases the rate of resistance and increases the cell's susceptibility (Xu *et al*., 2005; Zerp *et al*., 2009). Several plants were found to have chemical compounds that, when extracted, are of great benefit to human health; an example is Purpurogallin, which is a compound found in oak nutgall trees that possesses antioxidants, anti-inflammatory, and anticancer effects (Gao *et al*., 2018). Different types of cells were examined, such as keratinocytes, neurons, and epithelial cells. In esophageal squamous cell carcinoma (ESCC) cells, Purpurogallin acted as an inhibitor of the mitogen-activated protein kinase cascade (MAPK), reducing the expression of cyclins A and B therefore arresting the cell cycle and inducing apoptosis by activating the poly (ADP-ribose) polymerase-1, PARP-1 (Xie *et al*., 2019).

BCL2 inhibitors in nanoparticles

Drug delivery has always been a concern and many factors must be considered before administering any drug, such as side effects, cytotoxicity, biodegradability, drug absorption, drug dosage, etc (AlKhatib *et al*., 2006; Matalqah *et al*., 2020; Bustanji *et al*., 2023). A few decades ago, a new technology was discovered to help administer drugs more safely and with minimal dosage; this was referred to as nanoparticle drug delivery (Khdair *et al*., 2016). Nanoparticles are nanomaterials that bind drugs with high affinity and aid in their targeted delivery and controlled release (Patra *et al*., 2018; Alshaer *et al*., 2019; Gharaibeh *et al*., 2021; Lafi *et al*., 2021). Tumor-targeted nanoparticles were used to improve the therapeutic index of different BH3 mimetics. This technology showed impressive results as particles that cause dual inhibition of more than one BCL-2 protein were delivered, which increased the cancer remission rate. Mouse models with diffuse large B cell lymphoma were treated with S63845 and Venetoclax using two methods. In the first group, both drugs were administered systemically in the traditional route, while the other group took the same drugs but instead used nanoparticle technology. Traditional drug delivery showed excessive exposure of healthy organs to the drugs and weight loss. Moreover, the blood parameters, including RBC, WBC, platelet, and hemoglobin, were adversely affected.

On the other hand, the nanoparticle delivery method decreased the exposure rate of healthy organs and maintained the animal's weight (Tannan *et al*., 2021). Another promising cancer therapy approach is combining a chemotherapy-containing nanoparticle and a BH3 mimetic drug. In a study, MDA breast cancer cells were treated with doxorubicin nanoparticles along with BCL-2 inhibitor Navitoclax. Interestingly, the combination excreted synergism (Table 3). Therefore, mouse models of the same tumor type were treated following the same therapeutic strategy, and similar results were obtained (Kim *et al*., 2021). The use of metallic nanoparticles has become the center of attention, especially since it has shown potent antitumor effects as it increases the formation of reactive oxygen species (ROS), which regulates the expression of both BAX and BCL2. Moreover, this process triggers the caspase cascade activation by inducing mitochondrial damage and increased permeability (Daei *et al*., 2023). Silver nanoparticles (AgNPs) are the most used. They have been tested against different tumor cells, including bladder and colon cancers, where they induce apoptosis in those cells by stimulating the activation of the apoptotic intrinsic pathway (El-Deeb *et al*., 2020)

Dual BCL2 family inhibitors

Several types of inhibitors that target the BCL2 family members were developed to improve the efficiency of tumor therapy. Earlier in the review, we discussed many examples of inhibitors; some are chemical compounds, natural product extracts, microbial secondary metabolites, or even inhibitors that are incorporated in nanoparticles. Scientists started working on compounds targeting more than one member at a time. AZD4320 is a compound identified to bind BCL2 and Bcl-xL with high affinity. It has shown robust effects against hematologic malignancies in cell lines and patient samples with AML, ALL, CLL and MDS as it decreases cellular viability. In addition, it showed activity in xenograft models that have developed Venetoclax resistance, indicating that this kind of compound can act as a second line of therapy in cases where resistance is acquired (Balachander *et al*., 2020). Indole derivatives were designed and synthesized to bind BCL2 and MCL-1 via Van der Waals forces and hydrogen bonds. These were tested against cancer lines, including breast, prostate, and acute T-cell leukemia (Liu *et al*., 2023). A study has been carried out on WL-276, which is a BCL2 antagonist derived from the 1-phenyl-1H-indole molecule. The results indicated that BCL2 was suppressed by 71% and MCL-1 by 54%. The findings presented in this study offer an encouraging basis for the advancement of further inhibitors targeting BCL2/MCL-1 (Wang *et al*., 2008; Xu *et al*., 2017). Another BCL2/Bcl-xL inhibitor that was effective against lymphoma cells, DLBCL and Burkitt's lymphoma is BT-1197. The activation of the endogenous apoptotic pathway was seen upon treatment with BM-1197. It was found to alter Bak/Bcl-xL, Bim/Bcl-2, Bim/Bcl-xL, and PUMA/Bcl-2 protein interactions and induce a conformation change in the proapoptotic BAX leading to the release of apoptotic mediators such as cytochrome c hence cell death (Sun *et al*., 2020). Many other compounds are being synthesized daily, hoping to achieve the highest antiproliferative effect possible as this can either replace chemotherapy or exert synergism (Table 3).

Combination therapies involving BCL-2 inhibitors

The emergence and subsequent authorization of venetoclax, a distinct inhibitor of BCL2, represented a significant breakthrough in the field of precision cancer treatment. The efficacy of this medicine as a single treatment for CLL and other lymphoid neoplasms has been demonstrated to be exceptional. Unfortunately, the prolonged utilization of venetoclax has been associated with the emergence of secondary resistance a phenomenon that could be mediated by modifications in the apoptotic pathway. This implies that the efficacy of BCL2 inhibitors may be enhanced when delivered intermittently rather than as a sustained treatment regimen (Roberts *et al*., 2021). Nevertheless, when used in combination with other therapeutic approaches, it has demonstrated potential in the management of AML and may bypass potential resistance.

In parallel with the advancement of BCL2 inhibitors, the

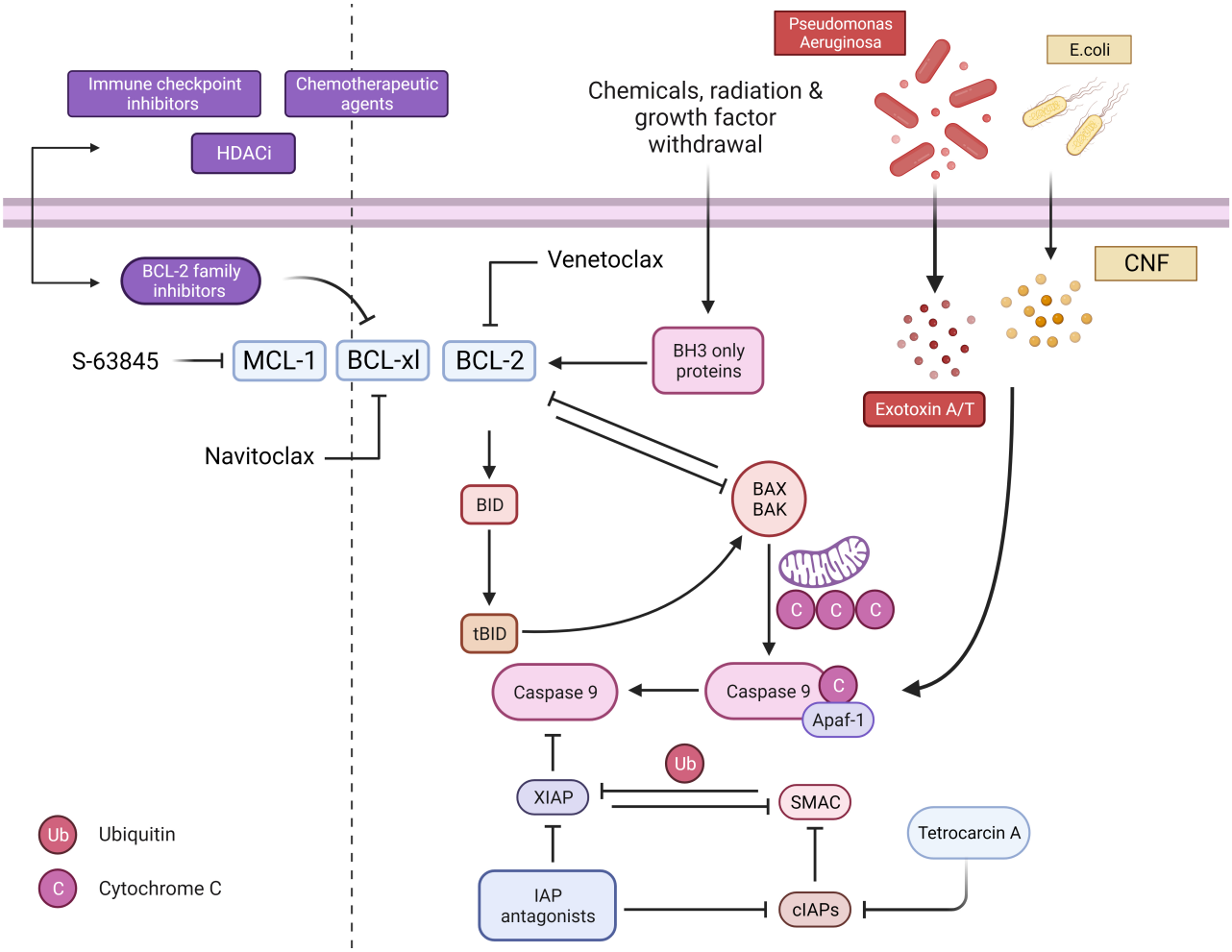

Fig. 3. Summary of different classes of BCL-2 family proteins inhibitors. Created with BioRender.com.

field of cancer therapeutics has observed the emergence of epigenetic treatments. Epigenetic alterations, characterized by their ability to enhance tumor aggressiveness without altering the DNA sequence, represent a promising class of therapeutic approaches in the field of cancer treatment. Histone deacetylases (HDACs) play a central role in epigenetic reprogramming, and their inhibitors (HDACis) have demonstrated promising anticancer effects; therefore, combining BCl2 inhibitors with HDACis can boost their therapeutic efficacy (Prado *et al*., 2021; Scheipl *et al*., 2021; Wang *et al*., 2021a).

The effectiveness of venetoclax has encouraged additional research in this field. It was combined with multiple anticancer drugs to target multiple pathways in the cancer cell cycle. Numerous examples were studied in both basic and clinical research, such as the combination of Venetoclax and Azacitidine or Decitabine for newly diagnosed acute myeloid leukemia (AML) (Aribi *et al*., 2023; Jin *et al*., 2023). Patients with newly diagnosed AML who are ineligible for intensive chemotherapy also have the option of receiving Venetoclax and Low-Dose Cytarabine (Asada *et al*., 2023). The combination of Venetoclax and Rituximab has been also approved for the treatment of CLL (Robak *et al*., 2022).

Kinase inhibitors, such as Bruton's tyrosine kinase (BTK) inhibitors in CLL, can induce cell death by inhibiting essential survival signaling pathways for cancer cells. By upregulating anti-apoptotic proteins, however, cancer cells can evade this. This resistance can be defeated by combining kinase inhibitors with BCL-2 inhibitors, resulting in synergistic cell death (Gifford *et al*., 2020).

Recent research indicates that BCL-2 inhibitors can modify the tumor microenvironment to make it more hospitable to immune cell infiltration. There is the possibility for enhanced anti-tumor responses when combined with immune checkpoint inhibitors, which unleash the immune system's power against cancer cells (Kohlhapp *et al*., 2021).

Bortezomib and other proteasome inhibitors induce cell death in cancer cells by disrupting protein homeostasis. Their combination with BCL-2 inhibitors can result in a dual attack on cancer cells, causing them to undergo apoptosis (Weller *et al*., 2022).

In CLL patients, a triple combination of Venetoclax, Ibrutinib, and Obinutuzumab has been investigated. With a high incidence of undetectable minimal residual disease in the peripheral blood and bone marrow, clinical trials suggest that this combination is highly effective (Huber *et al*., 2022).

The utilization of BCL-2 inhibitors in combination therapy

holds great potential in the field of cancer therapeutics. The objective of these therapy protocols is to concurrently address numerous pathways, with the potential to yield improved outcomes and overcome mechanisms of resistance. Through an in-depth understanding of the complex interaction between BCL-2 proteins and other biological pathways, it becomes possible to develop therapy protocols that are more efficient and customized for individuals diagnosed with cancer (Table 3).

CONCLUSION

Apoptosis is a strictly regulated process that helps maintain homeostasis and cellular degeneration. Due to the importance of this tumor suppressive machinery, scientists were working to understand how it works and what downstream effector proteins are involved. The BCL-2 protein family was found to be a key regulator of apoptosis. Moreover, aberrant expression of both pro-apoptotic and anti-apoptotic members has been correlated with various diseases, including cancer.

Cancer is known for its insensitivity to death signals, and its dysregulating BCL-2 proteins makes them druggable targets that carry great potential in halting tumor progression. Developing BCL-2 proteins inhibitors (BH3 mimetics) created a new opportunity for disease treatment (Table 3, Fig. 3). The FDA approved authentic BH3 mimetics for cancer therapy as they were proven to kill cancer cells and prevent recurrence or drug resistance. Several forms of these inhibitors were discussed in this review, such as chemical compounds either derived from former ones or designed using computational tools and artificial intelligence, natural product extracts from microorganisms, plants, marine inhabitants, and others. These include bacterial endo/exo-toxins, Perylene quinone mycotoxins, and gossypol. In the future, more BCL2 family inhibitor compounds are promised to be approved as either monotherapy or in combination with other remedies. Aside from hematologic malignancies, researchers are working on getting more solid tumors to respond to these kinds of treatments as it protects health cells from the damaging effect of chemo/radiation therapy.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to the University of Sharjah and the University of Jordan for their continuous support and for supplying the necessary resources for this study.

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