Anti-apoptotic proteins-targets for chemosensitization of tumor cells and cancer treatment*

Minireview

D. KOŠŤANOVÁ-POLIAKOVÁ, Ľ. ŠABOVÁ**

Laboratory of Molecular Biology, Cancer Research Institute, e-mail: exonsabo@savba.sk, Slovak Academy of Sciences, 833 91 Bratislava, Slovak Republic

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Apoptosis or programmed cell death is an essential process not only for the normal development and function of multi-cellular organisms but it is also an important phenomenon in tumor cells killing. Numerous studies have indicated that non surgical cancer therapies eliminate tumor cells by activating apoptosis. The central regulators of apoptosis are proteins of the Bcl-2 family. In a wide variety of human tumors, the increased expression of anti-apoptotic proteins (Bcl-2 and Bcl-$_{XL}$) was found. Moreover, it was revealed that high levels of these proteins block the action of many chemotherapeutic drugs. Due to this fact the inhibition of anti-apoptotic function of Bcl-2 proteins could be a strategy for either to restore the normal apoptotic process in tumor cells or to make them susceptible for chemo- and radio-therapeutic treatment. Three alternative therapeutic strategies for the repression of cytoprotective activity of anti-apoptotic proteins in tumor cells are reviewed in this article.

Key words: apoptosis, chemoresistance, cancer, Bcl-2 protein family, antisense oligonucleotides, BH3 peptides, BH3 mimetics

Several studies showed that abnormalities in apoptosis can lead to a number of human diseases [67]. Excessive apoptosis was found in neurodegenerative diseases, AIDS, cardiovascular diseases and stroke. On the other hand, the repression of apoptosis contributes to a wide variety of human cancers. As the deregulation of apoptosis plays a key role in the pathogenesis of many diseases, the search for novel drugs that could target the cell death machinery is accelerating. Two principal pathways leading to apoptosis were identified (Fig. 1), the mitochondrial – intrinsic [2] and the transmembrane – extrinsic [61]. Both involve the activation of cysteine proteases-caspases as the executors of apoptosis [68]. Caspases are divided into two groups, initiator and effector. Initiator caspases are activated by binding to adaptor molecule and subsequently activate effector caspases. Effector caspases then cleave a wide range of substrates to cause apoptosis [14]. Different initiator caspases are activated in dependence on apoptotic pathway. It was found that in mitochondrial pathway caspase-9, whereas in transmembrane pathway caspases-8 and 10 are activated [83, 4]. Despite the different initiator caspases, both pathways share common effector caspases-3, 6 and 7 [11].

Apoptotic response to the most of chemotherapeutic drugs in mammalian cells involves the induction of mitochondrial pathway in which mitochondrial membrane permeabilization, controlled by Bcl-2 protein family, is induced [3]. Permeabilization of the outer mitochondrial membrane allows the leakage of apoptotic mediators from the mitochondrial intermembrane space, such as cytochrome *c*, Smac/DIABLO, HtrA2/Omi, AIF and endonuclease G. These proteins are working in different ways to induce apoptosis. Smac/DIABLO and HtrA2/Omi suppress the ability of IAPs (inhibitors of apoptosis proteins) to inhibit caspases [65, 72]. Endonuclease G and AIF are involved in DNA fragmentation and AIF is involved also in chromatin condensation [40, 64]. The crucial role in this pathway plays cytochrome *c* whose presence in cytoplasm leads to the for-

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** Corresponding author

mation of apoptosome, ~1MDa complex, created by cytochrome *c*, Apaf-1 and a pro-caspase-9. In this complex caspase-9 becomes proteolytically active and subsequently activates effector caspase-3 [41, 83].

The extrinsic apoptotic pathway is triggered by the cell surface receptors and leads to the activation of pro-caspase-8, or in human cells also its close homologue pro-caspase-10 can be activated. Upon the ligation of death receptors, such as CD95 (FAS/APO1) or TNF-R1, by their ligands, the aggregated receptors recruit adaptor protein FADD. Subsequently, the effector domain (DED) of FADD interacts with DED domain within the pro-caspase-8. The high local concentration of pro-caspase-8 permits its autocatalysis resulted in caspase-8 which finally activates caspase-3 [48].

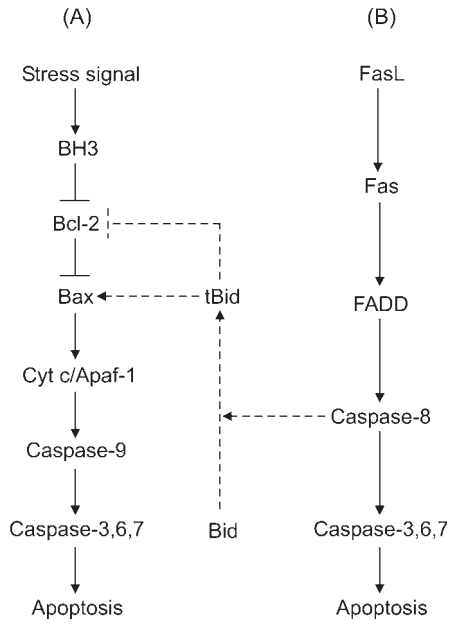

Figure 1. Two principal apoptotic pathways, (A) mitochondrial – intrinsic and (B) transmembrane – extrinsic. These pathways can be interconnected through BH3 only protein Bid.

However, in certain cell lines designated type II, the intrinsic and extrinsic pathways may intersect. It occurs during CD95-mediated apoptosis when little death-inducing signaling complex (DISC) and small amount of active caspase-8 is formed. In this case caspase-8 can by cleavage activate pro-apoptotic protein Bid and subsequently, truncated Bid (tBid) facilitates permeabilization of mitochondrial membrane [43]. Therefore, caspase-8 activation of Bid bridges the extrinsic and intrinsic apoptotic pathways [60]. However, physiological role and specificity of this crosstalk remains still elusive [28].

The critical regulators of intrinsic apoptotic pathway are proteins of the Bcl-2 family (Fig. 2) characterized by the presence of one to four conserved Bcl-2 homology (BH) domains [1, 11]. According to the mode of action these proteins can be divided into two groups, pro-apoptotic and anti-apoptotic. The pro-apoptotic proteins which promote apoptosis are divided into two subgroups; multidomains and so called BH3 only. The members of multidomain pro-apoptotic group (Bax, Bak, Bok, Bcl-$_{XS}$) share homology among BH1, BH2 and BH3 domains. However, BH3 only proteins (Bid, Bad, Bik, Noxa, Bim, Puma, Bmf, Hrk) share homology only within the BH3 domain and are functionally distinct. BH3 only proteins function as the sensors of cellular damage and presumably initiate the apoptosis [29]. Various apoptotic stimuli (Tab. 1) activate different BH3 only proteins by diverse modes of activation [2] which include both, transcriptional response (e.g. Bim, PUMA, Hrk and Noxa) and various post-translational mechanisms, such as dephosphorylation (Bad), cleavage (Bid) and phosphorylation (Bmf, Bim). Moreover, recent studies with BH3 peptides have suggested that there are two different subgroups in BH3 only proteins. One group is represented by Bid and Bim which are supposed to induce mitochondrial membrane permeabilization *via* Bax/Bak, as the direct interactions of Bid and Bim with Bax [7, 18]. The other group, including Bad and Bik, presumably sensitize the mitochondrial outer membrane

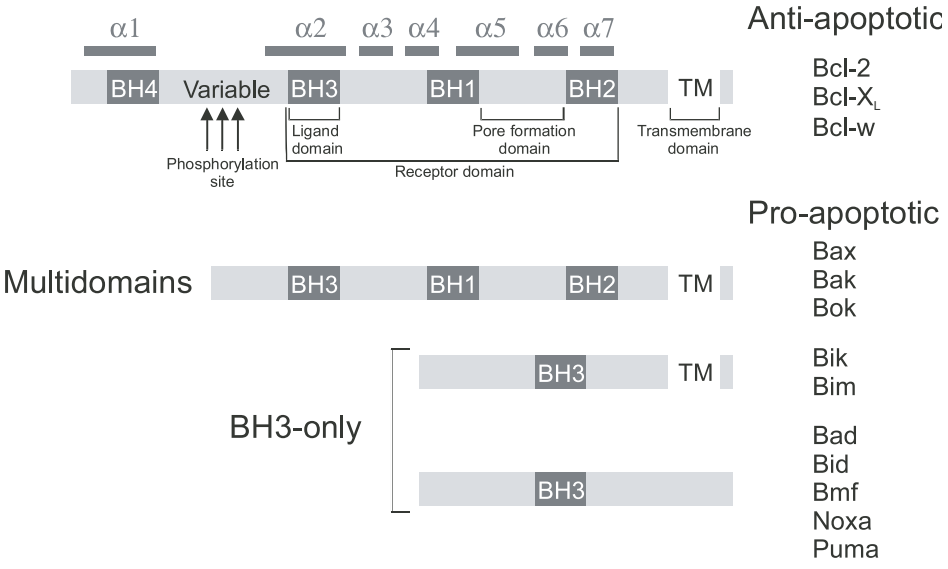

Figure 2. The members of Bcl-2 protein family. Bcl-2 homology domains (BH1-BH4), transmembrane domain (TM), α-helical regions (α1–α7) and potential phosphorylation site are indicated.

for permeabilization indirectly by binding to anti-apoptotic members of Bcl-2 family (Bcl-2 or Bcl-$_{XL}$) and in this way neutralizing their prosurvival function.

The anti-apoptotic proteins are characterized by the presence of four BH domains and their preferential localization in membranes. Whereas Bcl-2 is largely membrane associated [50, 30], most of Bcl-w and substantial portion of Bcl-$_{XL}$ is found in cytosol [19, 27, 53, 77].

It has been suggested that the ratio between anti-apoptotic and multidomain pro-apoptotic proteins determines the susceptibility of cells to death stimuli [16]. Different approaches including yeast two hybrid system, co-immunoprecipitation, *in vitro* binding assay and crosslinking experiments were used to demonstrate the formation of homo- and hetero-dimers between proteins of Bcl-2 family [25, 52, 80]. The ability of pro- and anti-apoptotic proteins to heterodimerize is given by their structure. NMR studies that allow to reveal three dimensional structure showed that three BH domains (BH1, BH2 and BH3) of anti-apoptotic proteins Bcl-2, Bcl-$_{XL}$ and Bcl-w form an elongated hydrophobic cleft that represents the binding site for BH3 domain of pro-apoptotic proteins [24, 47, 55].

Many studies have indicated that killing of tumor cells by commonly used treatments (chemotherapeutic drugs, radiation) is predominantly mediated by inducing apoptosis [22, 32]. Thus, the resistance of tumor cells to therapy can be caused by defect in the ability to initiate the apoptotic process. Overexpression of anti-apoptotic protein Bcl-2 is common in many types of hematological cancers and solid tumors. Moreover, high level of anti-apoptotic proteins in cancer cells is often associated with resistance to the traditional therapies that rely on the ability to induce apoptosis. Thus it seems likely that Bcl-2 and Bcl-$_{XL}$ would be good targets to modulate sensitivity of cells to apoptosis induced by chemotherapy. Modulation of apoptosis in cancer cells overexpressing anti-apoptotic proteins can be achieved by different approaches. Strategies that combine chemotherapeutics with molecules which neutralize the effect of Bcl-2 or Bcl-$_{XL}$ could improve the treatment of cancers.

Table 1. Different mode of activation of BH3 only proteins, transcriptional (A), post-translational (B)

(A)

Protein	Upstream signal	Transcriptional factor
Bim	Growth factor withdrawal	FKHR-L
Puma	DNA damage, ER stress	p53
Noxa	DNA damage, hypoxia	p53
Hrk	Growth factor withdrawal	unknown

(B)

Protein	Upstream signal	Protein modification
Bad	Growth factor withdrawal, glucose deprivation	Dephosphorylation
Bid	Activation by death receptor	Proteolytic cleveage by caspase-8
Bmf	UV irradiation, matrix detachment	Phosphorylation
Bik	?	Phosphorylation
Bim	Cytokine deprivation, calcium flux, UV and γ irradiation	Phosphorylation

Antisense approach

Antisense oligonucleotides bind to the selected m-RNA on the basis of their sequence and form RNA-DNA duplexes by Watson-Crick binding. RNA-DNA duplex is substrate for RNase H that cleaves the target m-RNA and by this way prevents m-RNA processing and subsequently the synthesis of protein [13]. The specificity of this approach is given by the fact that any DNA sequence longer than 17 nucleotides occurs only once within human genome. Activities of antisense oligonucleotides are limited by their susceptibility to nucleases and by their penetration to the cell. The sensitivity to nucleases can be overcome by using the phosphorothioate oligonucleotides that are resistant to nucleases. These oligonucleotides have phosphodiester backbone modifications where oxygen moiety of the PO is replaced by sulphur [63]. Another modification that increases the resistance to nucleases is the modification of ribose in oligonucleotides to 2'-O-metoxy-etoxy derivate [44]. Due to the hydrophilic nature of oligonucleotides their intracellular penetration is low and several attempts to achieve the direct permeation into cells were done. Cationic lipids have been successfully used for the delivery of oligonucleotides into cells in tissue culture [37]. However, it was reported that lipid carriers are necessary for efficient uptake of antisense oligonucleotides only *in vitro*. In animal models and in patients all therapeutically active antisense oligonucleotides have been administered in the form of naked DNA [23].

Due to the fact that Bcl-2 regulates the survival of cells and that its overexpression is associated with cellular resistance to chemotherapeutic drugs and gamma irradiation, Bcl-2 gene with its m-RNA and protein product represent an ideal target to design novel therapeutic strategies for cancer treatment. The first experiments demonstrated that an 18-mer oligonucleotide complementary to the first six codons of the Bcl-2 m-RNA was able to reduce the level of Bcl-2 protein in NIH 3T3 fibroblasts in which high levels of Bcl-2 proteins were achieved by ectopic expression from recombinant retrovirus and in DU-DHL-4 t(14;18) lymphoma cell line naturally expressing high level of Bcl-2 [34]. Later it was found that antisense Bcl-2 oligonucleotide not only induced reduction in Bcl-2 expression but also resulted in a significant enhancement of the sensitivity of human lymphoma cell lines to conventional chemothera-

peutic drugs, such as cytosine arabinoside and methotrexate [35]. Finally, the ability of antisense Bcl-2 oligonucleotide to suppress tumorigenicity was documented in animal model (SCID mice) of human malignancies. The cells derived from patient with B-lymphoma were prior to inoculation of the SCID mice treated with Bcl-2 antisense oligonucleotide. Results from experiments showed that mice injected with untreated cells developed lymphoma but the group of mice injected with antisense treated B-lymphoma cells, failed to develop lymphomas [12]. The pharmacokinetic study of a ^{35}S-labeled phosphorothioate oligonucleotide antisense to Bcl-2 (G3139) in mice showed that G3139 is widely distributed and slowly eliminated from plasma. Continuous subcutaneous infusion resulted in significantly more parent drug reaching the tissue and bone marrow than a single dose after intravenous administration [56].

Early clinical studies of Bcl-2 antisense therapy in patients with hematological malignancies, chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL), showed that G3139 used as a single agent was more effective in cancer therapy than cyclophosphamide alone and thus provided the proof for antisense treatment. Clinical trials using G3139 in combination with chemotherapeutic drugs have been conducted or are currently under way (Tab. 2). The phase I and phase II clinical trials using G3139 in combination with conventional chemotherapy have shown encouraging results in patients with CLL, NHL and acute myeloid leukemia (AML) [76]. The phase III clinical trials in patients with CLL and multiple myeloma have already been completed. Moreover, the study in CLL patients treated with chemotherapy and G3139, documented significant increase of the proportion of patients who achieved a complete or partial remission *versus* patients treated with chemotherapy only. The phase II trial of G3139 with flutarabine plus rituximab (chimeric murine/human monoclonal antibody directed against CD20 antigen) in patient with advanced CLL is currently under way. Despite the promising results obtained from phase I and II clinical trials in patients with advanced multiple myeloma treated with the high dose of dexamethasone with or without G3139 results from the phase III of clinical trial did not document a significant increase in time to disease progression in patients treated with G3139. Results from the phase II trial of G3139 combined with gemtuzumab ozogamicin showed that this combination could induce complete remission in patients with relapsed AML.

As it has been mentioned, over-expression of Bcl-2 protein is also associated with certain types of solid tumors (lung carcinoma, melanoma, hormone refractory prostate cancer). In the majority of small cell lung cancers (SCLC) elevated expression of Bcl-2 has been associated with radiation and drug resistance. To test if Bcl-2 expression level is critical for inhibition of apoptosis in SCLC cells, antisense strategy was used to reduce the amount of Bcl-2 protein. To identify the most effective sequence for reducing Bcl-2 protein level thirteen antisense oligonucleotides targeting various regions of Bcl-2m-RNA were tested in human SCLC cell lines. An antisense oligonucleotide (ODN 2009) was found to reduce Bcl-2 level and induced apoptosis [82]. Additionally, SCLC cell lines were used to investigate the combined effect of Bcl-2 antisense oligonucleotide with chemotherapeutic agents etoposide, doxorubicin and cis-platin. Obtained data showed that the use of ODN 2009 in combination with some of the tested chemotherapeutic drugs exerted synergistic cytotoxicity [78]. In a pilot trial, patients with chemorefractory SCLC were treated with G3139 antisense oligonucleotide combined with paclitaxel. Results of this trial showed that G3139 can be combined with paclitaxel, due to the fact that G3139 did not deteriorate the response to the therapy [58]. In phase I study, patients with SCLC were treated with G3139 in combination with etoposide and carboplatin. This combination was well tolerated and resulted in an encouraging response rate in patients with progredient forms of SCLC [57].

The effect of G3139 in combination with chemotherapeutic drugs was tested also for treatment of other types of cancer. The early experiments in SCID mice carrying human malignant melanoma xenografts showed that G3139 enhanced the efficacy of standard cytotoxic chemotherapy [31]. These results provided a basis to start clinical trials. In phase III trial of malignant melanoma, patients received either G3139 plus dacarbazine or dacarbazine alone. Obtained data were not sufficient to provide substantial evidence of efficacy and safety for the marketing approval of G3139. Combination of G3139 with mitoxantrone for the treatment of patients with metastatic hormone refractory prostate cancer demonstrated that in some patients stable prolonged disease was achieved [10]. Using combination of

Table 2. Clinical trials of Bcl-2 antisense oligonucleotide G3139 for different types of cancer

Type of cancer	Clinical trial	Chemotherapy	Status of trial
CLL	III. phase	Fludarabine and cyclophosphamide	Completed
Myeloma multiplex	III. phase	Dexamethasone	Completed
Malignant melanoma	III. phase	Dacarbazine	Completed
Non-SCLC and SCLC	III. phase	Docetaxel	Nearly completed
AML	II. phase	Gemtuzumab ozogamicin	
Esophageal and gastric carcinoma	I. and II. phase	Cisplatin and fluorouracil	
Colorectal carcinoma	I. and II. phase	FOLFOX4	
Hepatocellular carcinoma	I. and II. phase	Doxorubicin	
Breast carcinoma	I. and II. phase	Doxorubicin and docetaxel	
Prostate cancer (hormone-refractory)	I. and II. phase	Docetaxel	

G3139 with other chemotherapeutic agents (paclitaxel, docetaxel, etoposide, vinblastine, carboplatin, mitoxantrone) *in vitro* demonstrated marked increase in the sensitivity of prostate cancer cells to such type of therapy [38]. Bcl-$_{XL}$ protein, a close homolog of Bcl-2, also plays an important role in resistance of cancer cells to chemotherapeutic drugs. The functional inhibition of Bcl-$_{XL}$ by antisense oligonucleotides was tested *in vitro* in malignant pleural mesothelioma, colorectal carcinoma, prostate cancer, breast and lung cancer cell lines [20, 38, 54, 62]. It was revealed that down-regulation of Bcl-$_{XL}$ significantly enhanced the response to cytotoxic drugs.

However, most solid tumors are genetically heterogeneous cell populations and it is difficult to predict the anti-apoptotic protein which could be more relevant as molecular target for antisense therapy. It was reported that Bcl-2/Bcl-$_{XL}$ bispecific oligonucleotides down-regulated Bcl-2 and Bcl-$_{XL}$ expression on m-RNA and protein levels and simultaneously induced apoptosis in SCLC and non-SCLC cell lines [79]. Despite these findings, clinical studies with Bcl-$_{XL}$ or bispecific antisense oligonucleotides Bcl-2/Bcl-$_{XL}$ have not been performed yet.

Cell permeable BH3 peptides

An alternative approach to counteract the effect of anti-apoptotic proteins in tumor cells is to abolish their pro-survival functions by using small BH3 peptides. It has been suggested that the BH3 domains of pro-apoptotic Bcl-2 family members interact with the surface pocket of Bcl-2 or Bcl-$_{XL}$ protein. This interaction eliminates anti-apoptotic function of Bcl-2 and Bcl-$_{XL}$ proteins and triggers apoptosis. The study of Bcl-2/Bak peptide complex using NMR confirmed this assumption and showed that the Bak BH3 peptide is really inserted into hydrophobic cleft formed by BH1, BH2 and BH3 domains of Bcl-2 [59]. Furthermore, a short peptide containing the Bak BH3 domain induced apoptosis in HELA cells [26]. This result suggested that synthetic peptides encompassing minimal BH3 domain might be used in cancer therapy. Using an *in vitro* protein-protein binding assay the effect of different peptides derived from BH3 domains of Bad, Bax and Bak were tested for ability to block heterodimerization of Bcl-$_{XL}$ with pro-apoptotic proteins. These BH3 peptides were shown to inhibit Bcl-$_{XL}$-Bax and Bcl-$_{XL}$-Bad interactions in dose dependent manner [42]. In agreement with their high binding activity to Bcl-$_{XL}$, peptides derived from BH3 domain of Bad were more potent in blocking protein-protein interaction than BH3 peptides derived from other tested pro-apoptotic proteins [33]. Further investigations confirmed that synthetic BH3 peptides derived from Bax and Bad were sufficient to bind and functionally antagonize Bcl-$_{XL}$ protein but did not stimulate apoptotic activity of Bax or Bad [46]. Peptide derived from BH3 domain of Bad acted as a selective death ligand also for Bcl-2 protein. This death ligand entered HL-60 tumor cells and was bound to Bcl-2 protein. Moreover, in SCID mice injected with HL-60 cells this ligand was able to reduce the growth of human myeloid leukemia xenografts [75].

Although the anti-apoptotic proteins were assumed to bind all to BH3 only proteins, recently published data revealed marked differences in their binding ability. Detailed *in vitro* binding studies with BH3 peptides derived from different BH3 only proteins, together with analysis of proteins association in cells, indicate that most BH3 peptides exhibit preferences in binding to certain anti-apoptotic proteins. Only Bim and Puma were able to neutralize all the pro-survival proteins, whereas the less potent BH3 only proteins showed a more restricted binding spectrum. Bad and Bmf preferred Bcl-2, Bcl-$_{XL}$ and Bcl-w, whereas Bik and Hrk exerted the highest binding activity to Bcl-$_{XL}$, Bcl-w and A1 and Noxa bound only Mcl-1 and A1 [9]. The question whether BH3 proteins only sequester the anti-apoptotic proteins or they can also activate pro-apoptotic proteins is not completely elucidated. Recent results showed that BH3 domains of some proteins (Bid, Bim) demonstrated dual function. They directly activated Bax and indirectly *via* binding to anti-apoptotic proteins were able to abolish the inhibition of Bax [36].

Although BH3 peptides are attractive candidates for disrupting interactions between pro- and anti-apoptotic proteins, their usage *in vivo* is problematic. These small peptides have little ability to penetrate the cell membrane and reach their intracellular targets, the anti-apoptotic proteins. To improve the delivery of synthetic peptides into cell, several approaches were used. BH3 peptides were linked with transporter peptide or non-peptidic molecules. The internalization domain of the Antennapedia (ANT) protein was fused with BH3 peptide derived from Bak and this fusion peptide caused the activation of caspases and induced apoptosis in α-FAS treated HELA cells [26]. Another approach how to help peptides to cross the cell membrane is to attach them to fatty acids. To generate a cell permeable ligand, decanoic acid was attached to a synthetic peptide derived from BH3 domain of Bad. This construct induced apoptosis *in vitro* and it was able to suppress the growth of human myeloid leukemia in SCID mice [75]. Besides the difficulty to penetrate intact cells, the efficacy of BH3 peptides *in vivo* is severely influenced also by susceptibility to proteolytic degradation and the loss of their secondary structure.

Recently, a promising strategy termed hydrocarbone stapling was used to generate BH3 peptides with improved pharmacologic properties. The stapled peptides proved to be helical, protease resistant and have cell permeable molecules that bound multidomain Bcl-2 members with increased affinity. The stapled BH3 peptide derived from Bid with the affinity for binding to Bcl-$_{XL}$ was able to inhibit the growth of leukemia cells *in vivo* [73].

In order to design more effective cancer therapy it will be important to know in detail how these peptides interact with all Bcl-2 family members.

Non-peptidic Bcl-2 and Bcl-$_{XL}$ antagonists-BH3 mimetics

The use of antisense oligonucleotides and small BH3 peptides to suppress the anti-apoptotic function of Bcl-2 or Bcl-$_{XL}$ is associated with potential limitations (stability, high cost, non-selective toxicity). Therefore, there is an intense interest to discover small non-peptidic molecules that can inhibit Bcl-2 and Bcl-$_{XL}$ activity. The advantage of such low molecular weight compounds is that they have usually high membrane permeability. Up to now, more than ten compounds that might prevent the interaction of Bcl-2 or Bcl-$_{XL}$ with pro-apoptotic proteins have been reported.

The first small molecule inhibitor (HA14-1) was identified by a computation screen based on the predicted structure of BH3 binding site of Bcl-2 [74]. The ability of HA14-1 to prevent binding of BH3 peptides to Bcl-2 protein was confirmed by a competition fluorescence polarization assay. Moreover, *in vitro* experiments showed that this compound induced apoptosis in malignant hematopoietic cell lines overexpressing Bcl-2. Competitive binding assay based on the fluorescence polarization was used to screen chemical library of 16 320 compounds. In this assay DEGTEREV et al. [15] identified two classes of small molecules termed BH3I-1 and BH3I-2 able to bind Bcl-$_{XL}$ and to induce apoptosis in Jurkat cells overexpressing Bcl-$_{XL}$. Recently it was found that BH3I-2 was able to induce apoptosis in TRAIL resistant cell lines, lymphoblastic leukemic line CEM, HL-60 and U937 [17].

In addition to the non-natural molecules, natural products, tetrocarcin A, antimycin A, gossypol and chelerythrine, have been reported to induce apoptosis in cell lines overexpressing Bcl-2 and Bcl-$_{XL}$. Tetrocarcin A, a natural product of *Actinomycete,* was originally discovered as an antibiotic active against Gram positive bacteria [66]. Although the mechanism of its action was unknown this compound showed also antitumour activity in murine experimental models [45]. Recently, tetrocarcin A was shown to preferentially suppress the anti-apoptotic functions of Bcl-2 family of proteins in apoptosis triggered by various death stimuli [49].

Another natural product able to bind to Bcl-$_{XL}$ and inhibit its anti-apoptotic function is antimycin A, an antibiotic from *Streptomyces* species. In early studies, antimycin A was found to inhibit mitochondrial oxidative phosphorylation through the binding to cytochrome *b* [6]. During the testing of known agents with mitochondrial toxicity for the induction of apoptosis in the murine hepatocyte cell line TAMH overexpressing Bcl-$_{XL}$ antimycin A was revealed to sensitize these cells to apoptosis. Further investigation using fluorescence spectroscopy showed that antimycin A was able to bind to Bcl-2 hydrophobic cleft and to compete for binding with BH3 peptides. To exclude that the inhibition of respiration is involved in antimycin effect, 2-methoxy-antimycin A_3, inactive as an inhibitor of respiration, was used. This compound displayed the ability to bind to Bcl-$_{XL}$ and to sensitize the cells to death [70].

Recently it was found that gossypol, a constituent of plant *Gossypium*, is a potent inhibitor of Bcl-2 and Bcl-$_{XL}$ function [81]. Gossypol is a polyphenolic compound and its anticancer effect *in vitro* was demonstrated in many different carcinoma cell lines [69]. Later studies showed that gossypol promoted apoptosis in human tumor cell lines *via* an ability to bind Bcl-2 or Bcl-$_{XL}$ [51]. Additionally, this product displayed anti-tumor activity in tumor xenografts studies in SCID mice and it has been tested in clinical trials involving patients with advanced malignancies. The clinical study that used gossypol in treatment of refractory metastatic breast cancers revealed its function as a modulator of cell cycle [71]. Several side-effects of gossypol, probably due to the presence of reactive aldehydes in its structure, limit its usefulness. Apogossypol, an analog lacking aldehydic groups was synthesized and showed drug-like properties comparable to gossypol [5].

The product of plant *Bocconia vulcanica*, chelerythrine was identified as an inhibitor Bcl-$_{XL}$ function in a high throughput screening of 104 423 extracts derived from natural products. Interestingly, chelerythrine was originally identified as a selective inhibitor of protein kinase C (PKC) [21]. Chelerythrine also exhibited cytotoxic activity against human tumor cell lines tested *in vitro*. Recent experiments further revealed that chelerythrine inhibited binding of Bak BH3 domain to Bcl-$_{XL}$ and it was able to displaced Bax from binding to Bcl-$_{XL}$. Additionally, mammalian cells treated with chelerythrine underwent apoptosis with apoptotic markers. However, Bcl-$_{XL}$ overexpressing cells that were completely resistant to apoptotic stimuli remained sensitive to chelerythrine [8].

Conclusion

At present it is accepted that at least some of the conventional chemotherapeutic drugs kill cancer cells by inducing apoptosis. Cancer cells have the ability to evade apoptosis and a natural consequence is that they are resistant to therapy. The over-expression of anti-apoptotic proteins Bcl-2 or Bcl-$_{XL}$ is one of the mechanisms that contribute to resistance of cancer cells to cytotoxic therapies. Therefore, the anti-apoptotic proteins represent one of the new targets for rational therapeutic approach strategy. The mechanisms for overcoming the resistance to apoptosis include direct targeting of anti-apoptotic proteins. Except for the described approaches *via* Bcl-2 proteins many alternative strategies aimed at other proteins in signaling pathways of apoptosis are under investigation. In the future is expected that sensitization of tumor cells to established therapy protocols can contribute to the progress in cancer therapies.

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