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Triggering apoptosis in tumors: an overview of potential approaches in treatment of leukemia

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Abstract

Apoptosis, as a well-studied process of a programmed cell death, is essential for the maintenance of cell homeostasis and integrity of organisms. This process occurs normally during development and aging and it is a balance of the sustainability of the tissue cell population. In addition, apoptosis also occurs as a defensive mechanism such as an immune response or after cell damage as a consequence of a pathological condition or the action of harmful agents. Apoptotic activation tends to be less responsive with aging, causing accumulation of non-functional cells and pathological changes such as degenerative diseases or tumor transformation. This overview aims to provide summarized facts about different approaches of apoptosis research, targeting and regulation in tumors especially in leukemic cells as a way of pharmacological manipulation with a potential therapeutic benefit.

Keywords

cell death, hematological malignancy, tumor cell elimination

Introduction

In a broad sense apoptosis is a mode of programmed cell death which is genetically controlled. Generally, the factors of apoptosis are one of the most thoroughly studied biological phenomena which are evolutionarily conserved and immanent to metazoan organisms. Primary physiological role of apoptosis is in physical sculpting of species specific body organs shape at early stage of development. Apoptosis is also the way of elimination of non-functional cells, for example cells that did not establish functional synaptic network in neural system. Normally, in humans billions of cells are being eliminated daily as a part of this "sweeping process". Although, there is a wide range of stimuli and conditions which can cause apoptosis, both physiologically and pathologically not all cells undergo elimination in response to the same stimulus. Radiation or chemotherapy drugs cause damage of DNA in some cells, which can lead to apoptosis induction (Elmore, 2007). Understanding the importance of the apoptosis process as well as

the way of its regulation can contribute to the clarification of key factors that affect cell proliferation and differentiation. Therefore, the overall cells survival has crucial importance in some pathological conditions, especially malignancy. In fact, defects of apoptotic pathways are believed to contribute to numerous human diseases, from neurodegenerative disorders to various types of malignancy (Lowe & Lin, 2000).

Apoptosis activation and mechanisms of control

Molecular machinery of apoptosis is strictly and genetically regulated. It engages BCL-2 (antiapoptotic B cell lymphoma 2) family of tens of genes, classified as pro-apoptotic or anti-apoptotic (pro-survival) genes. There are also many "independent" genes with critical role in regulation of cell-death such as TP53 (*tumor protein 53*) or other genes of cell-cycle regulation which is clearly shown in many genes knock-out studies (Chin & Fu, 1998; Salleh et al. 2004). Critical components of apoptotic process are also caspases, a unique family of cysteine proteases. The initiation of the apoptotic process activates a cascade series of regulatory proteins, caspases (*cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases*). Depending on the way in which apoptosis is induced, as well as the caspase position in the apoptotic signal cascade, various caspases will be activated. Generally, caspases are divided into initiatory (e.g., caspase-9) and effectors' caspases (e.g. kaspaza-3 and -7). Due to inability of the cell to modulate caspase activity, the apoptosis process can be disturbed and potentially lead to carcinogenesis, autoimmunity, neurodegeneration and immunodeficiency (Parrish et al, 2013).

Mechanism of apoptosis is very complex and involves two main pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial mediated pathway. The extrinsic pathway is activated by ligation of different plasma membrane receptors such as FAS (*Fas cell surface death receptor*), TNF (*tumor necrosis factor*) or TRAIL (*TNF-related apoptosis-inducing ligand*) (Sheikh & Huang, 2004). The intrinsic pathway is activated by mitochondrial dysfunction and release of death promoting proteins: AIF (*apoptosis inducing factor*), EndoG (*endonuclease G*), Smac/DIABLO (*second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI*), Omi/HtrA2 (*mitochondrial serine protease*) (Green & Reed, 1998). Different proteins regulate these pathways including p53, PI3K (*phosphoinositide 3-kinase*), NF-κB (*transcription factor*) and the ubiquitin proteasome system (Ghobrial et al, 2005). There is an additional perforin/granzyme pathway mediated by cytotoxic T cell for induction of apoptosis through either granzyme B or granzyme A (Elmore, 2007).

Regardless of the biochemical differences of activation of apoptosis, the final result is cell death accompanied by the characteristic morphological changes of the plasma membrane, mitochondrial dysfunction, cell shrinkage, chromatin lysis and condensation, nuclear fragmentation, dissociation of cell organelles and formation of apoptotic bodies. In further development of this review we will focus to the research of apoptosis as a key reason for tumor development and progression and the proposed ways for their therapeutic restoration to normal level in leukemia.

Link between apoptosis and tumorigenesis with an emphasis on leukemia

In tumor cells, the normal cell cycle control is dysfunctional, causing over-proliferation of cells and/or decreased cells elimination (King & Cidlowski, 1998) that is fundamental principle of immanent anti-tumor activity. Apoptosis as a biological phenomenon has long been associated with elimination of tumor cells and tumor progression (Kerr et al, 1972). Different changes and defects in apoptotic mechanisms play important roles in tumor pathogenesis accumulating the genetic alterations (Hassan et al, 2014). Deregulation of apoptosis causes misbalance between cell proliferation, cell survival and cell death and plays a major role in the initiation and progression of solid and hematological tumors. Over-expression of apoptosis inhibitors as well as inactivation of apoptosis promoters is observed in human cancers (Kaufmann & Vaux, 2003; Hassan et al, 2014). According to Wong (2011) different

mechanisms contribute to tumor cell evasion of apoptosis and carcinogenesis (Figure 1). However, there is a growing interest for development of therapeutic strategies based on apoptosis regulation and induction in different tumors. Leukemia is heterogeneous group of haemopoietic cancers and represents the 11th and 10th most frequent cause of cancer occurrence and death worldwide (Miranda-Filho et al. 2018). Its development is a multistep process characterized by progressive genetic alterations that leads to the transformation of normal hematopoietic stem cells into leukemic derivates (Testa & Riccioni, 2007). One of the most widespread malignancies of myeloid cells is acute myeloid leukemia (AML) characterized by an accumulation of immature myeloid blasts in the bone marrow. Generally, AML is associated with a poor prognosis and overall survival, especially in the older population and current treatment has been improved only modestly for the past 30 years (Peña-Martinez et al, 2017). The leading approach in leukemia treatment is targeting leukemic cells via induction of apoptosis (Cassier et al, 2017). Currently, targeted therapies designed to induce apoptosis in leukemia are the most promising anti-leukemic strategies for targeting and elimination of the tumor cell, especially without or with limited collateral damage to normal hematopoietic progenitor cells (Testa & Riccioni, 2007).

Multidrug resistance (MDR) in leukemia

An important factor that limits the successful treatment of a wide range of malignancy is tumor resistance to chemotherapeutic agents or to multiple drugs. This phenomenon is known as multidrug resistance (MDR), which is a main reason why chemotherapy treatment outcome vary in cancers. Resistance to multiple drugs is clinically recognized as the development of tumor resistance to a wide variety of antitumour drugs after exposure to a single drug. This phenotype is well known in clinical practice and has been extensively studied, especially in AML (Hunault et al. 1997; Mahadevan & List, 2004). One of the basic mechanisms underlying this MDR phenotype is the active cellular extrusion of chemotherapeutic agents via p-glycoprotein, or resistance to multiple drug-coded MDR1 gene. Today, there are outstanding efforts to improve measuring of MDR in clinical samples, which is a critical step in the correct determination of MDR phenotypes in patients with malignancies (Leith, 1998). MDR mechanism may be developed by increased release of the drug outside the cells and reduced absorption of the drugs (Mansoori et al. 2017). Recent studies (Li & Dalton, 2006; Chapuy et al. 2008; Mansoori et al. 2017) suggest that cell-to-cell interactions, tumor microenvironment, signal transduction molecules or lysosomal drug sequestration could also be factors of MDR

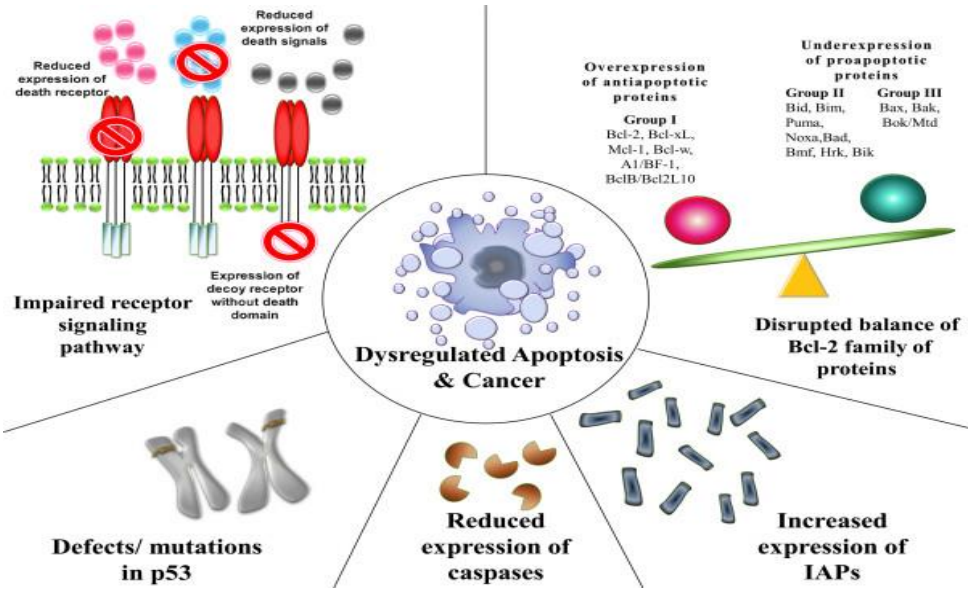

Figure 1. Various mechanisms that contribute to the deregulation of apoptosis and cancer development (Wong, 2011)

development. Interaction between leukemia and stromal bone marrow cells (fibroblasts, adipocytes, myocytes, chondrocytes, etc.) is associated with regulation of stem cell maintenance and localization that stimulate proliferation, self-renewal and differentiation. These cells are involved in the regulation of normal and tumor hematopoiesis due to production of wide range of stimulus and signaling molecules such as cytokines, chemokines, growth factors that activate hematopoiesis precursors. Therefore, it has been shown that this interaction is related and contributes to the development of chemo-therapy resistance *in vitro* and *in vivo* (Macanas-Pirard et al., 2017). All MDR mechanisms are potential targets in development of new personalized treatments. Great progress represent new scientific knowledge about the expression level of certain genes and/or proteins in various types of leukemia cells, especially those of primary cultures (Hlozková & Starková, 2018), which offers the new way of creating metabolic profiles and directions of therapeutic action even in individual cases.

In vitro and *in vivo* methods for apoptosis research

In researches of antitumor activity, different types of leukemia cells or cell lines with very heterogeneous characteristics are frequently used. All of these characteristics include differences in their morphology and expression for specific markers that make them universal models of research, especially considering the complexity of leukemia and all of its subtypes. Lymphoblastoid cell lines used in leukemia research are developed by infecting peripheral blood lymphocytes with Epstein Barr virus to immortalize B cells *in vitro* giving rise to an actively proliferating B cell population (Neitzel, 1986). Epstein Barr virus encoded crucial proteins for cell immortalization which is successful method from the last few decades with minimal amendments and provides an excellent *in vitro* model system as these cells are relatively easy to prepare and maintain, somatic mutation rate is low and represent an unlimited source of biomolecules (Hussain & Mulherkar, 2012). In addition to primary cell-lines, there is a wide range of commercially available leukemic cell lines and panels which are used in different studies (Table 1). The cytotoxic effects of potential new treatments in leukemia can be studied using several standardized methods for cell viability assessment: vital dye exclusion examines individual cells in a population, whereas the MTT assay for example, provide information about relative proportion of live and dead cells. The most used vital dyes are trypan blue, nigrosin, eosin Y, fast green and neutral red (McCarthy & Evan, 1998). However, these methods cannot distinguish the nature of cell death. Analysis of cyto-morphological alterations in the apoptosis monitoring implies studying of apoptosis by standard light microscopy using histological dyes that enable distinguishing between apoptosis and necrosis. Using fluorescence dyes (Hoechst 33258, acridine orange, Annexin V-FITC/PI) contributes to extended range of fluorescence microscopy, flow cytometric and immunofluorescence assays in apoptosis detection (Ji & Yu, 2015; Zhuo et al, 2015). Analysis of apoptosis can also be conducted using electron microscopy that clearly reveals DNA condensation in the nucleus and other morphologic changes characteristic for apoptosis (McCarthy & Evan, 1998). Also, they utilized a time-lapse video microscopy where cells are viewed microscopically over an extended period making them suitable for various forms of analysis. Czarnota et al. (1999) have described ultrasound imaging of apoptosis; the new non-invasive detection method *in vitro*, *in situ* and *in vivo*. *In vivo* detection of apoptosis may be very helpful and even crucial in monitoring tumor progression and prediction of the responses to antitumor treatment.

One of the useful methods for assessing apoptosis and monitoring drug response in hematological tumors is based on fluorodeoxyglucose-based positron emission tomography (FDG-PET) described by Newbold et al. (2014). Analysis of apoptosis is also possible using rhodamine 123 fluorescence dye intensity to measure mitochondrial membrane potential via flow cytometry (Ji and Yu, 2015). In addition, DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. Observations of DNA fragments include the most commonly used TUNEL (Terminal dUTP Nick End-Labeling) method (Darzynkiewicz et al. 2008). Notably, molecular techniques are developed

to detect caspases, cleaved substrates, regulators and inhibitors and include various types of caspase activity assays, western blot analysis, immunoprecipitation and immunohistochemistry. Additionally, apoptosis PCR microarray methodology uses real-time PCR to profile thev expression of genes that encode key ligands, receptors, intracellular modulators, and transcription factors involved in the regulation of programmed cell death (Elmore, 2007).

Table 1. The most common human leukemic cell lines in cytotoxicity studies

Cell line	Type of leukemia	Antitumor approach	Reference
HL-60	Acute myeloid leukemia	cell cycle regulation, induction of apoptosis	Sanchez-Gonzales et al. 2006
		cytotoxic and pro-apoptotic activity	Stanojković et al. 2018
		induction of apoptosis	Nakamura et al. 2001
		cytotoxic and antiangiogenic activity	Jackson et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
		inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
		inhibition of proliferation and apoptosis induction	Shashi et al. 2006
Jurkat	Acute T cell leukemia	cytotoxicity induction	Ramage et al. 2003
		cell cycle arresting and induction of apoptosis	Spinozzi et al. 1994
		induction of apoptosis	Lee et al. 2009
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
K-562	Chronic myeloid leukemia	cell growth inhibition, induction of apoptosis	Zhang et al. 2006
		cytotoxic and pro-apoptotic activity	Stanojkovic et al. 2018
		cell growth inhibition, cell cycle arresting, apoptosis induction	Chun-Guang et al. 2010
		cytotoxic and antiangiogenic activity	Jackson et al. 1998
		apoptotic response and cell differentiation	Benito et al. 1996
		inhibition of cell viability, cell cycle arresting, apoptosis induction	Zhang et al. 2017
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
NB4	Acute promyelocytic leukemia	induction of apoptosis	Rubio et al. 2014
		cytotoxicity induction	van der Weide et al. 2012
		inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
HEL	Erythroleukemia	apoptotic response and cell differentiation	Benito et al. 1996
		partial differentiation and apoptosis induction	Roboz et al. 2000
UT-7	Acute myeloid leukemia	inhibition of angiogenesis, growth arresting, apoptosis induction	Spiekermann et al. 2002
		regulation of cellular proliferation and differentiation	Drexler et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		cytotoxicity induction	Ramage et al. 2003
TF-1	Erythroleukemia	regulation of cellular proliferation and differentiation	Drexler et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		inhibition of cell signal transduction	Kobune et al. 2009
Kasumi-1	Acute myeloblastic leukemia	inhibition of angiogenesis	Zhang et al. 2013
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
		inhibition of cell signal transduction	Kobune et al. 2009
MOLT-4	Acute lymphoblastic leukemia	cell growth inhibition and apoptosis induction	Broggini et al 2003
		inhibition of proliferation and apoptosis induction	Shashi et al. 2006
		induction of apoptosis, reduction of cell growth	Mertens-Talcott and Percival 2005
TPH-1	Acute monocytic leukemia	inhibition of cell viability, cell cycle arresting, apoptosis induction	Zhang et al. 2017
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
KG-1	Acute myelogenous leukemia	inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
RS4;11	Acute lymphoblastic leukemia	apoptosis induction	Uckun et al. 1995
		apoptosis induction	Dörrie et al. 2001

Development of new therapeutic models based on modulation of apoptosis

Understanding of the apoptotic process has led to the development of new therapeutic strategies in the treatment of various types of cancer. Creating innovative methodology and approaches in apoptosis induction in leukemia and other malignancies, increase the possibility of discovering more effective anticancer therapeutics.

In order to develop and implement more efficient anticancer therapeutics, previous studies have provided a lot of useful information about apoptosis targeting in lymphoid and myeloid leukemia cells by various compounds of different origin. Induction of apoptosis in myeloid cells via activation of caspases under different conditions and treatments has been described (Huang et al, 1999; Yinjun et al, 2004). In this type of tumor cells, regulation of apoptosis by activation or suppression of some signaling pathways is also observed (Pomares et al, 2016; Zhou et al, 2017). There are many natural and synthetic products known as apoptosis inducers in leukemias (Huang et al, 1999; Shashi et al, 2006; Billard 2014; Rubio et al, 2014; Zhou et al, 2017) with different mode of action. Leukemia cells undergo apoptosis in the presence of some plant product, such as some flavonoids (Jihed et al, 2012; Ruela-de-Sousa et al., 2010) and algae extracts (Bechelli et al., 2011). Generally, numerous natural products are recognized as apoptosis inducers in different cancers (Taraphdar et al, 2001).

However, the most antitumor approaches that aim to induce apoptosis are mainly based on regulation of apoptotic pathways in leukemic cells. There are various strategies via activation of the extrinsic apoptotic pathway. Some members of the TNF-family (*three ligands TNF-α, FasL and TRAIL*) directly trigger apoptosis and together with their respective four receptors (TNF-R1, Fas, TRAIL-R1 and TRAIL-R2) have been considered as potential anticancer therapeutics (Testa & Riccioni, 2007). According to Samudio et al. (2009) the majority of leukemia cells express TRAIL receptors, but these samples are notoriously resistant to apoptosis induction by TRAIL. However, in different types of leukemia, especially in AML, many directions in apoptosis mediation and induction are described. That includes regulation of apoptotic process through p53-dependent manner and targeting MDM2/p53 pathway (Cassier et al. 2017) as well as development of XIAP (*X-linked inhibitor of apoptosis protein*) inhibitors (Testa & Riccioni, 2007). XIAP is the most potent endogenous inhibitor of caspase activity which overexpression confers resistance to both mitochondrial and death receptor pathway of apoptosis activation (Samudio et al, 2009). Mitochondrial or intrinsic apoptotic pathway is regulated by pro- and antiapoptotic members of the BCL–2 family. It is confirmed that the most genotoxic chemotherapeutics activate this pathway of apoptosis via activation of p53 signaling, resulting in the expression of proapoptotic target genes (Chipuk et al, 2004). An additional difficulty is affecting the specific fusion proteins, characterized in approximately 50–55% of AML cases that play a key role in the development of leukemia through their effect on cell proliferation, survival and apoptosis (Frohling et al, 2005).

Most cytotoxic drugs kill cells by modulating the process of apoptosis based on the fact that apoptotic markers may be indicators of tumor chemosensitivity. This concept has been investigated in AML by Ong et al. (2000) suggesting that high bax expression was associated with significantly improved survival, emphasizing bax to be an independent predictor of survival. Del Poeta et al. (2008) also confirmed impact of the bax/bcl-2 ratio, determined by flow cytometry, on AML prognosis. Clinical studies of Bcl-2 antisense drug in combination with cytotoxic therapy of AMLs, showed promising results.

BH3 mimetics in modulation of apoptosis

Targeting inhibition of anti-apoptotic factors, especially the membres of BCL-2 family proteins, is one of the leading approaches to activating the apoptosis in tumors. This family of proteins includes pro- and anti-apoptotic crucial factors in apoptosis regulation and control of cell death primarily by interacting with direct binding that regulates the permeabilization of the mitochondrial outer membrane. This leads to irreversible release of intermembrane spatial proteins and caspases thus causing apoptosis (Kale et al, 2018).

Understanding the regulation of apoptosis by BCL-2 factors has led to the development of new class of antitumor drugs targeting anti-apoptotic members by mimicking their natural BH3 proteins (*BCL-2 homologous proteins, BH3-only proteins*), also called BH3 mimetics. BH3 mimetics represent a new class of small molecule blockers with a promising potential in developing targeted therapies based on apoptotic regulation (apoptosis-based targeted therapy). These drugs directly activate apoptosis by binding and inhibiting selected anti-apoptotic protein members from the BCL-2 family (Delbridge & Strasser, 2015). Example of synthetic peptide venetoclax that belongs to a class of BH3 mimetics (Figure 2) is currently approved by the

Malignant stem cells as potential therapeutic target

In the last 50 years, intensive studies have confirmed that one of the leading antitumor strategies in many malignancies, especially in AML, is targeted therapy, or the elimination of malignant stem cells (Dick, 2005). Leukemia stem cells (LSCs) are defined as cells that can initiate the disease when they are transplanted into immunodeficient animals and can be self-renewed to maintain leukemia in series of transplantation. These cells may be partially differentiated into non-leukemic stem cells that resemble progenitor cells and original disease but are not able to self-regenerate (Thomas &

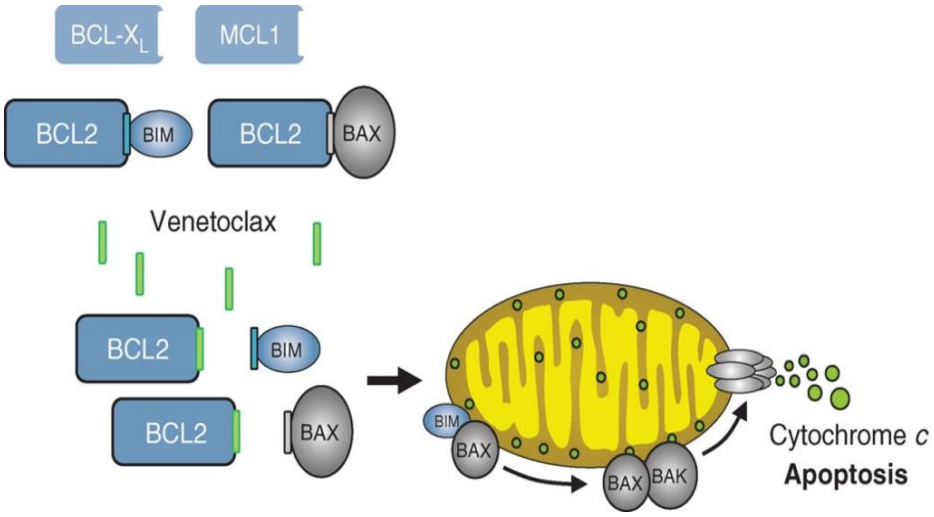

Figure 2. Example of BCL2 inhibition by venetoclax and apoptosis induction via release of proapoptotic proteins (Konopleva et al. 2016)

Food and Drug Administration for the treatment of relapsed/refractory chronic lymphocytic leukemia (CML) (Chung, 2018). Activation and/or re-establishment of apoptosis offer the potential for elimination of tumor cells in all stages of tumorigenesis. As BH3-mimetics become a part of clinical practice, they could significantly improve the outcome of therapy and overall survival (Campbell & Tait, 2018). Moreover, preclinical studies announce the potential for development of BH3 mimetics that target other BCL-2 members, especially in leukemia of myeloid cell. Thus, BH3 mimetics appear to be destined to become a powerful new weapon in the tumor arsenal (Cory et al, 2016).

Majeti, 2017). The earliest conceptual idea that leukemia is hierarchically organized, returns to the fields of this type of research to identify clonogenic progenitors of AML *in vitro*.

It has been proven that AML, similar to hematopoiesis, is hierarchically organized *in vivo* (Bonnet & Dick, 1997). Although analogy with stem cells of normal hematopoiesis can be very instructive and give some research guidance, it is important to highlight the very dynamic and unstable nature of LSCs. This is crucial in the development of improved and effective therapy (Pollyea & Jordan, 2017). From a clinical point of view, the tumor stem cell model implies that, in order to eradicate the disease and achieve long-term remissions, potential

treatments must be aimed to eliminate the LSCs population (Reya et al. 2001). In previous studies, the detailed characterization of LSCs has shown the properties of self-renewal, relative rest, resistance to apoptosis and reduced susceptibility to conventional therapy. Certainly, genetic analyzes and profiling of DNA methylation has contributed to expanding knowledge about the epi/genetic profile of this disease (Papaemmanuil et al. 2016). However, the application of these data and their association with the *in vivo* biology of LSCs is still in the intensive research. Two basic strategies for the target treatment of malignant stem cells in leukemia are divided into: therapies that selectively remove LSCs, also called specific therapies and therapies that remove the widespread population of leukemia cells and part of active stem cells. Target therapy may have multiple approaches, among which the most commonly strategies are based on immunological, antigen-specific or metabolic modulation as well as epigenetic regulation (Pollyea & Jordan, 2017).

In conclusion, targeting apoptosis process is one of the promising antitumor strategies, that specifically refers to hematological tumors and its resistance to conventional chemotherapy occurs. Today, induction and modulation of the apoptosis, especially in the cells of various types of leukemia, is applicable as one of the leading anti-leukemic strategies with high potential for developing and promoting targeted therapy.

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