

Targeting of Bcl-2 family proteins for treatment of acute leukaemia

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Abstract. Many studies suggest that killing of tumour cell by commonly used therapies (chemotherapy, radiotherapy) is mediated primarily by induction of apoptosis. Therefore, resistance of tumour cells to therapy can be caused by a failure in the ability to initiate apoptosis. Defects in apoptosis signalling pathways are also one of the main features of cancer, and particularly acute leukaemia. Malignant cells constantly resist the effects of cellular stress (e.g. DNA damage, oncogene activation), which would cause death of normal cells through apoptosis. Dysregulation of apoptosis has therefore give rise to tumour growth, disease progression and resistance of malignant cells to chemotherapy. Structural analysis of Bcl-2 family proteins playing a key role in regulation of mitochondrial apoptosis together with studies of their biochemical functions have outlines strategies for generation of drugs, resulting in numerous novel chemical entities with potential to reverse resistance of malignant cells to apoptosis. The use of these therapeutic approaches may in the future represent a new way in cancer therapy with high potential to improve clinical outcome and prognosis of acute leukaemia patients.

Key words: Apoptosis — Cancer therapy — Leukaemia — Resistance

Introduction

Apoptosis is physiological, genetically controlled process that leads to a cell death, which is not accompanied by an inflammatory response. It provides fast and effective removal of unwanted, damaged and potentially dangerous cells such as cells infected with viruses and tumour cells in order to maintain tissue homeostasis (Taylor et al. 2006). Induction of apoptosis may be caused by variety of stimuli from external or internal environment of cells. These include genotoxic and physical shock, hypoxia, expression of oncogenes or exposition of cells to cytotoxic drugs used in treatment of cancer (Haupt et al. 2003). Despite the diversity of stimuli that lead to apoptosis, the process of programmed cell death is associated with characteristic morphologic changes, as first described by Kerr et al. (1972). Cell shrinkage, condensation of nuclear chromatin, cleavage of DNA at internucleosomal sites, resulting in the generation of a characteristic pattern of DNA fragments, are typical features of apoptosis (Jin and El-Deiry 2005). Blebbing of the cell surface results in the release

of membrane-bound apoptotic bodies, which are recognized by macrophages, or neighbouring cells that consume them, without inflammatory reaction of surrounding tissue. The majority of the morphologic changes during apoptosis are caused by a family of intracellular cysteine proteases, called caspases, involved in the enzymatic cascade entailing the cell death. Caspases are synthesised as inactive zymogens and are proteolytically processed to an active form following apoptotic stimulus (Schultz and Harrington 2003; Jin and El-Deiry 2005). Caspases involved in apoptosis are according to their functions divided into two groups to initiator caspases (caspase 2, 8, 9, 10), which are capable of autocatalytic activation and mediate the activation of second group of effector caspases (caspases 3, 6, 7), which are capable to cleave many cellular substrates, thereby induce cell death (Testa and Riccioni 2007). Molecular mechanisms of apoptosis, particularly at the level of initiation, are diverse. At present, the extrinsic (receptor) and intrinsic (mitochondrial) pathway are considered as dominant mechanisms of apoptosis (Reed and Pellecchia 2005). The extrinsic pathway is triggered by the binding of an extracellular ligand to a receptor on the plasma membrane, which induces the formation of the death-inducing signalling complex (DISC). Formation of DISC triggers cleavage of procaspase 8 to its active form, which subsequently activates effector caspases, including

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caspase 3 (Haupt et al. 2003; Schultz and Harrington 2003; Elmore 2007). The intrinsic pathway is activated in response to intracellular stress signals (e.g. activation of oncogenes, DNA damage, oxidative stress, disturbances in protein folding) but also to the effects of stimuli from the external environment (e.g. removal of growth factor, ischemia and viral infection) (Elmore 2007). These stimuli lead to the release of cytochrome *c* from mitochondria into the cytoplasm, due to changes in mitochondrial outer membrane permeability, formation of apoptosome and consequent activation of initiator caspase 9 which in turn activates effector caspase 3 (Chipuk et al. 2008).

Apoptosis and leukaemia

Deregulation of apoptosis plays an important role in the development of a variety of human pathologies, including autoimmune diseases, neurodegenerative disorders and cancer. It is generally accepted that cancer progression is not the result only of increased rate of cell proliferation but also of decreased rate of cell elimination through apoptosis (Hanahan and Weinberg 2000). Precise identification of the different components of apoptotic pathways allowed the detection of various defects of apoptosis associated with leukaemia (Kitada et al. 2002). These defects provide a survival advantage of leukaemic blasts over their normal counterpart and are frequently associated with worse response to standard chemotherapy and with poor survival of patients (Letai et al. 2004). Since the main goal of anticancer chemotherapy is to induce apoptosis of malignant cells, the failure of apoptosis may also reduce the sensitivity of malignant cells to treatment and cause resistance (Wong and Puthalakath 2008). Identification of the molecular mechanisms of apoptosis deregulation in malignant cells has led to cancer therapies directly targeting the apoptotic pathway. Many of the drugs having potential to restore the sensitivity of leukaemic cells to apoptotic stimuli are currently under investigation at a clinical level (Schuler and Szende 2004).

The effect of chemotherapy may result in activation both mitochondrial and receptor pathway of apoptosis. However, recent analysis of targeted disruption of genes involved in the mitochondrial pathway point to a crucial and indispensable role of mitochondrial pathway of apoptosis in response to anticancer therapy (Testa and Riccioni 2007). Therefore, deregulation of mitochondrial apoptosis in acute leukaemia will be the focus of this review.

Bcl-2 family of proteins

Proteins of Bcl-2 family that control and affect the permeability of mitochondrial outer membrane are the key regu-

lators of mitochondrial apoptosis pathway (Chipuk et al. 2008). The Bcl-2 proteins contains at least one of the four Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4. Proteins, through the BH domains, specifically affect each other to form homo- and heterodimers, to ensure their pro- or antiapoptotic function (Kim et al. 2006). To date, 25 members of the Bcl-2 family of proteins, located predominantly in the outer mitochondrial membrane, have been identified. Proteins of Bcl-2 family can be divided into three subfamilies based on structural and functional features (Youle and Strasser 2008) (Table 1).

Antiapoptotic proteins (Bcl-2, Bcl-xl, Bcl-w, A1 and MCL1) contain all four BH domains and their main function is to promote cell survival. In the 3-dimensional structure, the BH domains are arranged to form a hydrophobic groove on the surface of antiapoptotic proteins. Integrity of hydrophobic groove is essential for their antiapoptotic function and for binding to proapoptotic proteins. This fact forms the basis for the development of inhibitors of antiapoptotic Bcl-2 proteins that bind with high affinity into the hydrophobic groove, thus block their antiapoptotic function (van Delft and Huang 2006).

Another group consist of proapoptotic proteins (BAX, BAK) containing three BH domains (BH1, BH2 and BH3) and are termed "multidomain proteins". In viable cells, the „multidomain“ proapoptotic members are present in the

Table 1. Survey of Bcl-2 family proteins (according to Strasser 2005)

| Protein | Domains | Function |
|---------|------------------------|--------------------------------------|
| Bcl-2 | BH1, BH2, BH3, BH4, TM | Pro-survival/anti-apoptotic proteins |
| Bcl-w | BH1, BH2, BH3, BH4, TM | |
| Bcl-xl | BH1, BH2, BH3, BH4, TM | |
| A1 | BH1, BH2, BH3, BH4 | |
| BOO | BH1, BH2, BH3, BH4, TM | |
| MCL1 | BH1, BH3, TM | |
| BAX | BH1, BH2, BH3, TM | Pro-apoptotic BAX/BAK-like proteins |
| BOK | BH1, BH2, BH3, TM | |
| BAK | BH1, BH2, BH3, TM | |
| Bcl-xs | BH3, BH4, TM | |
| Bcl-gl | | Pro-apoptotic proteins |
| BFK | | |
| BAD | BH3 | Pro-apoptotic BH3-only proteins |
| BID | BH3 | |
| BIK | BH3, TM | |
| HRK | BH3, TM | |
| BIM | BH3, TM | |
| Noxa | BH3, BH3 | |
| PUMA | BH3 | |
| BMF | BH3 | |

form of inactive monomers at the mitochondria (BAK), where are bound to antiapoptotic proteins Bcl-xl or MCL1, or in the cytosol (BAX) (Willis et al. 2005; van Delft and Huang 2006). Apoptotic stimuli lead to translocation of BAX to mitochondrial membrane, followed by homooligomerisation of BAX protein. BAK also homooligomerises into multimers and such both proteins form their active conformations. Oligomerised BAX and BAK form pore in mitochondrial membrane and thus facilitate the release of cytochrome *c* from mitochondria to cytosol, causing the initiation of apoptosis (Youle and Strasser 2008).

The last group consists of proteins containing only BH3 domain. These proteins are termed "BH-3 only" proteins. They function as primary sensors of apoptotic stimuli arising from various cellular processes. This group includes: BIK, HRK, BIM, BAD, BID, PUMA and Noxa (Wong and Puthalakath 2008). The shape of the BH3 domain of these proteins allows its insertion into the hydrophobic groove of antiapoptotic proteins and such inhibit their function. Therefore, the main task of this group is to promote cell death by formation of heterodimers with antiapoptotic proteins, thereby indirectly activate proapoptotic BAX and/or BAK and release of cytochrome *c*. Some "BH-3 only" proteins

(e.g. Bid and Bim) may activate BAX and BAK also directly (Kim et al. 2006).

Ratio between pro- and antiapoptotic proteins of Bcl-2 family determines whether cell survives or dies. Disruption of this balance may cause either over induction or in contrast regression of apoptosis (Kim et al. 2006).

In addition to the regulation of cell cycle and DNA repair, p53 is also a dominant regulator of mitochondrial pathway of apoptosis (Fig. 1). p53 can initiate apoptosis by transcriptional activation of the expression of proapoptotic proteins of Bcl-2 family (e.g. BAX, BAK, PUMA and Noxa) (Sax and El-Deiry 2003), as well as transcriptional repression of antiapoptotic proteins of Bcl-2 family (Wu et al. 2001). Molecular mechanism of transcription-independent pathway by which p53 activates apoptosis involves the direct binding of p53 to several antiapoptotic proteins of Bcl-2 family (e.g. Bcl-xl); thereby p53 inhibits their antiapoptotic function (Mihara et al. 2003; Chipuk et al. 2004). According to its function in cell death and the maintenance of genome stability, p53 is frequently mutated in solid tumours (Soussi 2007), but in haematological malignancies it is a rare feature (Krug et al. 2002). Although p53 mutations in haematological malignancies are infrequent, they are generally associated with

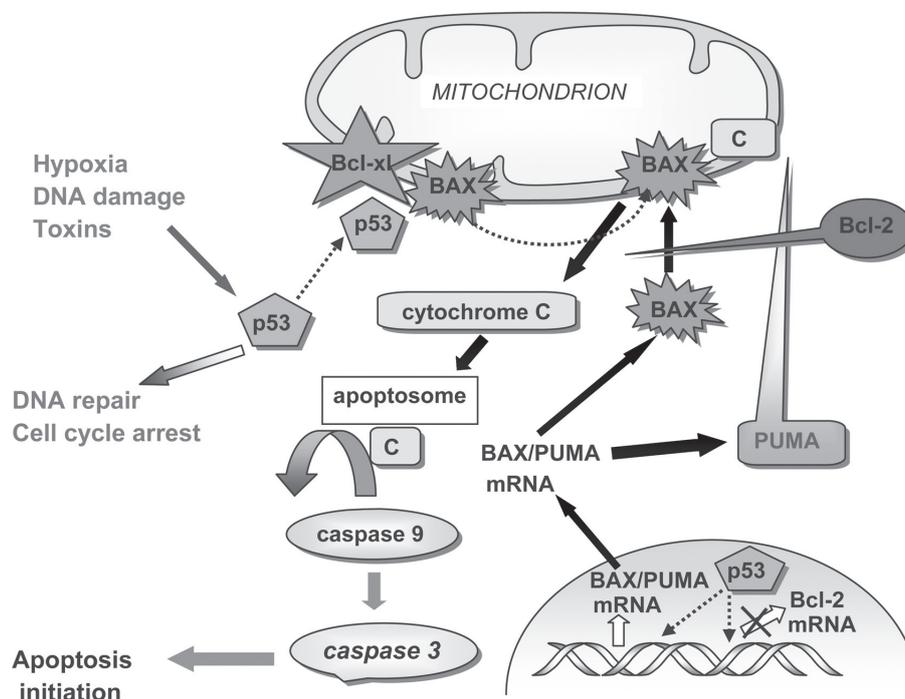


Figure 1. Intrinsic mitochondrial apoptotic pathway (modified according to Polster and Fiskum 2004). Various stressful stimuli, e.g. damage to DNA or hypoxia, stimulate the activation of p53 by different types of posttranslational modifications. Active p53 induces transcription of several pro-apoptotic genes, including BAX, BID, NOXA, and PUMA as well as represses transcription of anti-apoptotic genes, e.g. Bcl-2. Transcription-independent mechanism involves direct binding of active p53 to anti-apoptotic mitochondrial proteins, e.g. Bcl-xl, inhibiting their ability to block outer membrane pore formation by BAX and BAK.

worse disease progression and drug resistance (Haferlach et al. 2008; Nahi et al. 2008). Mutations of *TP53* gene also contribute to resistance of leukaemic cell lines to irradiation due to delay of induction of apoptosis (Aldridge and Radford 1998; Mareková et al. 2003).

Expression of antiapoptotic Bcl-2 proteins in acute leukaemia

Increased expression of several antiapoptotic Bcl-2 proteins was observed in various haematological malignancies, including both acute lymphocytic leukaemia (ALL) and acute myelogenous leukaemia (AML). The best studied is expression of Bcl-2 (Klobusicka et al. 2001). High levels of Bcl-2 may occur as a result of the t(14;18) translocation, present in a high percentage of follicular lymphomas (Tsujiimoto et al. 1985). This translocation places the *Bcl-2* gene into juxtaposition with powerful enhancer elements associated with immunoglobulin heavy chain locus, which results in elevated levels of Bcl-2 mRNA and protein. Overexpression of Bcl-2 was also observed in a wide range of haematologic malignancies in the absence of t(14;18) translocation, however, the exact cause of high levels of Bcl-2 protein is not known. It is expected that altered expression or function of transcription factors (e.g. WT1) controlling the promoter region of *Bcl-2* gene might contribute to elevated levels of Bcl-2 protein (Campos et al. 1993). Elevated expression of Bcl-2 has also been shown to be responsible for resistance of P-glycoprotein (P-gp) overexpressing L1210 cells to cisplatin without any contribution from the drug efflux activity of P-gp (Gibalová et al. 2009).

Several studies suggested that elevated levels of Bcl-2 protein may be associated with adverse clinical outcome (reduce rate of complete remission, shorter disease free or overall survival) as well as with more aggressive malignant phenotype and/or resistance to different classes of chemotherapeutic agents (Campos et al. 1993; Nuessler et al. 1999; Andreef and Konopleva 2002). Although high levels of Bcl-2 are commonly found at the diagnosis of disease, increased levels of Bcl-2 were not observed during relapse of acute leukaemia (Wuchter et al. 2000). However, some researchers observed no relation between levels of Bcl-2 and response of AML or ALL patients to initial therapy. The differences in measured levels of Bcl-2 and its different prognostic significance may arise as a result of inclusion of non-homogenous groups of patients, in which predominate younger patients with favourable types of leukaemia, as well as of significant differences in Bcl-2 levels between individual samples. Another reason for the differences may be complex interactions between proteins of Bcl-2 family, which highlights the need for a comprehensive study of Bcl-2 family proteins (Kaufmann et al. 1998). It has been suggested that the BAX/

Bcl-2 ratio may be a good prognostic factor in AML. Higher BAX/Bcl-2 ratio correlated significantly with a higher complete remission rate, longer overall and disease-free survival (Del Poeta et al. 2003).

In addition to Bcl-2, increased expression of Bcl-xl is often observed in AML blasts. Previous studies suggest that increased expression of Bcl-xl mediates resistance to a variety of treatments and drugs, including etoposide, doxorubicin, cisplatin, vincristine, bleomycine, paclitaxel and ionizing radiation (Datta et al. 1995; Minn et al. 1995). In case of ALL, elevated Bcl-xl may explain relative resistance of ALL cells to p53-dependent apoptosis (Račay et al. 2008). Prognosis of patients with AML might also be influenced by Bcl-xl/Bcl-xs ratio. It was reported that ratio of antiapoptotic Bcl-xl to proapoptotic Bcl-xs is higher in chemoresistant cells (Ray et al. 1996; Yamaguchi et al. 2002).

The analysis of MCL1 protein expression in AML showed great heterogeneity, but no correlation was found between protein levels and response to standard chemotherapy. However, it was observed a two-fold increase of MCL1 levels at the time of leukaemic relapse (Kaufmann et al. 1998).

Leukaemia therapy targeted to Bcl-2 family of proteins

Since, high levels of antiapoptotic Bcl-2 family proteins are associated with resistance to chemo- or radiotherapy due to blocking of the mitochondrial pathway of apoptosis, currently targeted strategies that overcome the cytoprotective effects of Bcl-2 and its related proteins have a leading position in development of new therapeutic options for acute leukaemia (Galluzzi et al. 2006). The options to avoid unwanted influence of antiapoptotic Bcl-2 proteins include: inhibition of gene transcription, induction of mRNA degradation or the direct attack of proteins by inhibitors (Reed and Pellecchia 2005).

Drugs regulating gene expression

Several types of drugs altering the expression of antiapoptotic Bcl-2 family genes were identified. Some synthetic retinoids reduce levels of Bcl-2 or Bcl-xl mRNA in leukaemia cells (e.g. fenretinide) (Reed 1999; Faderl et al. 2003). Inhibitors of histone deacetylases (HDACs), chromatin-modifying enzymes, have also the ability to decrease the expression of *Bcl-2*, *Bcl-xl* or *MCL1* at transcriptional level. Sodium butyrate is HDAC inhibitor that induces apoptotic cell death which is accompanied by up-regulation of proapoptotic BAX and down-regulation of antiapoptotic Bcl-2 and Bcl-xl (Choi 2006). Clinical trials of HDAC inhibitors are in progress, with hints of activity documented for lymphoma and some solid tumours (Kelly et al. 2003). The use of valproic acid, another HDAC inhibitor, has shown promising results in AML and

myelodysplastic syndrome patients. Testing of effectiveness of valproic acid in other haematologic malignancies as well as optimisation of treatment schedules may provide valuable and essential knowledge for its clinical application (Kuendgen and Gattermann 2007).

Drugs attacking mRNA

The effort to avoid the influence of antiapoptotic proteins of Bcl-2 family has led to attempts to reduce the levels of their mRNA using antisense oligonucleotides, short sequences of single-stranded synthetic deoxyribonucleotides that bind to specific complementary coding regions on mRNA. Formed RNA-DNA heteroduplex is a substrate for RNase-H, which cleave the target mRNA and prevent its processing and subsequent protein synthesis (Moreira et al. 2006). The specificity of this approach lies in the fact that each DNA sequence longer than 17 nucleotides occurs only once within human genome. Oblimersen sodium (Genasense; G3139), an 18-mer phosphorotiated oligodeoxynucleotide, binds to the first six codons of open reading frame of Bcl-2 and such downregulates its expression (Klasa et al. 2002). Phase I and II of clinical trials of oblimersen alone and in combination with standard chemotherapy have shown encouraging results in chronic lymphoblastic leukaemia (CLL), non-Hodgkin lymphoma (NHL) and AML patients (Marcucci et al. 2005; Rom et al. 2009). Phase III of clinical trials in CLL patients has been already completed and significantly higher percentage of patients who achieved complete or partial remission of the disease compared to patients treated with chemotherapy alone was observed (O'Brien et al. 2009a). The combination of oblimersen with conventional first-line therapy based on cytarabine/anthracycline yielded a 48% rate of complete remission of *de novo* AML (Marcucci et al. 2005). Unfortunately, a randomized phase III trials of older AML patients failed to show improved outcomes for those receiving the combination with oblimersen (Marcucci et al. 2007).

Small-molecule inhibitors of the Bcl-2 family of proteins

The observation that antiapoptotic proteins of Bcl-2 family bind BH3 domains of proapoptotic proteins in a hydrophobic groove as a mean to suppress apoptosis initiation revealed the opportunity for the design of small molecules that bind directly into groove, thereby promoting apoptosis. Since these designed molecules mimic the function of "BH-3 only" proteins, they are termed BH3 mimetics. BH3 mimetics, destroying cancer cells by targeting specific pathways that allow survival of cancer cells, have been tested in clinical trials to improve therapy of malignant diseases including acute leukaemia (Zhang et al. 2007). The main advantage of these molecules is that they are highly permeable through

membranes due to their low molecular weight. Until now, it was discovered more than ten substances that may prevent the interaction between Bcl-2 or Bcl-xl and proapoptotic proteins (Fig. 2). Currently, the most studied ABT-737 binds with high affinity ($K_i \leq 1$ nM) to the BH3 binding pocket of Bcl-2, Bcl-xl and Bcl-w, but not to MCL1 ($K_i > 1$ μ M), similarly to the "BH-3 only" protein Bad (Oltersdorf et al. 2005). ABT-737 does not directly activate BAX or BAK, but binds to the antiapoptotic proteins and such prevents them to block apoptosis. ABT-737 exhibits single agent activity against a panel of haematological malignancies, especially in those that critically dependent on antiapoptotic Bcl-2 proteins for survival (Oltersdorf et al. 2005; Konopleva et al. 2006; Del Gaizo Moore et al. 2008; Vogler et al. 2008). Simultaneously, ABT-737 displays synergism with chemo- and radiotherapy. It was observed 2–4-fold reduction of the median effective concentration (EC_{50}) of etoposide, doxorubicin, cisplatin and paclitaxel in the presence of ABT-737 in a variety of cell lines (Oltersdorf et al. 2005). Since ABT-737 does not bind to MCL1, high levels of MCL1 may contribute to the development of resistance to this agent. Several reports have demonstrated that MCL1 represents a key determinant of ABT-737 sensitivity and resistance of cancer cells (van Delft et al. 2006; Chen et al. 2007; Kang et al. 2008; Chen et al. 2009). Downregulation of MCL1 by CDK inhibitors (e. g. roscovitine, flavopiridol, seliciclib) or Raf/Mek inhibitors (sorafenib) may dramatically increase ABT-737 cytotoxicity in malignant cells (Chen et al. 2007; Hikita et al. 2010; Yecies et al. 2010). Poor pharmaceutical properties (low solubility in water) of ABT-737 considerably limit its use as therapeutic agent. The second-generation inhibitor ABT-263, that is orally bioavailable, is currently in phase I clinical trials (Tse et al. 2008).

The efficacy of ABT-737 can also be increased by using its combination with obatoclox mesylate. Obatoclox (GX15-070) mesylate is besides Bcl-2, Bcl-xl and Bcl-w capable to prevent MCL1/BAK binding (Nguyen et al. 2007). Phase I studies focused on haematological malignancies demonstrated biological activity, evidence for single-agent activity and good tolerability. Clinical response to obatoclox mesylate was observed in AML (Schimmer et al. 2008) and CLL patients (O'Brien et al. 2009b). In addition, recent study showed that the combination of HDAC inhibitors and obatoclox has synergistic antileukaemic activity mediated by induction of both apoptosis and autophagy (Wei et al. 2010).

Antimycin A and gossypol are the most promising from a number of natural compounds that have potential to inhibit antiapoptotic proteins of Bcl-2 family. Antimycin A, a streptomyces-derived inhibitor of ubiquinol-cytochrome c reductase of the mitochondrial respiration chain, promotes cell death by binding to Bcl-2 (Tzung et al. 2001). Gossypol, capable to bind in submicromolar concentrations to Bcl-2 and MCL1, exhibits anticancer effects *in vitro* in different

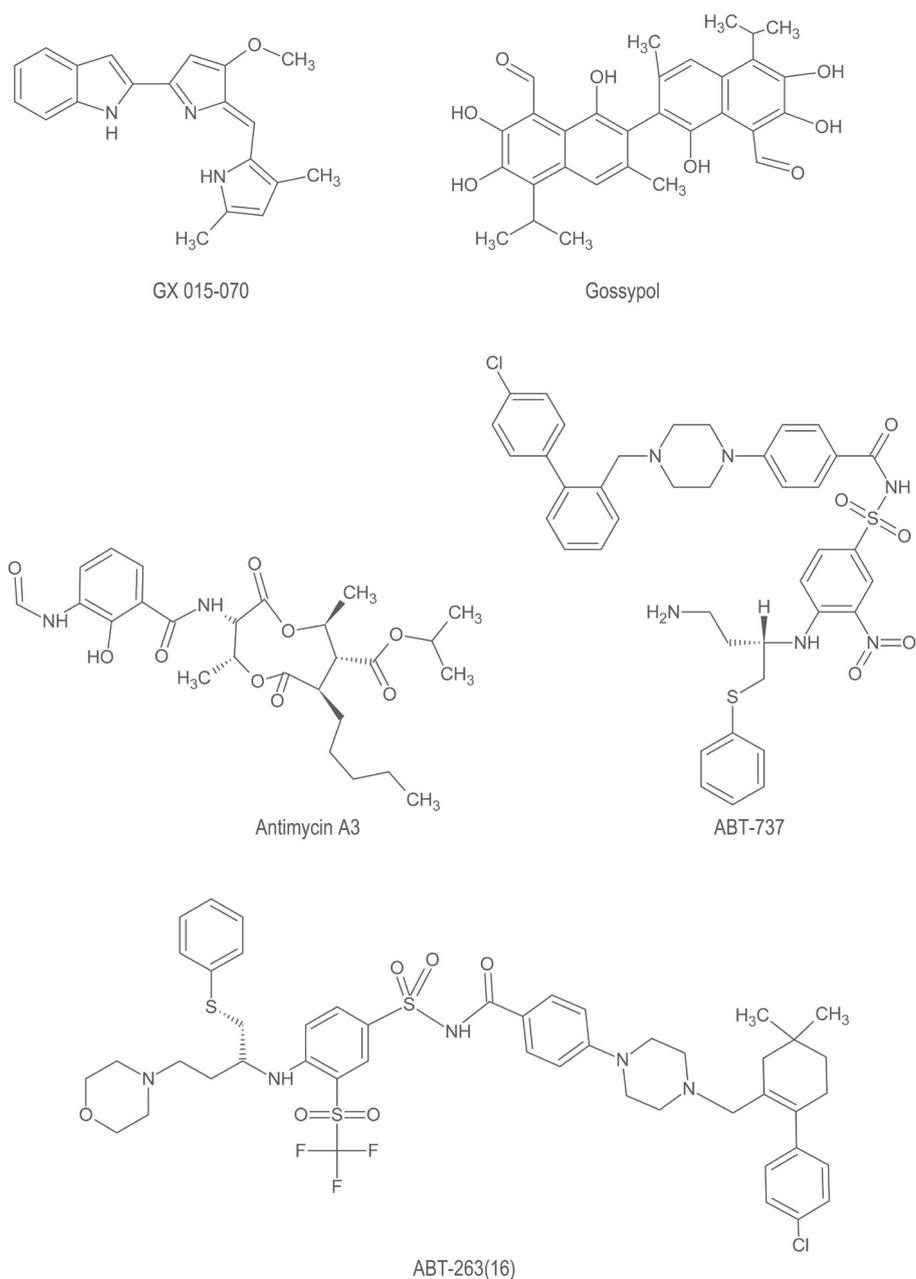


Figure 2. The structures of small-molecule inhibitors of Bcl-2 and Bcl-xL (BH3 mimetics).

cancer cell lines (Oliver et al. 2004). However, clinical use of gossypol is significantly limited by its gastrointestinal toxicity (Kang and Reynolds 2009).

Due to complex interaction between Bcl-2 proteins, it is likely that efficient recovery of sensitivity of malignant cells to apoptosis may require the use of combination of drugs targeting several antiapoptotic proteins. For such reason, recent research is directed at identification of new small molecules that could make stable complexes simultaneously

with Bcl-2, Bcl-xL and MCL1. A few new designed molecules are waiting for evaluation of clinical efficiency (Dalafave and Prisco 2010).

Conclusions

Acute leukaemia is aggressive haematologic malignancy, which arise as a consequence of malignant transformation of

haematopoietic cells at early stage of differentiation. Despite of improvements in treatment strategies, prognosis of acute leukaemia is still poor. Efficacy of current approaches to cure leukaemia, including chemo-, radio- and immunotherapy critically depends mainly on the proper functioning of cell death programs. Therefore, deregulation of apoptosis is often associated with disease progression and resistance to chemotherapy. Recently, much attention is paid to investigate the options to restore the sensitivity of leukaemic cells, as well as the possible ways to increase the efficiency of chemotherapeutic treatment. Since the effects of chemotherapy are not strictly specific to tumour cells, but they are more or less toxic to normal cells as well, new targeted therapies could simultaneously help to alleviate the side effects of chemotherapy. Detailed study of the molecular mechanisms of apoptosis has helped to identify several potential targets for developing new drugs. These are particularly the proteins that block apoptosis, which increased expression is often observed in malignant cells and is associated with worse prognosis of disease and resistance to chemotherapy.

More detailed research of the link between apoptosis deregulation and cancer development as well as translation of acquired knowledge into clinical practice may lead in the future to fundamental changes in prognosis of acute leukaemia patients.

Acknowledgment. This work was supported by APVV VVCE 0064-07 Biomembranes. Authors are grateful to Pavol Kupec for his excellent help with figures. J. J., J. H., A. Š., D. D. and P. R. have no conflict of interest and no financial interest in the publication of this manuscript.

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Received: December 6, 2010

Final version accepted: January 20, 2011