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ORIGINAL ARTICLE

Endoplasmic reticulum stress associated with caspases-4 and -2 mediates korbazol-induced B-chronic lymphocytic leukemia cell apoptosis

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Summary

Purpose: *B-cell chronic lymphocytic leukemia (B-CLL) is an incurable disease that rapidly develops drug resistance. Therefore there is a need for identifying new agents that will improve the therapeutic outcome. Korbazol is a natural product known to exert cytotoxic effect on the in vitro survival of leukemic cells. The aim of this study was to investigate the mechanism of korbazol-induced apoptosis in B-CLL leukemic cells.*

Methods: *Peripheral blood mononuclear cells from 10 B-CLL patients were used for assessing the effect of caspase inhibitors and chelator of intracellular Ca^{2+}.*

Results: *Cell death rate induced by the tested compound was decreased with the caspase-3 inhibitor Ac-DEVD-CHO, and the inhibitors of caspase-2 (Z-VDVAD-FMK) and -4 (Z-YVAD-FMK), but not with the caspase-9 inhibitor z-LEHD-FMK and caspase-8 inhibitor z-IETD-FMK. No significant release of cytochrome C (cyt C) from mitochondria to the cytosol of B-CLL cells treated with korbazol was observed. Moreover, chelating of intracellular Ca^{2+} with BAPTA-AM almost completely abolished the cytotoxic effect of korbazol.*

Conclusion: *Engagement of caspases-2 and -4 and mobilization of intracellular Ca^{2+} indicate involvement of endoplasmic reticulum (ER) stress in apoptosis induced by korbazol.*

Key words: apoptosis, chronic lymphocytic leukemia, endoplasmic reticulum stress, korbazol

Introduction

B-CLL is a hematopoietic disorder that results in the accumulation of clonal CD5-positive B-cells with a low proliferation rate in the blood, bone marrow, lymph nodes and spleen [1]. In most patients, the circulating mature B-lymphocytes are largely quiescent G_0 phase cells, which accumulate due to their longer survival compared with normal B cells rather than to increased proliferation [2]. B-CLL represents an example of a malignancy caused by failure of programmed cell death and the defects in the apoptotic machinery not only increase spontaneous cell survival but also contribute to chemoresistance of the neoplastic clone.

Killing of cancer cells by various cytotoxic approaches such as anticancer drugs is predominantly mediated through induction of apoptosis in target cells [3].

Apoptosis or programmed cell death is a tightly regulated physiological process defined by orderly and characteristic sequence of structural changes resulting in cell detachment and rounding, chromatin aggregation, nuclear and cytoplasmic condensation, and eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies) that often contain morphologically intact organelles. The execution of apoptosis is mediated through consecutive activation of the members of the caspase family by cleaving specific substrate molecules [4].

The caspases, key components of this self-destruction machinery, are a group of intracellular cysteine proteases that are synthesized in the cells as inactive zymogens (procaspases) containing an N-terminal regulatory prodomain and a large (20 kDa) and a small (10 kDa) enzymatic subunit. Caspase recognition sequences exist between each of these domains, permitting proteolytic activation to occur either by self-processing or through

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cleavage by another caspase. Consistent with this, inhibition of caspases with small peptide inhibitors, such as pancaspase inhibitor Z-VAD-FMK, can prevent apoptosis. Caspase cascades are initiated by diverse death signals through assembly of the active multiprotein complexes that trigger autolytic processing and activation of the initiator caspases including caspases –2, -8, -9, -10, -12, which, upon their release, are responsible for the cleavage and the activation of downstream effector caspases including caspases-3, -6, and -7. Upon activation, effector caspases can directly cleave and destroy diverse arrays of essential cellular proteins leading to the coordinated destruction of the cell, or in some cases, effector caspases may function indirectly by amplifying a suicide signal. Two best studied apoptotic signaling pathways involve the activation of the extrinsic death receptors by their ligands and the intrinsic mitochondrial stress leading to the release of apoptogenic factors from this organelle. The best studied extrinsic pathway, death inducing signaling complex (DISC), begins outside the cell through the activation of the specific TNF family of death receptors (DR4, DR5, CD95/Fas) at the cell surface. Following binding to their cognate cytokine ligands, these death receptors trimerize leading to the recruitment, assembly and activation of initiator caspases -8 [5] and -10 [6]. In type I cells, strong caspase-8 and -10 activation bypasses mitochondria and directly leads to the activation of other downstream effector caspases and subsequently to apoptosis [7]. In type II cells, this initial activation of caspases leads to cleavage and activation of the pro-apoptotic Bcl-2 family member BID. The resulting truncated BID (tBID) fragment translocates to mitochondria and induces the release of cyt C from this organelle to the cytosol which triggers the formation of the apoptosome complex containing cyt C, pro-caspase-9 and Apaf1. Activation of the apoptosome subsequently leads to the cleavage of downstream caspases, such as caspase-3 and possible further activation of initiator caspases as part of a feedback amplification loop [8,9]. Moreover, various inducers of apoptosis can directly influence the permeability of the outer mitochondrial membrane, ultimately leading to the release of cyt C and other proapoptotic molecules (apoptosis inducing factor/AIF, endonuclease G, Smac/DIABLO, Omi/HtrA2 and many others) from the mitochondrial intermembrane space, which further stimulate caspase activation in the cytosol.

A number of recent studies have provided direct evidence that programmed cell death cascades can be initiated at other sites within the cell, in particular organelles such as the ER and Golgi apparatus [10]. Golgi complex, like mitochondria, senses and integrates unique local conditions, and transduces pro-apoptotic signals through local caspases. Caspase-2 is expressed at the Golgi complex and cleaves its substrate goldin-160, suggesting a caspase-2-dependent transduction of apoptotic signaling through the Golgi complex [11]. Moreover, caspase-2 is localized in the cytoplasm [12], mitochondria [13], and in the nucleus from where it regulates mitochondrial function [14]. In addition. emerging evidence has suggested the involvement of caspase-2 in ER stress, where it functions as an initiator caspase [15].

The ER is the first stop on the secretory pathway wherein chaperone-assisted polypeptide folding and modification ensures that proteins obtain the proper mature formation. When the capacity of the ER to properly fold proteins is comprised or overwhelmed, a highly conserved unfolded protein response (UPR) signal transduction pathway is activated, known as ER stress and it requires both the nucleus and the Golgi apparatus for signal transduction. A variety of toxic insults (including an oxidative stress, perturbation of calcium homeostasis with the chemical toxicant thapsigargin, which inhibits the sarcoplasmic/endoplasmic Ca^{2+}-ATPase [SERCA] resulting in ER Ca^{2+} depletion, and inhibition of N-linked protein glycosylation with tunicamycin) can cause ER stress and ultimately lead to cell death [16]. Persistent or excessive ER stress induces the activation of various apoptotic pathways, and the predominant apoptotic pathway may differ among cell types. Three major apoptotic pathways have been reported in the ER stress-mediated cell death: two of them, namely ASK/JNK pathway and the activation of caspase-8 by its interaction with BAP31 [17], induce cyt C release from mitochondria and caspase-9 activation. The third one is the apoptotic pathway in which caspase-12 in rodents (i.e. caspase-4 in humans) functions as the initiator caspase [18]. In rodents, upon ER stress caspase-12 is activated via processing by intracellular proteases named calpains that are activated by Ca^{2+} released in the vicinity of the ER [19], and caspase-7 which translocates from cytosol to ER [20]. Activated caspase-12 directly cleaves caspase-9 without the involvement of cyt C, leading to the caspase-9-dependent activation of caspase-3 [21]. A direct activation of caspase-3 by caspase-12 through a protein complex formation has been described in other experimental systems [22]. In addition, processed caspase-12 can translocate from the ER into the nucleus and directly participate in apoptotic events in the nuclei [23]. Very recently, it has been shown that in humans, caspase-4 is localized at the ER membrane, and is activated by ER stress, but not by the other apoptotic signals [24]. Cleavage of caspase-4 is not affected by overexpression of the anti-apoptotic Bcl-2 that prevents apoptotic signal transduction to the mitochondria, suggesting that activation of caspase-4 in ER stress-induced apoptosis is upstream of mitochondria.

The ER represents the main site of storing intracellular Ca^{2+} that is a major second messenger implicated in the signal transduction pathways regulating cell cycle, proliferation and apoptosis. SERCA pumps located in the ER membrane maintain the Ca^{2+} concentrations in the ER up to three orders of magnitude higher than the Ca^{2+} concentration in the cytosol. Previous studies demonstrated that the release of Ca^{2+} from the ER and the elevation of intracellular Ca^{2+} are key components for the variety of the apoptotic signaling pathways including those induced by dexamethasone, ionizing radiation, T cell receptor and FAS stimulation. Increased cytosolic Ca^{2+} can mediate apoptosis through activation of a wide variety of Ca^{2+}- sensitive enzymes, such as calpains. Upon their activation by Ca^{2+} these enzymes may generate signaling molecules, such as the proapoptotic protein BAX, for the recruitment of mitochondria to the apoptotic cascade or for the direct activation of the caspase enzymes [25].

Our previous *in vitro* studies (J BUON, in press) showed that the extract of the natural product korbazol strongly induced apoptosis in B-CLL cells, but not in normal mononuclear cells. This cytotoxic effect is dose-dependent and is not dependent on *de novo* protein expression. Identifying korbazol as a potential therapeutic agent for the treatment of B-CLL, we investigated the mechanism underlying its anticancer activity.

Methods

Cell isolation and culture

Clinical information was obtained from 10 untreated B-CLL patients at the Clinical Center, Kragujevac. The local Ethics Committee approved the study and prior to initiation written informed consent was obtained from all subjects according to the Declaration of Helsinki. Diagnosis and staging were based on standard clinical, morphologic and immunophenotyping criteria. Peripheral blood (10 ml) was collected in heparin-coated tubes from untreated patients fulfilling the diagnostic criteria for B-CLL. Mononuclear leukocytes were separated by density gradient centrifugation (Histopaque 1077, Sigma, Germany). Cells were washed three times in RPMI 1640 culture medium (20 mM Hepes, Sigma, Germany) and finally suspended in the supplemented RPMI 1640 culture medium (10% autologous serum, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin, all from Sigma, Germany). Cell number and viability were determined by using acridine orange/ethidium bromide (AO/EB) double staining (Sigma, Germany).

Tested compound

The natural product korbazol (Biofarm Group, Serbia, SCG), registered as dietary supplement (Department of Preventive Medicine, MMA, Belgrade, SCG: Korbazol HA108/05), contains plant extracts (*Echinacea purpurea, Paullinia cupana*), micronized zeolite, pollen, propolis, all preserved in honey. To produce the extract, 3.2 g of korbazol was dissolved in 10 ml of water/5% DMSO (Merck) and water soluble fraction of korbazol was filtered through nitrocellulose filters (Millipore, USA). The sterile extract was stored at -20° C. All experiments were performed with extract dilution 1:8.

MTT cell viability assays

Cultured cell viability was determined by assaying the reduction of MTT to formazan. In brief, after incubation of cells (0.5×10^6 cells/well) in 96-well plates with the tested compound and inhibitors of caspases or BAPTA-AM, media were removed and MTT (0.5 mg/1 ml of PBS) was added to each well. The cells were then incubated at 37° C for 4h, and DMSO (100 μl/well) was added to dissolve the formazan crystals. Absorbance was measured at 550 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria).

Effect of caspase inhibitors on korbazol-induced apoptosis

For studies using caspase inhibitors 0.5×10^6 cells in culture medium were preincubated with 50 μM Z-VAD-FMK, pancaspase inhibitor (Promega), 100 μM Ac-DEVD-CHO, caspase-3 inhibitor (Promega), 50 μM Z-IETD-FMK, caspase-8 inhibitor (Calbiochem), 100 μM Ac-LEHD-CMK, caspase-9 inhibitor (Calbiochem), 50 μM Z-VDVAD-FMK, caspase-2 inhibitor (R&D) or 100 μM Z-YVAD-FMK, caspase-4 inhibitor (R&D) for 2 h prior to the addition of korbazol extract. Cells were further incubated for 22 h at 37° C in an atmosphere of 5% CO_2 and absolute humidity and analyzed for cell death using MTT cell viability assay.

Analysis of cytochrome C release from mitochondria

Cells were plated on 1 mm glass coverslips in RPMI 1640 containing 10% autologous serum in the presence or the absence of korbazol extract. After 24h incubation at 37° C, cells were rinsed with PBS and then fixed (15 min) with ethanol/acetone (1:1) at room temperature. Fixed cells were washed with PBS and permeabilized with 0.2% Triton® X-100 in PBS for 2 min. After

washing with PBS and blocking with 10% FBS/PBS (30 min) cells were stained with Anti-Cytochrome C mAb (Promega) diluted in PBS (1:1000) for 30 min in a moist chamber at room temperature. Cells were washed with PBS twice and incubated with secondary antibody (Alexa 488-conjugated anti-mouse Ab, Molecular Probes Inc.) (1:200) for 20 min at room temperature. Finally, cells were washed with PBS and the coverslips were mounted onto microscope slides and analyzed by fluorescence microscopy (Polywar, Reinchard Jung). A minimum of 300 cells was counted in every sample.

Effect of calcium chelation on korbazol-induced apoptosis

Cells were pretreated with increasing concentrations of the intracellular calcium chelator BAPTA-AM (0.5 μM, 15 μM and 25 μM) for 30 min at 37° C. In two separate experiments, cells were further incubated with or without korbazol extract in medium either with Ca^{2+} or in Ca^{2+}-free medium, respectively. After 22 h at 37° C in an atmosphere of 5% CO_2 and absolute humidity cell viability was determined by MTT assay.

Statistics

Statistical analyses were performed using commercially available software (SPSS version 13.0; SPSS Inc., Chicago, IL). The distributions of data were evaluated for normality using the Kolmogorov-Smirnov test and then retested with the chi-square test. Comparison of quantitative parametric data between two study groups was done by using unpaired t-test. Differences between the paired data were evaluated using the paired t-test. In case of nonparametric data and two study groups the Mann Whitney test was used. A p-value <0.05, from two-sided tests, was considered statistically significant.

Results

Korbazol induced caspase-dependent apoptosis of B-CLL cells

To determine whether korbazol-induced apoptosis was dependent upon caspase activation, B-CLL cells were pretreated with broad-spectrum caspase inhibitor. Pretreatment with Z-VAD-FMK (50 μM) resulted in statistically significant inhibition of korbazol-induced apoptosis (56% inhibition; p<0.001; Figure 1), indicating that its ability to induce cell death is dependent on caspases.

To determine whether apoptosis induced by the tested extract was associated with the activation of caspase-3, cells were pretreated with caspase-3 inhibitor. As shown in Figure 1, Ac-DEVD-CHO (100 μM) prevented korbazol-induced cell death (14% inhibition), and that inhibition was statistically significant (p<0.005).

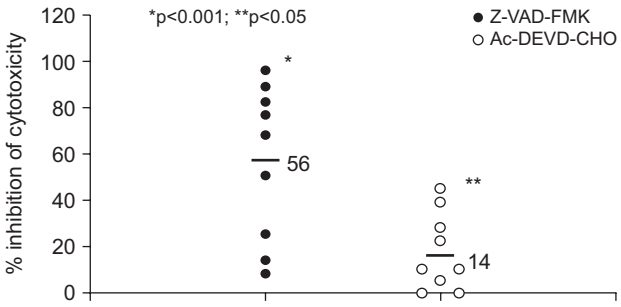

Figure 1. Korbazol-induced apoptosis of B-CLL cells is caspase-dependent. CLL cells were pretreated with caspase paninhibitor Z-VAD-FMK (50 μM, 2h) and caspase-3 inhibitor Ac-DEVD-CHO (100 μM, 2h) respectively, and then treated with korbazol for 22h. Cell viability was determined by the MTT assay. Results are presented as percent of inhibition of korbazol-induced apoptosis in the presence of caspase inhibitors, and every single value is the average of triplicate for each independent experiment. The horizontal bars and numbers near the bars represent the mean values for every group of data. Both caspase inhibitors significantly protected against cell death, suggesting caspase dependency and involvement of caspase-3 in the korbazol-induced apoptotic pathway.

Caspase-8 and -9 inhibitors did not prevent korbazol induced apoptosis

To further characterize korbazol-induced apoptosis and examine whether it activates the extrinsic or intrinsic apoptotic pathway, we pretreated cells with caspase-8 or -9 inhibitors. Neither Z-IETD-FMK (a caspase-8 inhibitor) nor Ac-LEHD-CMK (a caspase-9 inhibitor) blocked apoptotic events in korbazol-treated B-CLL (Figure 2). These results suggest that the initiating stimulus emanates from an origin other than death receptors or mitochondria.

Korbazol did not induce cytochrome C-release from mitochondria

Although our results have shown that caspase-9, which is activated by Cyt c/Apaf-1 complex, is not the upstream caspase in korbazol-induced apoptosis, we attempted to assess whether korbazol induces mitochondrial dysfunction. As Figure 3 shows, cyt C release was observed in the same percent age in treated and untreated cells, probably due to spontaneous apoptosis. This result clearly demonstrates that cyt C leakage from mitochondria is not accompanied with this apoptotic pathway.

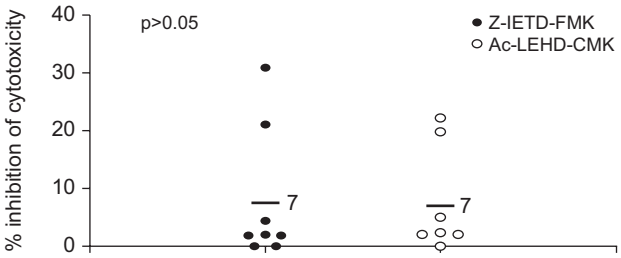

Figure 2. Caspase-8 and -9 inhibitors did not prevent Korbazol induced apoptosis. CLL cells were pretreated with caspase-8 inhibitor Z-IETD-FMK (50 μM, 2h) and caspase-9 inhibitor Ac-LEHD-CMK (100 μM, 2h) respectively, and then treated with korbazol for 22h. Cell viability was determined by the MTT assay. Results are presented as percent of inhibition of korbazol-induced apoptosis in the presence of caspase inhibitors, and every single value is the average of triplicate for each independent experiment. The horizontal bars and numbers near the bars represent the mean values for every group of data. None of these inhibitors have blocked apoptotic events in korbazol-treated B-CLL, demonstrating involvement of signaling pathways bypassing death receptors and mitochondria.

Korbazol-induced apoptosis is caspase-2 and -4 dependent

Since korbazol does not induce apoptosis via death receptors or mitochondria, we assumed that initial death signal may come from the other cell organelles, namely ER and/or Golgi complex. Thus, inhibition and silencing of either caspase-2 or caspase-4 could suppress ER stress-induced apoptosis. Pretreatment with both caspase-2 inhibitor Z-VDVAD-FMK and caspase-4 inhibitor Z-YVAD-FMK counteracted korbazol-induced apoptosis (Figure 4). Exposure to these inhibitors resulted in statistically significant decrease of the extent of cell death induced by korbazol. Caspase-2 inhibitor decreased the cytotoxic effect of korbazol by 27% ($p<0.005$) and caspase-4 inhibitor by 24% ($p<0.001$).

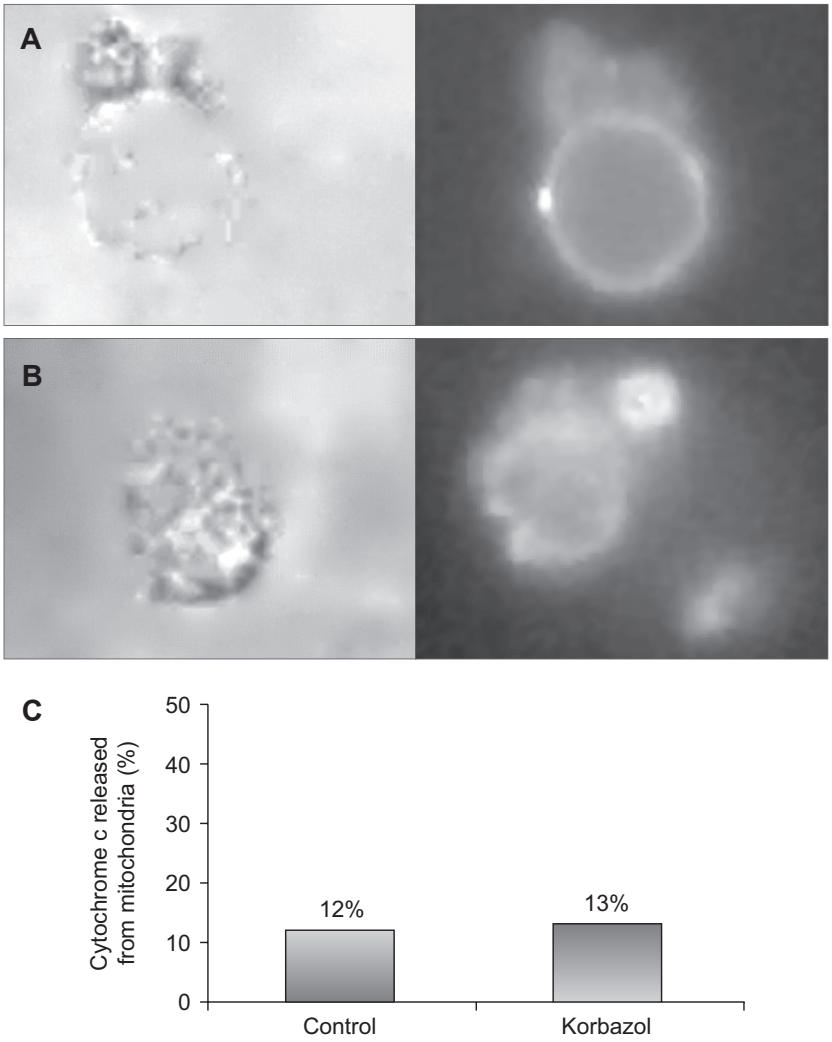

Figure 3. Korbazol did not induce cytochrome c release from mitochondria. CLL cells were treated with media alone and with korbazol for 24h and then fixed, permeabilized and stained with anti-cytochrome c antibody as described in "Methods". **A:** bright granulated fluorescent staining pattern indicates mitochondrial localization of cytochrome c; **B:** Diffuse staining with cytochrome c antibody indicates cytochrome c release from mitochondria to the cytosol; **C:** percent of cells with released cytochrome c.

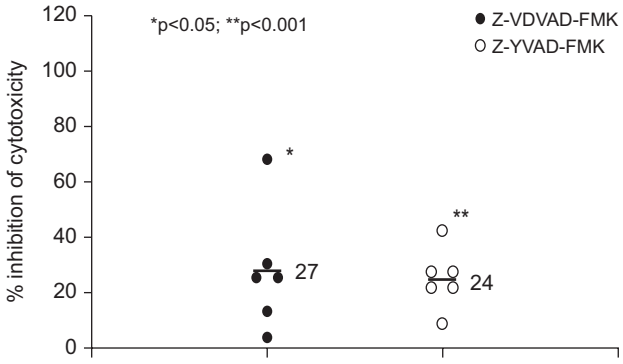

Figure 4. Korbazol induced apoptosis is caspase-2 and -4 dependent. CLL cells were preincubated with caspase-2 inhibitor Z-VDVAD-FMK (50 μM, 2h) and caspase-4 inhibitor Z-YVAD-FMK (100 μM, 2h) respectively, and then treated with korbazol for 22h. Cell viability was determined by the MTT assay. Results are presented as percent of inhibition of korbazol-induced apoptosis in the presence of caspase inhibitors, and every single value is the average of triplicate for each independent experiment. The horizontal bars and numbers near the bars represent the mean values for every group of data. Both caspase inhibitors significantly protected against cell death, suggesting caspase-2 and -4 dependency and involvement of ER stress in korbazol-induced apoptotic pathway.

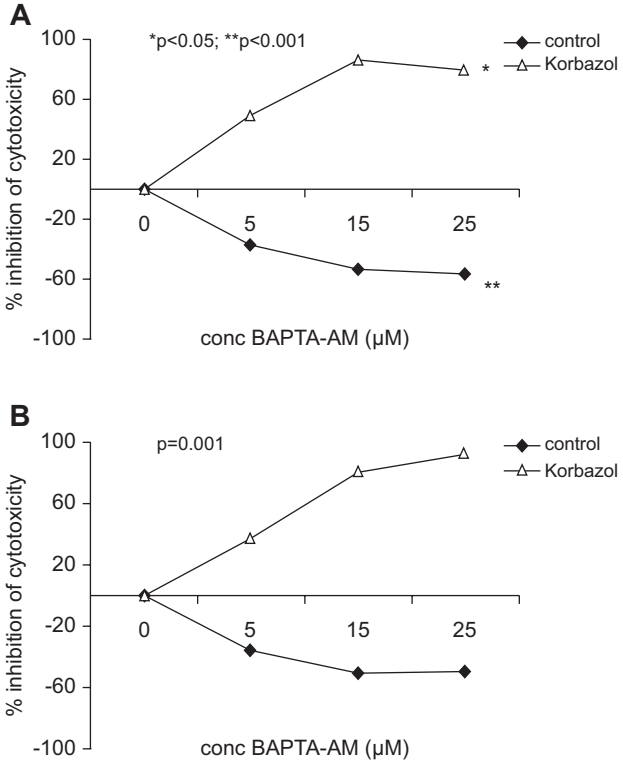

Figure 5. Ca^{2+} depletion protected B-CLL cells from korbazol-induced cytotoxicity. CLL cells were pretreated with raising concentrations of intracellular calcium chelator BAPTA-AM and then incubated with or without korbazol. In two separate experiments cells were further incubated with or without korbazol extract in medium either with Ca^{2+} or in Ca^{2+}-free medium. While BAPTA-AM induced cell death in untreated control cells, decrease of intracellular Ca^{2+} in cells treated with korbazol abrogated its cytotoxic effect, either in the medium with Ca^{2+} **(A)**, or in Ca^{2+} free medium **(B)**.

Ca^{2+} depletion protected B-CLL cells from Korbazol-induced cytotoxicity

To identify the contribution of the Ca^{2+} mobilization from intracellular and extracellular pools in korbazol-induced apoptosis, we pretreated the cells with raising concentrations of intracellular calcium chelator BAPTA-AM. While BAPTA-AM induced cell death in untreated control cells, decrease of intracellular Ca^{2+} in cells treated with korbazol abrogated its cytotoxic effect (Figure 5A). The same effect was observed when the experiment was performed in Ca^{2+}-free medium (Figure 5B), suggesting that Ca^{2+} extracellular influx is not necessary for the apoptotic activity of korbazol. These results support the crucial role of the Ca^{2+} mobilization from intracellular stores, but not the extracellular Ca^{2+} entry in the korbazol-induced cytotoxic process.

Discussion

Cells death by apoptosis occurs normally at different stages of morphogenesis, including growth and development of metazoans, and in normal turnover in adult tissue. Apoptosis can be triggered by an array of extra- or intracellular stimuli. Imbalances between cell death and survival may result in premature death, uncontrolled proliferation or tumor formation. B-CLL represents a typical example of malignancy caused by alterations in the pathways of programmed cell death. B-CLL originates from clonal expansion of long-lived B-lymphoid cells, most of which are in G_0/G_1 phase. Disease progression involves the persistent accumulation of monoclonal B cells, most likely due to defects in apoptosis that leads to immortal clones of cells rather than increased cell proliferation [26]. Deregulation of programmed cell death pathways also contributes to resistance to conventional anticancer agents, especially in later stages of the disease. Chemoresistance has been attributed to a variety of factors such as high expression of Bcl-2 antiapoptotic proteins [27], loss of functional p53 [28], mitochondrial defects, and *in vivo* exposure to exogenous prosurvival cytokines in the microenvironment. There is currently no curative treatment for CLL. First line therapy in CLL is the alkylating agent chlorambucil or the purine analog fludarabine. Other agents used include the alkylating agent cyclophosphamide, the purine analog cladribine, as well as combinations therapies. Although response rates to conventional therapy are relatively high, recurrence and subsequent resistance to chemotherapy frequently occur. Since the agents with different primary intracellular targets can trigger apoptosis through similar mechanisms, defects in

apoptosis programs may result in multi-drug resistance. Thereby, novel strategies are clearly required to improve the clinical outcomes of patients with this malignancy.

Natural products have been the source of many useful drugs, and their importance in the prevention and treatment of cancer is becoming apparent. Our previous study (J BUON, in press) that korbazol, natural product registered as dietary supplement, induced apoptosis in isolated CLL lymphocytes *in vitro*. Interestingly, the same concentration of this product failed to induce apoptosis in normal human lymphocytes. The aim of the current study was to elucidate the apoptotic pathway employed by the tested compound. An important role of caspases in korbazol extract-induced apoptosis can be hypothesized from our data that the broad-spectrum caspase inhibitor Z-VAD-FMK blocked this cell death program. Caspases activation is probably only a part of the apoptotic execution machinery since Z-VAD-FMK, even at a high concentration of 50 μM, although with high statistical significance, only partially inhibited the apoptotic process. Caspase-9, the critical player of the apoptotic stimuli acting through mitochondrial dysfunction, and caspase-8, critical for the apoptotic pathways generated by death receptors, seem not to be the essential inducer caspases since neither z-IETD-FMK, the specific inhibitor for caspase-8, nor z-LEHD-FMK, specific caspase-9 inhibitor, protected B-CLL cells from apoptosis. Furthermore, in the present study we showed that korbazol kills CLL cells without significant cyt C release from mitochondria. Both treated and untreated cells showed a similar percent of cyt C release in the cytosol. Pretreatment of cells with caspase-3 inhibitor, Ac-DEVD-CHO, inhibited korbazol-induced apoptosis, indicating engagement of this effector caspase. The role of caspases-2 and -4 in korbazol-mediated cell death was confirmed with specific inhibitors, Z-VDVAD-FMK (caspase-2) and Z-YVAD-FMK (caspase-4), since both significantly decreased the extent of the cell death. The ER plays a prominent role in intracellular Ca^{2+} homeostasis. It regulates not only the Ca^{2+} within its lumen, but also the cytosolic Ca^{2+} and the Ca^{2+} permeability of the plasma membrane (store-operated Ca^{2+} influx) [29]. Here, we demonstrated that the inhibition of cytosolic Ca^{2+} increase achieved by chelating intracellular Ca^{2+} resulted in significant abrogation of korbazol-induced cytotoxicity. It was further demonstrated that the increase in cytosolic Ca^{2+} is a result of the release of Ca^{2+} from intracellular stores, but not of the entry of Ca^{2+} into the cell from the external solution. These findings suggest that korbazol causes ER stress, which induces the activation of caspases and subsequent cell death without engagement of mitochondria and cyt C release into the cytosol.

It appears that the action of korbazol activates a cell death pathway independent of the canonical regulators of apoptosis. Considering that CLL cells, like many other malignant cells, have evolved mechanisms that render them less sensitive to chemostimulation of their mitochondrial death machinery, the identification of new agents that activate signaling pathways bypassing the currently known essential regulators of sensitivity to spontaneous and/or chemotherapy-induced apoptosis in B-CLL may have potential therapeutic promise. Given its ability to overcome mitochondrial apoptosis resistance mechanisms, korbazol may also be effective in other hematopoietic malignancies and solid tumors. Thus further investigations and understanding of their molecular targets and activity toward malignant cells may yield more effective therapies in the future.

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