Structure, Function and Inhibition of Bcl-2 Family Proteins: A New Target for Anti-Tumor Agents

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Abstract: Proteins of Bcl-2(B-cell lymphoma-2) family are key regulators of programmed cell death. Scientist found that some members of this family are over-expressed in malignant tumors and influence the sensitivity of tumor cells to chemotherapy and radiotherapy. This report reviews the recent progress of structures, functions and inhibition of Bcl-2 family proteins, especially the development of Bcl-2 inhibitors in past decades as anticancer agents.

Keywords: Apoptosis, Bcl-2, structure, function, inhibitors, anti-tumor.

1. INTRODUCTION

Apoptosis, a form of programmed cell death, is an important physiological process that plays an essential role in normal development and homeostasis of organism. B-cell lymphoma-2 (Bcl-2) family proteins include proapoptotic (prodeath) and antiapoptotic (prosurvival) members, which are key regulators of programmed cell death and the anti-apoptotic members could act as a pro-oncogene in follicular B-cell lymphoma. In the past two decades, more and more evidence showed that some anti-apoptotic members of this family, such as Bcl-2 and Bcl-xl, are over-expressed in some cancer cells and are related with the resistance of cancers to chemotherapy and radiotherapy. Along this line, inhibiting Bcl-2 and related proteins should selectively eliminate the cancer cells with elevated Bcl-2 proteins. Therefore, these proteins are attractive targets for cancer therapies. To date, there is an increasing interest in the development of Bcl-2 inhibitor-based anticancer agents. This review will focus on the recent progress of the structures and functions of Bcl-2 proteins, and then give a brief discussions of Bcl-2 inhibitors in clinical test.

2. APOPTOSIS PATHWAY AND MECHANISMS

Apoptosis is an important physiological process and helps body to remove unwanted cells. It is characterized with a variety of morphological changes, including cell shrinkage, nuclear fragmentation, cell membrane blebbing and chromatin condensation. This process plays an essential role in normal development and homeostasis in higher organism. Deregulation of apoptosis is involved in many human diseases. For example, excess of apoptosis can lead to cardiovascular diseases and neurodegenerative diseases; reduced apoptosis will contribute to tumorigenesis and autoimmune diseases [1].

There are two major pathways to induce apoptosis. One is the intrinsic pathway (also known as mitochondrial pathway) and the other is the extrinsic pathway (also termed as "death receptor" pathway). Intrinsic pathway can be initiated by multiple signals (such as microtubule disruption, DNA damage, and growth-factor deprivation), which leads to the activation of proapoptotic Bcl-2 family proteins (e.g. Bak, Bax). This process promotes inter-membrane space of mitochondria (IMS) to release cytochrome c (Cyt C) and apoptotic protease activating factor-1 to the cytosol. These cytosolic Apaf-1 and Cyt C interact with procaspase-9, leading to the formation of the apoptosome [2] . The procaspase-9 is activated by apoptosome and induce caspase-3, 6, 7 to initiate protein degradation [3,4]. The extrinsic pathway is evoked by multiple signals and lead to the ligation of death receptors (such as tumor necrosis factor receptors, TNF). Interaction of death receptors with its ligand (physiologic ligand, FASL) [5], adaptor protein FADD (Fas-associated via death domain) [6] would activate procaspase-8 to form a death-inducing signaling complex and then the caspase-8 could be activated by autocleavage. Finally apoptosis occur after caspase-8 activates downstream effectors caspases-3, 6, 7. The details of apoptotic pathway are shown in Fig. (**1**). Generally, Bcl-2 proteins have significant effect on the intrinsic pathway with little impact on the extrinsic pathway [7].

3. STRUCTURES OF BCL-2 FAMILY PROTEINS

The Bcl-2 (B-cell lymphoma-2) gene was discovered at the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas. Now, more than 25 members of Bcl-2 family proteins have been identified in mammals. These proteins can be divided into two subfamilies according to their functions. One subfamily, named anti-apoptotic (prosurvival) Bcl-2 family proteins, is required for cell survival which include Bcl-2, Bcl-xl, Mcl-1, Al, Bcl-w, Bcl-RAMBO and Boo [8]. Members in this subfamily usually have four

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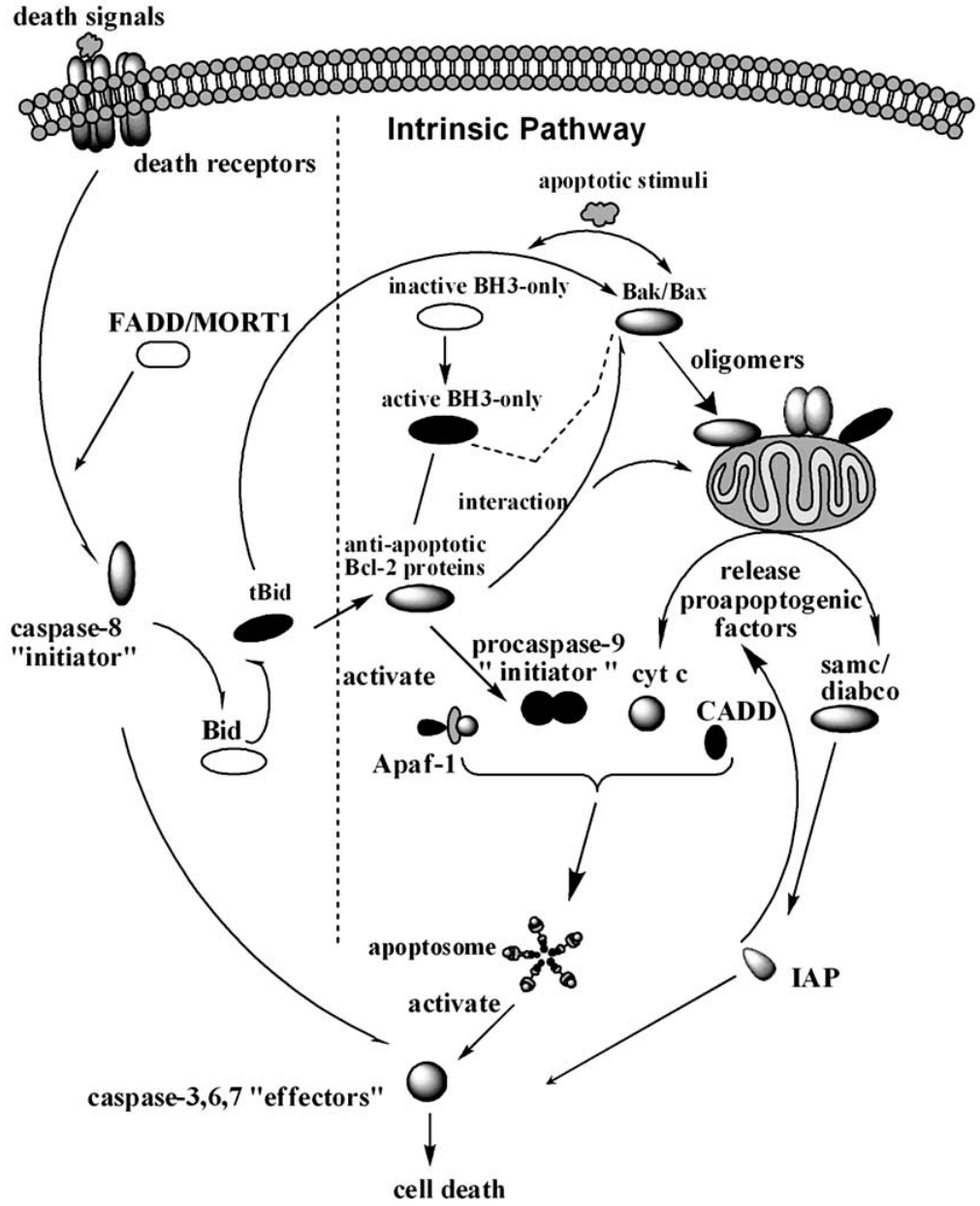


Fig. (1). The intrinsic and extrinsic apoptosis pathway in mammal.

specific homology domains, named BH1, BH2, BH3, and BH4 domains, except A1 and Mc1-1. The other subfamily, named pro-apoptotic Bcl-2 family proteins, is required for apoptotic cell death which are further divided into two subgroups based on their structures [6]. One subgroup, such as Bax, Bak, Bcl-x_S, Bok/Mtd and Bcl-G_L, contain two or three conserved domains; whereas the other subgroup, named BH3-only proteins, including Bim, Bad, Bid, Bmf, Noxa, Puma and Hrk, only have one conserved BH3 domain. The BH3-only proteins are believed to fine tune apoptotic process in mammalian cells. (Fig. **2**) [8,9].

It has been demonstrated that BH1, BH2, and BH3 domains play an important role in the interactions between anti-apoptotic members (e.g. Bcl-2 and Bcl-xl) and pro-apoptotic proteins (e.g. Bax) [10]. BH3 domain is essential for BH3-only proteins binding to anti-apoptotic Bcl-2 family proteins and blocking their anti-apoptotic function [11]. The structures of BH1-BH3 domains in anti-apoptotic Bcl-2 family proteins were identified as a long hydrophobic groove on the surface which could act as the binding site for the BH3 domain of pro-apoptotic partners [12]. The BH4 domains exist mainly in the anti-apoptotic Bcl-2 family proteins and only one pro-apoptotic protein (Bcl-xs). This domain usually locate in the N-terminal region and is required for the protein-protein interaction with regulatory proteins (e.g. phosphatase calcineurin and the protein kinase Raf-1) rather than the Bcl-2 family [13]. One study also demonstrated that, in Bcl-2 and Bcl-xl, BH4 domains played an essential role in preventing apoptotic mitochondrial changes [14].

Many Bcl-2 family members have a conserved carboxy-terminal transmembrane domain by which the proteins anchor on the membrane of the different organelles, such as

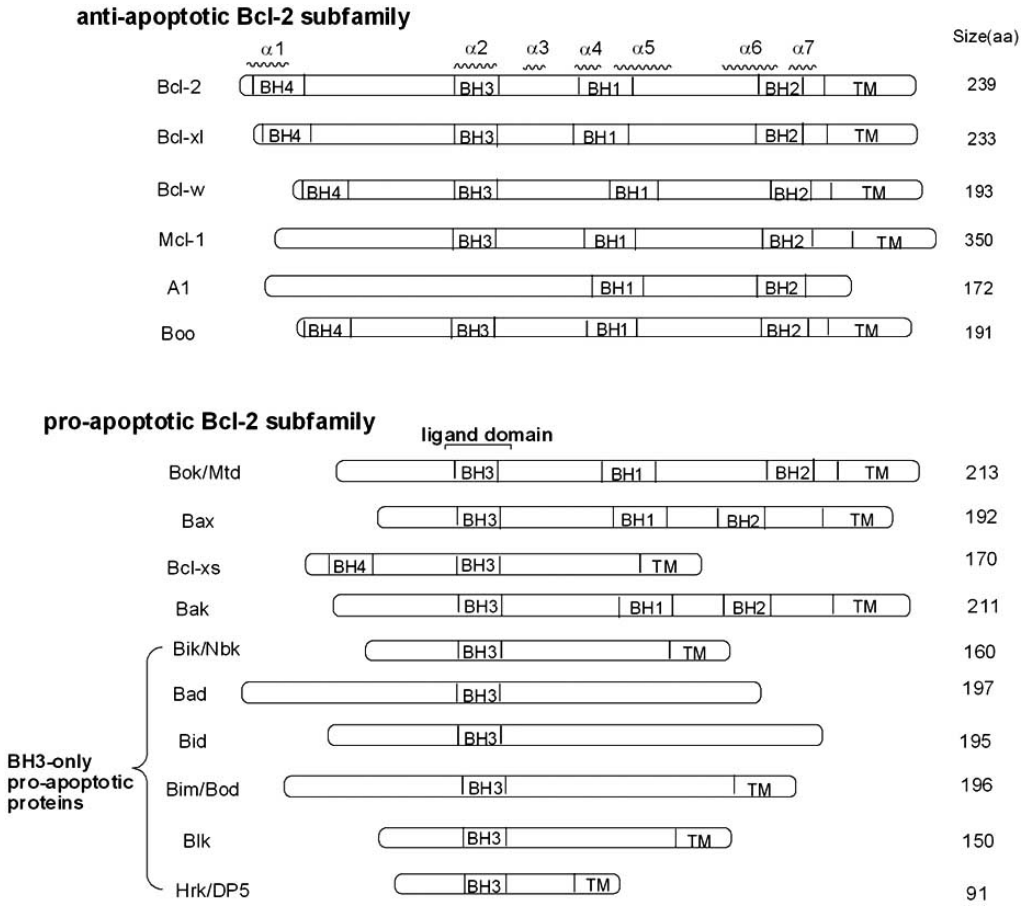


Fig. (2). The Bcl-2 homology domain (BH) of pro-apoptotic and anti-apoptotic Bcl-2 family proteins.

endoplasmic reticulum, nuclear envelope and the out mitochondrial membrane [15]. Anti-apoptotic Bcl-2 proteins could transfer to cytosol after C-terminal tail was cleavage [16].

In 1996, the three-dimensional structure of Bcl-xl was determined by X-ray crystallography and NMR spectroscopy [12]. Structure of Bcl-xl is homologous with the pore-forming domains of certain bacterial toxins which known to form ion channels in bacteria membranes.The Bcl-xl protein contains eight α-helices and shows similar 3D structure in solution and crystal [17]. The helices α5 and α6 with the length of 30Å are identified as predominant core and pore-forming domains. Surrounded by five amphipathic helices, helices α5 and α6 that comprise of predominant hydrophobic residues arranged in an antiparallel style. Bcl-xl and Bcl-2 also consist of a 60-residues flexible loop located at N-terminal of the BH3 domain, which connects helices α1 and α2. This loop contains several potential phosphorylation sites [10] and is suggested to be the region with negative regulation of anti-apoptotic activity in Bcl-2 and Bcl-xl [18].

Although the anti-apoptotic and pro-apoptotic Bcl-2 family proteins showed greatly different in primary structures and biological functions [7], their three-dimensional structures of Bcl-xl [12], Bcl-2 [19], Bcl-w [20], Bid [21,22], Bax [23] are considerably similar. For example, structure of Bax determined by combination of nuclear Overhauser effects [24] (NOEs) and dipolar coupling restraints showed that Bax contains nine α-helices which arrange in the same way as Bcl-xl [23]. In addition, the large loop connected α1 and α2 helices is similar to that of Bcl-xl.

4. THE FUNCTIONS OF BCL-2 FAMILY PROTEINS

Multi-domain Bcl-2 family proteins mainly locate in the membrane of mitochondria, endoplasmic reticulum (ER), and nuclear. Generally, the relatively balance between anti-apoptotic and pro-apoptotic Bcl-2 family members will determine the fate of cell [25]. For example, a relatively high abundance of pro-apoptotic Bcl-2 proteins will lead cell to apoptosis while more anti-apoptotic Bcl-2 family members will restrain apoptosis.

The anti-apoptotic Bcl-2 family members can prevent the release of apoptogenic factors and regulate endoplasmic reticulum (ER) Ca^{2+} dynamics [26] . The protective function of Bcl-2/Bcl-xL seems to be related with their function to form inactivating heterodimers with Bax/Bak [27]. The pro-apoptotic Bcl-2 family members show the ability to perturb the membrane permeability of mitochondria and result in the release of numerous apoptogenic proteins (e.g. cytochrome c) from the mitochondrial intermembrane space [28,29]. In addition, Bax and Bak can also control apoptosis by regulating ER Ca^{2+}-dependent apoptotic stimuli [30].

BH3-only protein was reported to be an upstream point of an apoptotic signal-transduction cascade that leads mitochondria release of cytochrome c and activates the caspases

[31]. In response to certain death signals, BH3-only proteins bind to anti-apoptotic Bcl-2 proteins and block their function [32]. Then the accumulation of pro-apoptotic Bax and Bak on the ecdoplasmic reticulum and mitochondrial members lead to the release of apoptogenic proteins such as cytochrome c from mitochondria to trigger activation of caspases and ultimately cells die.

Because the regulation of apoptosis by Bcl-2 proteins has been thoroughly reviewed recently, it will not be discussed herein. Detailed information can be found in recent reviews by Letai *et al.* [33] , Adam *et al.* [34], van Delft *et al.* [35] and Skommer *et al.* [36].

5. BCL-2 INHIBITION IN CANCER THERAPY

More and more evidence suggested that radiation and chemotherapeutic drugs eliminate tumor cells through induction of apoptosis. Apoptotic deregulation in cancer cells appears to affect the signaling pathways upstream of Bax/Bak and mitochondria through the over-expression of anti-apoptotic Bcl-2 family proteins. This presents the opportunity to restore apoptosis in cancer cells by manipulating the balance between pro- and anti-apoptotic Bcl-2 famiy members. Therefore, the strategies of antagonizing anti-apoptotic Bcl-2 family proteins will no doubt help us find a new way to solve the resistance in the treatment of cancers. Three strategies have been used to achieve inhibition for anti-apoptotic Bcl-2 proteins members, which include anitsense oligonucleotides, BH3 domain mimetic peptides and small molecule inhibitors.

5.1. Antisense Oligonucleotides

A feasible strategy to inhibit the anti-apoptotic Bcl-2 proteins is to decrease their expression through antisense or RNAi therapies [37]. So far, four antisense oligonucelotides [38-40] have been reported to target Bcl-2,Bcl-xl or related homologous proteins (Fig. **3**). Among them, G3139 has entered Phase III trials [41] and OGX-011 is now being estimated in Phase II studies, whereas oligonucleotide 4625 and ISIS 20408 are still in the preclinical studies. Their potential clinical activity remains unclear. Antisense oligonucleotides that are short, single-stranded DNA molecules can target at the open reading frame of mRNA of the Bcl-2 [39], which cause the reduction of gene expression and induction of apoptosis.

Genasense	5' -TCTCCCAGCGTGCGCCAT- 3'
GX-011	5' -CAGCAGCAGAGTCTTCATCAT -3'
4625	5' -AAGGCATCCCAGCCTCCGTT -3'
ISIS 20408	5' -TTGGCTTTGTGTCCTTGGCG -3'

Fig. (3). Four antisense oligonucelotides reported.

5.2. BH3 Peptide

Theoretically, BH3 peptides should effectively bind and antagonize the anti-apoptotic members to overcome drug resistance, because the BH3 domains of pro-apoptotic proteins directly binding to hydrophobic grooves of the anti-apoptotic proteins with high affinity to form heterodimer and initiate apoptosis. BH3 peptide mimetics are usually designed according to the BH3-only proteins structure. In the cell-free assay, BH3 peptides can bind to antiapoptotic Bcl-2 proteins and disrupt the interaction between the proapoptotic and antiapoptotic Bcl-2 family members [42]. However, the physical and chemical properties of peptides limited their application as therapeutic agents. Chemical modification is a useful strategy to peptide optimization. For example, Wang and colleagues synthesized a cell permeable Bcl-2 binding peptide (CPM-1285) by attaching a fatty acid to a peptide derived from proapoptotic protein Bad [43]. This peptide can bind to Bcl-2 with an IC_{50} of 130 nM and enter HL-60 tumor cells to induce the apoptosis *in vitro*. Further studies also showed that CPM-1285 can slow human myeloid leukemia growth in severe combined immunodeficient mice.

5.3. Small Molecular Antagonist of Bcl-2

Design of non-peptidic, small-molecule inhibitors to regulate protein-protein interactions is one of the most challenge works for medicinal chemist in recent decades. Different modern technology, such as virtual screening [44,45], nuclear magnetic resonance technique (NMR), structure-based design have been used. To date, many compounds [46] have been identified as lead compounds of Bcl-2 inhibitors.

5.3.1. Chromene Derivatives

HA14-1 was found by Huang *et al.* [47] in 2000 using virtual screening from 193,833 compounds in MDL/ACD 3D database. It is the first reported small-molecule inhibitor of Bcl-2 proteins with a IC_{50} value of 9 μM in a competing Bcl-2 binding assay based on Fluorescence polarization principle. Furthermore, HA14-1 can interfere the interaction between Bcl-2 and the BH3-only protein Bim [48] and Bax [49], which selectively induces apoptosis in malignant hematopoietic cell lines that over-express Bcl-2 [50]. HA14-1 can be used in combination therapy for cancer treatment to synergize some antitumor agents, such as doxorubicin , dexamethasone [51].

The structure-activity relationship studies of HA14-1 have shown that the 6-bromo of HA14-1 has little effect on its bioactivity and can be replaced by a variety of alkyl groups [52]. Considering HA14-1 shows poor stability, easily decomposes, and generates reactive oxygen species (ROS), Xing's group designed and synthesized the sHA14-1 which is an analogue of HA14-1 by introducing phenyl group in 6 position and removing cyano substituent. This compound displayed higher potency to antagonize the binding interaction between Bak peptide and Bcl-2/Bcl-xl compared to HA14-1 [53].

5.3.2. Polyphenol Derivatives

Gossypol is a polyphenolic compound isolated from cotton seeds and roots. This compound showed anti-tumor activity [54,55] and has been evaluated in human clinical trials involving many malignancies. Gossypol was identified as Bcl-2 inhibitors using structure-based database screening. Later, an enantiomer of gossypol, (-)-gossypol, was found to

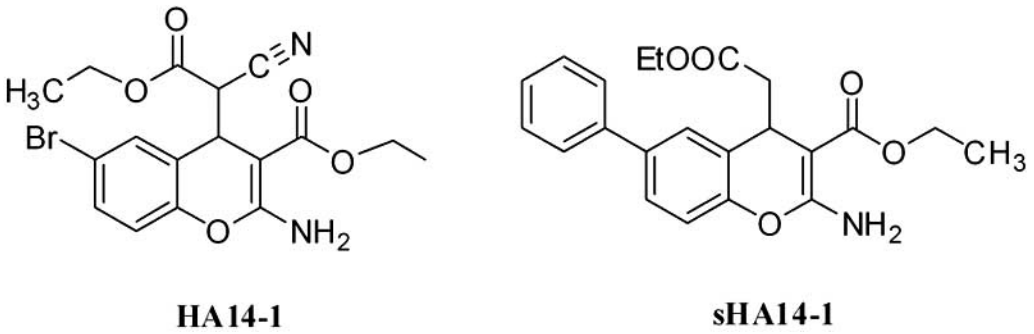


Fig. (4). Structures of HA14-1 and sHA14-1.

be more active than its racemate and able to overcome the resistance by the overexpression of Bcl-2 or Bcl-xL in Jurkart T leukemia cells [56].

However, several side-effects may occur *in vivo* because the two reactive aldehydes in gossypol is able to form Schiff's base with primary amines in nucleic acids and proteins. To overcome this problem, two reactive aldehydes in gossypol were removed to generate compound ApoG2. This compound has high potency to inhibit Bcl-2 and Mcl-1 proteins with Ki values of 36 and 25 nM respectively. ApoG2 also can induces apoptosis of tumor cell lines and show much better toxicity and efficacy profile compared with gossypol in a transgenic mouse mode [57].

Wang and colleagues designed and synthesized a series of new polyphenol compouds based on the interaction between gossypol and BH3 domain. Among them, TW-37 [58], TM-1206 [59], TM-179 [60] are reported to display high affinity in sub-micromolar range. Preclinical studies showed that TW-37 combined with MEK inhibitors potently inhibits the growth of melanoma cells [61], and is effective against chemoresistant diffuse large cell lymphoma cells with little toxicity to normal peripheral blood lymphocytes [62].

5.3.3. Acylsulfonamide Derivatives

In 2005, ABT-737 was developed by Abbott Laboratories with the help of NMR-based screening, structure-based design and parallel synthesis [63]. As the most potent inhibitor of the anti-apoptotic Bcl-2 proteins, ABT-737 showed high binding affinity for Bcl-2 , Bcl-xl and Bcl-w with Ki of no more than 1 nM. ABT-737 is efficacious against small-cell lung carcinoma (SCLC) and several lymphoid malignancies *in vivo* [63]. It exhibited synergistic effect when combined with many clinically anticancer agents such as doxorubicin, cisplatin, arabinoside, paclitaxel and vincristine [63-66]. Currently, this compound is in Phase II clinical trial. However, recent studies showed that ABT-737 can not effectively inhibit Mcl-1 [66] which is expressed in variety of tumors and lead to the resistance of some solid tumors to this compound. In addition, low oral bioavailability also is a problem for ABT-737. Abbott Laboratories therefore developed an analogue of ABT-737, ABT-263, an orally available drug and showed complete regression in SCLC tumor xenografts models upon oral dosing [67]. It is currently in Phase I clinical.

5.3.4.BH3Is Derivatives

In 2001, Degterev *et al.* screened a library of 16,320 compounds using fluorescence polarization competition as-

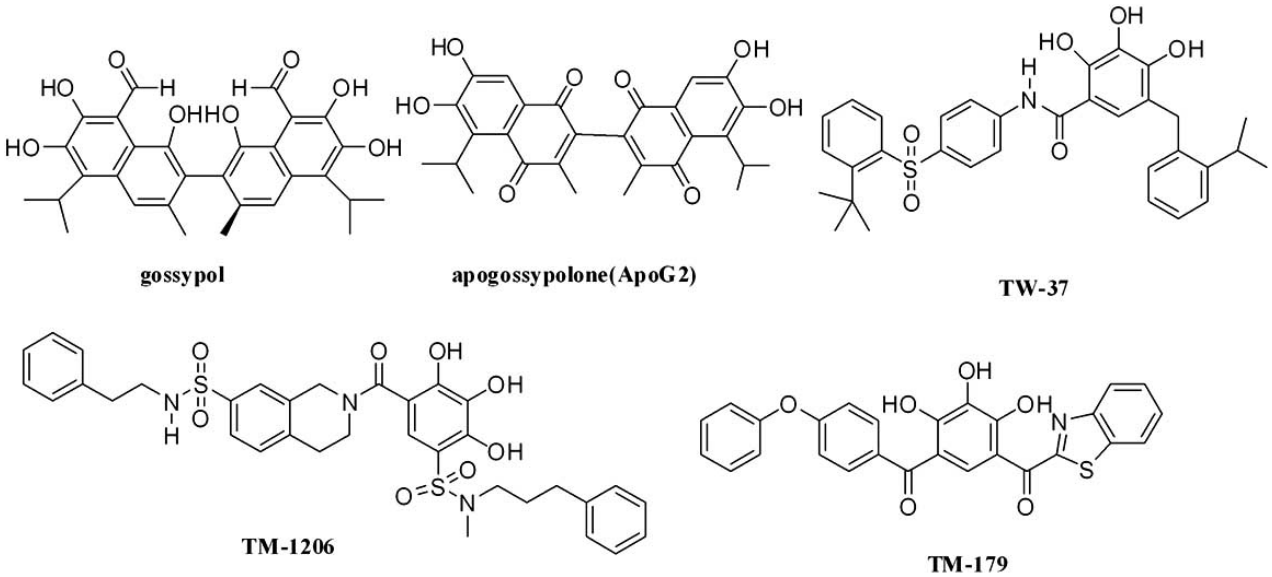


Fig . (5). Polyphenol derivatives.

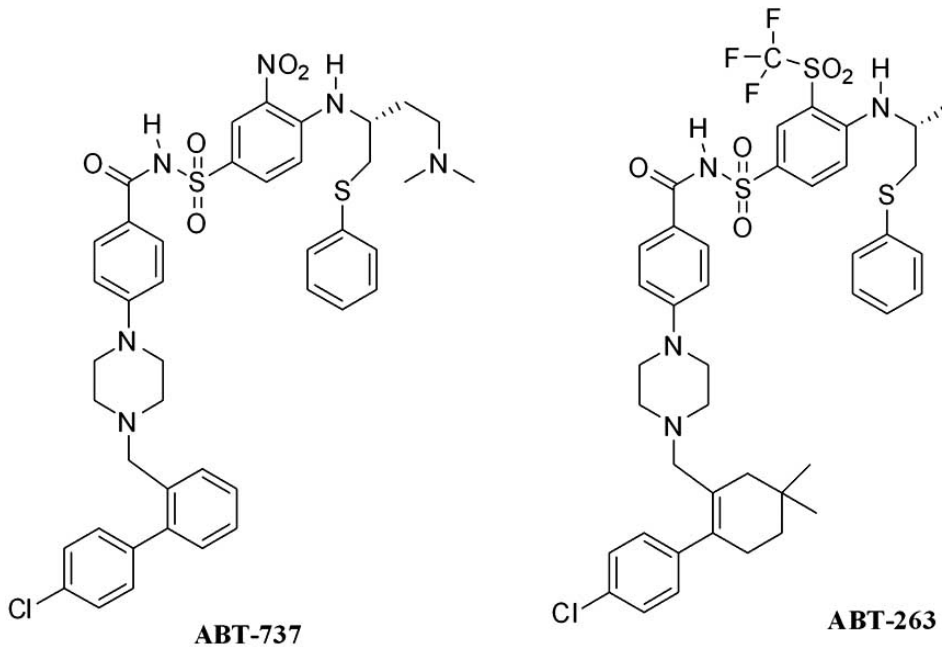


Fig. (6). Structures of ABT-737 and ABT-263.

say [68]. Two series of compounds, including thiazolidine derivatives BH3I-1 and benzene sulfonyl derivatives BH3I-2, have been identified to inhibit binding of Bak BH3 peptide to Bcl-xl. These compounds were named as BH3Is due to their inhibiting the BH3 interaction. The most active compound, BH3I-1, showed binding constant value Ki about 2.4 μM and now was in preclinical test.

5.3.5. Other Small Molecule Inhibitors in Clinical Trial

Some compounds from natural products, for example Chelerythrine and Antimycin A3, were in preclinical test. Chelerythrine is a natural benzophenanthridine alkaloid and was identified as an inhibitor of Bcl-xl in 2003 [69]. Subsequently, some experimental results demonstrated that Chelerythrine does not bind the BH3 hydrophobic groove on the Bcl-xl surface [70].

GX15-070 (Obatoclax) is an indole bipyrrole compound developed by Gemin X Biotechnologies. As a BH3-mimetic, it can induce apoptosis by inhibiting the interaction between antiapoptotic and proapoptotic proteins [71]. It had been reported that [^{1}H]-labelled GX15-070 can bind to Bcl-xl, Bcl-w and Mcl-1 with K_d values in the 0.5 range μM [72] and has been used to synergize other anticancer agents such as cisplatin and bortezomib. This compound is being assessed for solid tumors and hematological malignancies in Phase I and II clinical trials [73].

6. CONCLUSION AND PROSPECT

Recently, anti-apoptotic Bcl-2 proteins have become promising targets for cancer therapy. This decade has witnessed a tremendous progress in the area of Bcl-2 inhibitors. Currently, different approaches have been used in the design and development of Bcl-2 inhibitors, such as antisense oligonucleotides, BH3 peptides and small molecule inhibitors. Peptides are generally considered to have shortcomings of low orally bioavailability [74], instability, non-specificity and poor membrane permeability [75]. Therefore, there is an urgent need for developing small molecular inhibitors against Bcl-2 proteins. To date, several small molecule in-

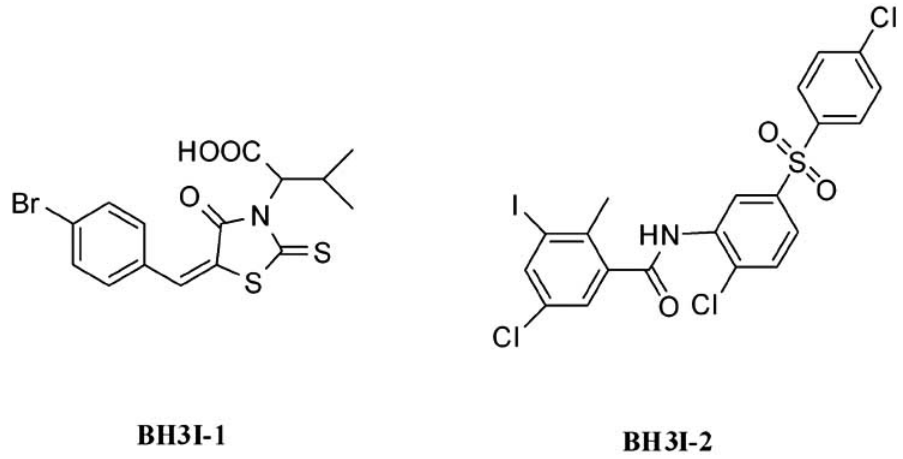
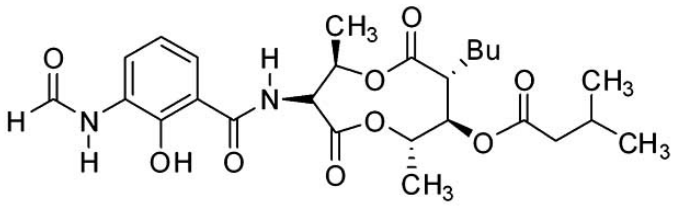
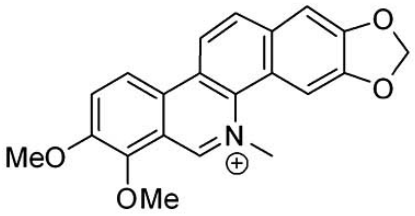


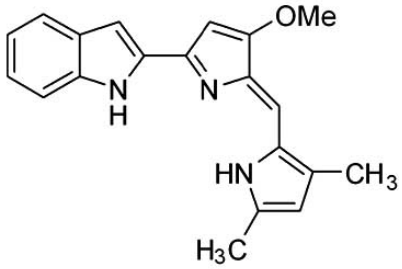
Fig. (7). Structures of BH3I-1and BH3I-2.



antimycin A3



chelerythrine



GX15-070

Fig. (8). Other small molecule inhibitors**.**

hibitors of Bcl-2 family proteins have been in the stage of preclinical and clinical trial to synergize many anticancer agents to overcome drug resistance in cancer treatment. With the development of structure biology and screening technology improvement, more and more Bcl-2 inhibitors will become drug candidates for clinical application in the future.

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ABBREVIATIONS

Smac	=	Second mitochondria-derived activator of caspases
TNF-R1	=	Tumor necrosis factor receptor-1
CLL	=	Chronic lymphocytic leukemia
Cyt C	=	Cytochrome c
FADD	=	Fas-associated via death domain
SCID	=	Severe combined immunodeficiency
SAHB	=	Stabilized Alpha-Helix of Bcl-2 Domains
PPIS	=	Protein-protein interactions
NMR	=	Nuclear magnetic resonance
ROS	=	Reactive oxygen species
FP	=	Fluorescence polarization

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