*Journal of BUON* **15:** 732-739, 2010


ORIGINAL ARTICLE

# *In vitro* induction of apoptotic cell death in chronic lymphocytic leukemia by two natural products: preliminary study

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## Summary

***Purpose:*** *B cell chronic lymphocytic leukemia (B-CLL) is an neoplastic disorder characterized by alterations in the pathways of programmed cell death (apoptosis). Deregulation of apoptosis pathways also contributes to chemoresistance of B-CLL cells. Therefore, it is not surprising that induction and acceleration of apoptosis represent key point in novel B-CLL therapeutic protocols. The present study was designed to investigate the effects of two natural products, Immunarc forte and Korbazol on the in vitro survival of leukemic cells.*

***Methods:*** *Peripheral blood mononuclear cells (PBMC) from 20 B-CLL patients and 20 healthy donors were used for cytotoxicity studies. Cytotoxic activity of the tested products were assessed by the MTT colorimetric assay and the type of cell death was determined by flow cytometry.*

***Results:*** *We found that Korbazol was selectively cytotoxic against B-CLL cells, but the cytotoxic activity of Immunarc forte was much weaker. Of note, synergy was shown between these two drugs, and this effect was also selective, without affecting the normal mononuclear cells. According to Annexin-V binding, Korbazol and Immunarc forte induced apoptotic type of cell death in B-CLL cells. Moreover, treatment with Korbazol, but not with Immunarc forte, decreased spontaneous apoptosis in cultured normal polymorphonuclear cells.*

***Conclusion:*** *Our findings imply that Korbazol is as potential therapeutic agent that induces apoptosis of B-CLL cells. The resistance of normal mononuclear cells and anti-apoptotic effects on normal polymorphonuclear cells, as well as its ability to synergize with Immunarc forte, warrants further investigation and supports their therapeutic application in the treatment of B-CLL.*

**Key words:** apoptosis, chronic lymphocytic leukemia, immunarc forte, korbazol, natural products

## Introduction

Apoptosis, also called programmed cell death, has been introduced on the basis of observations that cells which die during development also have a characteristic set of structural changes notably different from necrosis. However, these characteristics may be observed on cells that die in different conditions: Natural killer (NK) cells, dendritic cells or T cells cytotoxicity [1-3]; negative selection of immune cells in the thymus [4]; normal cellular turnover in tissues [5,6]; in tumors and normal tissues when they are exposed to low doses of ionizing radiation [7,8]; chemotherapeutics [9,10] and even hypoxia [11,12]. Apoptosis essentially represents controlled breakdown of cells. Today, we know that apoptosis is involved in many physiological processes and that there is hardly any disease whose pathogenesis can be explained without apoptosis. Generally, there are diseases with too little apoptosis and diseases with too much apoptosis. For example, autoimmune diseases are characterized by impaired apoptosis of T lymphocytes, whereas cancer could be looked upon as a disease where the net increase in tumor burden is the sum of an increased growth rate and a decreased apoptotic rate.

Programmed cell death plays a central role in the selection and differentiation of lymphoid cells [4,6], as well as in regulating the size of the mature lymphocyt-

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Received 03-03-2010; Accepted 02-04-2010

ic population [5]. Imbalances in genetic mechanisms that either promote or block physiological cell death can slow or even stop normal cell turnover resulting in progressive accumulation of the malignant clone. A large amount of evidence indicates that malignancies of the lymphoid tissue could be caused by deregulation of apoptosis [13-16]. B-CLL represents a typical example of malignancy caused by failed programmed cell death. B-CLL originates from the clonal expansion of long-lived B-lymphoid cells, most of which are in $G_0$/$G_1$ phase [17-19]. The high level of bcl-2 expression, generally observed in B-CLL, has been implicated in the deregulation of apoptosis in the leukemic cells, as well as a loss of functional p53 and mitochondrial defects [20-24]. There is also evidence that c-myc oncogene, implicated both in the induction and prevention of apoptosis, can be relevant for dysfunction of apoptotic events in B-CLL [25,26].

Therefore, induction and acceleration of apoptosis become major aims in novel B-CLL therapeutic protocols. Although characterized by an extended lifespan *in vivo* [27], B-CLL cells spontaneously die *in vitro* [28,29] and in response to stimuli such as chlorambucil and other cytotoxic drugs, steroids, calcium ionophore, and purine nucleoside analogs [30-32]. Protein kinase C phosphorylation may prevent apoptosis [33], whereas cytokines prevent (IL-4, IL-9, interferon-γ and -α) or induce (IL-5 and IL-10*)* apoptosis of B-CLL cells [34-38].

Although initially responsive to a number of chemotherapeutic drugs, B-CLL cells rapidly develop drug resistance and ultimately cause treatment failure, regardless of the drugs used [39-44]. In this regard, B-CLL remains an incurable disease and therefore it is not surprising that there is still substantial interest in identifying new drugs that induce B-CLL apoptosis with greater selectivity.

We report herein the induction of apoptosis in B-CLL cells cultured with the natural product Korbazol and the recruitment of its action with another natural product, Immunarc forte. The DNA cleavage pattern, Annexin-V binding, effect of inhibitors, and synergy between these two products are discussed, and the clinical implications of these observations are addressed.

## Methods

### *Patients and samples*

Peripheral blood was obtained from untreated B-CLL patients referred to the CLL out-patient clinic at Kragujevac University Hospital. 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.

PBMC from 20 B-CLL patients and from 20 healthy donors were isolated from heparinized blood samples by centrifugation over a Histopaque 1077 layer (Sigma, Germany) at a density of 1.077 g/ml. The separated cells were washed 3 times in RPMI 1640 culture medium (20 mM Hepes, Sigma, Germany) and finally suspended in the supplemented culture medium RPMI 1640 (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 using Trypan blue and Acridine orange/Ethidium bromide staining (all from Sigma, Germany). Freshly isolated cells were used for cytotoxicity studies.

### *Test compounds*

Immunarc forte and Korbazol (all from Biofarm Group, Serbia) are natural products registered as dietary supplements (Department of Preventive Medicine, MMA, Belgrade, Serbia: Immunarc forte N435/04, Korbazol HA108/05; Table 1). To produce the extract, 3.2g of Korbazol were dissolved in 10 ml of dd$H_2O$/5% DMSO (Merck) and filtered through nitrocellulose syringe filters (Millipore, USA). The sterile extract was

**Table 1.** Composition of Immunarc forte and Korbazol

| *Immunarc forte (capsule)* | *Korbazol (250 g)* |
|---|---|
| Phaeophycea digitata Khorbi (Brown Seaweed) 2.0 mg | Guarana (liquid extract) 1.19 ml |
| Echinacea purpurea pulvis 90.0 mg | Propolis (dry extract) 595.24 mg |
| Germanium - 132 (Ge 132) 1.5 mg | Pollen P2 1071.43 mg |
| Nicotinic acid (B3) 8.0 mg | Zeolit 4761.9 mg |
| Retinol (A) 80.0 IUs | Echinacea purpurea 1190.48 mg |
| Pyridoxine hydrochloride (B6) 1.0 mg | Honey ad 250 g |
| Tocopherol adsorbate (E) 20.0 IUs | |
| Ascorbic acid (C) 12.0 mg | |

stored at -20° C. Immunarc forte was dissolved in water (10 mg/ml dd$H_2O$) to produce stock solution.

*Cytotoxicity*

The assay was performed in 96-well flat-bottom microtiter plates. PBMC from B-CLL patients or healthy donors were seeded into the microwells ($4\times10^5$/200 µl/well) in culture medium with or without various concentrations of Immunarc forte (30, 60, 120, 250, 500 and 1000 µg/ml) or decreasing dilutions of Korbazol extract (1:256, 1:128, 1:64, 1:32, 1:16 and 1:8). Cells were incubated for up to 48 h at 37° C in an atmosphere of 5% $CO_2$ and absolute humidity. Eight wells per plate with B-CLL cells and normal PBMC cultured in 200 µl of standard medium with corresponding concentration of DMSO were used as control. Cultured cell viability was determined by assaying the reduction of MTT to formazan. In brief, after incubation of cells, 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).

*Analysis of cell death*

After cultivation with Korbazol (extract dilution 1: 8) or Immunarc forte (1000 µg/ml), cells were harvested by centrifugation, suspended in RPMI 1640 culture medium and aliquoted for flow cytometry as well as for electrophoresis.

*Flow cytometry*

The apoptotic and viable cells were detected using Annexin-V-FITC/7-AAD kit (Beckman Coulter, USA) according to the manufacturer's instructions. Annexin-V binding detects relocation of membrane phosphatidyl serine from the intracellular surface to the extracellular surface, which is a hallmark of apoptosis. Flow cytometric analysis of labeled cells was performed by FACS Caliber flow cytometry (Becton Dickinson, Mountain View, CA). Analysis of the percentage of viable cells or Annexin-V positive cells (apoptotic cells) was calculated using software package CellQuest software (Becton Dickinson, USA).

*DNA fragmentation: Analysis using agarose gel electrophoresis*

This procedure is based on the internucleosomal DNA cleavage, one of the most important biochemical hallmarks for the apoptotic mode of cell death. Specifically, when DNA extracted from apoptotic cells was analyzed using gel electrophoresis, a characteristic internucleosomal "ladder" of DNA fragments was found. DNA fragmentation was analyzed by the gel electrophoresis method. Briefly, cells were lysed with 1% sodium dodecyl sulfate (SDS) in TE buffer (10 mM Tris, pH 8.0, 0.5 mM EDTA and 1% SDS) and digested by proteinase K (100 µg/ml; Oncogene, Germany) for 4h at 56° C. Samples were extracted with phenol and chloroform and the DNA was precipitated with a 1/10 volume of 3M sodium acetate and an equal volume of ethanol. DNA was pelleted at 13,000 × g and resuspended in TE buffer and 10 µg/ml of DNase free RNase (Oncogene, Germany) for 30 min at 37° C. DNA samples were analyzed on 2% agarose gel (Agarose, Eurogentec, Belgium) in TAE buffer (0.04M Tris-acetate, 2mM EDTA) by electrophoresis at 50V for 120 min. The DNA was stained with ethidium bromide (EB) (0.5 µg/ml) and visualized with 312 nm UV light.

*Statistical analyses*

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 Kolmogorov-Smirnov test and then retested with $x^2$ test. Comparison of quantitative parametric data between two study groups was done using unpaired t-test. With nonparametric data and two study groups Mann-Whitney test was used. Data were analyzed by Kruskal-Wallis test in case of more than two groups and nonparametric data. One-way ANOVA was performed in case of more than two groups and parametric data. When this test indicated significant differences, the Bonferroni test was used to identify intergroup differences. A p-value $<0.05$, from two-sided tests, was considered statistically significant.

## Results

*Flow cytometry*

To characterize the type of cell death induced by Korbazol and Immunarc forte, treated and untreated cells were labeled with Annexin-V-FITC and assayed on flow cytometer. Whereas 23% of untreated cells were Anexine-V positive (Figure 1A), due to spontaneous apoptosis after cultivating B-CLL lymphocytes *in vitro*, 36% of Annexin-V-positive cells were recorded after treatment with Immunarc forte (Figure 1B) and 85% after treat-

ment with Korbazol (Figure 1C). These results explicitly revealed that both Korbazol and Immunarc forte induced apoptotic type of cell death in B-CLL lymphocytes.

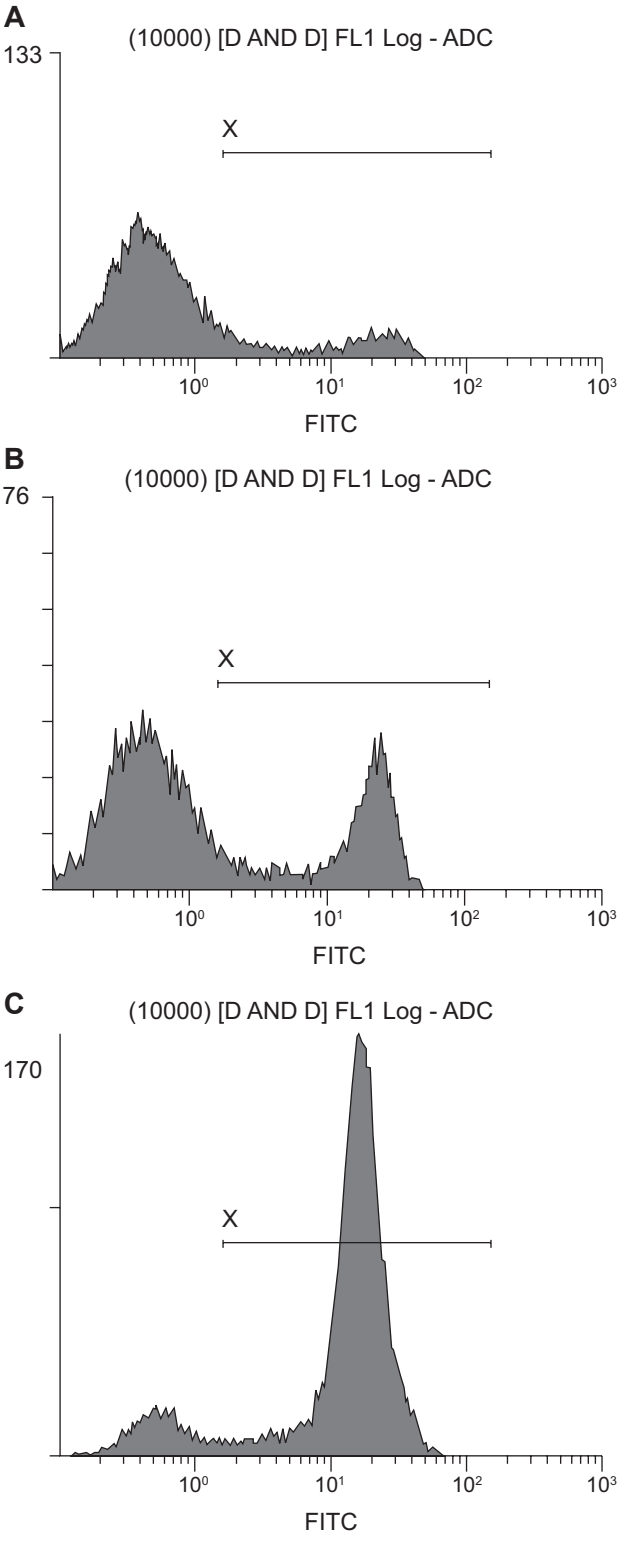


**Figure 1.** Flow cytometric analysis of Annexin-V FITC staining. CLL cells were incubated for 48h without the tested compounds **(A)** or with 1000 μg/ml Immunarc forte **(B)** and Korbazol extract dilution 1:8 **(C)**. The percent of apoptotic and viable cells were determined by analysis of Annexin-V-FITC binding on flow cytometer.

*DNA fragmentation: Analysis using agarose gel electrophoresis*

To determine whether morphological changes observed in B-CLL cells treated with the tested compounds were in agreement with chromatin degradation, DNA extracted from apoptotic cells was electrophoresed on 2% agarose gel. Despite several cases investigated, Korbazol did not induce any visible ladder formation in B-CLL cells. Yet, a substantial amount of high-molecular-weight DNA was present in samples treated with higher doses (Photo 1).

*Korbazol and Immunarc forte are selectively cytotoxic toward B-CLL cells*

Our investigation was aimed to determine potential the cytotoxic effects of Immunarc forte and Korbazol on B-CLL mononuclear cells. As shown in Figure 2A, we found that normal PBMC were resistant to Immunarc forte at all tested concentrations. Next, we observed that Immunarc forte had weak cytotoxic effect on B-CLL mononuclear cells (8% cytotoxicity at 500 μg/ml, 13.0% cytotoxicity at 1000 μg/ml).

B-CLL cells were also sensitive to Korbazol and

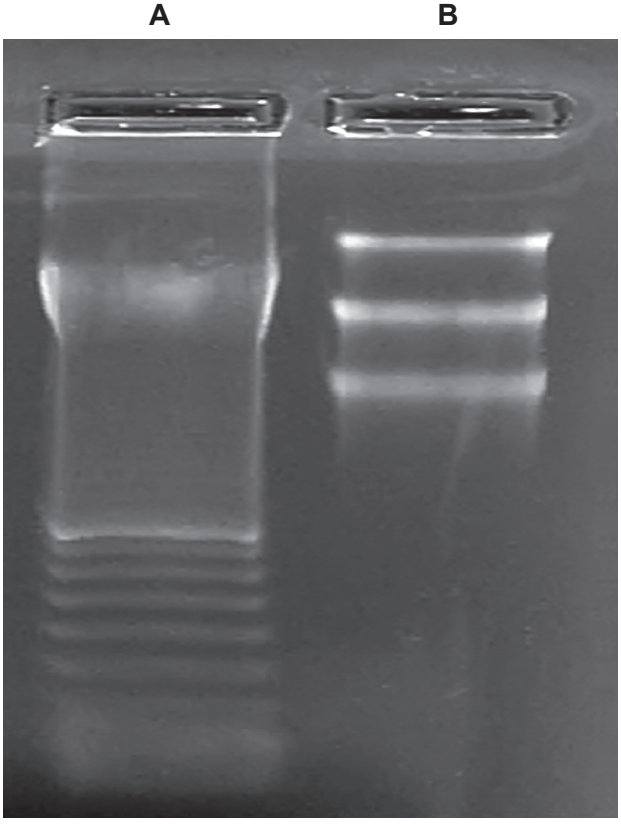


**Photo 1.** DNA electrophoresis of Korbazol-treated CLL cells. Lane **A:** 100 bp molecular weight marker. Lane **B:** CLL cells exposed to Korbazol extract dilution 1:8.

this drug was considerably cytotoxic and showed potent proapoptotic effects on B-CLL cells. This property was dose-dependent (increases in concentration markedly induced apoptosis with concomitant decrease of viable cells). Korbazol demonstrated significant cytotoxic effects (Kruskal-Wallis, $p<0.001$), starting with lower doses (extract dilution 1:128), with maximal cytotoxicity at higher doses: 36.0% at dilution 1:16 (Bonferroni test, $p<0.001$) and 74.2% at dilution 1:8 (Bonferroni test, $p<0.001$). Nevertheless, control PBMC were substantially less sensitive to Korbazol and only the highest concentration of the tested compound showed weakly cytotoxic effects (6.0% at 1:8) (Figure 2B). This finding was confirmed using an additional 40 B-CLL patients (data not shown).

These results unequivocally demonstrated that Korbazol effectively and very selectively induced apoptosis in leukemic cells. To confirm selective effects of Korbazol we compared quantitative parametric data between two study groups (CLL cells and normal PBMC) by application of unpaired t-test and the differences were highly significant (dilution 1:16, t-test; $p=0.016$; dilution 1:8, t-test; $p<0.001$).

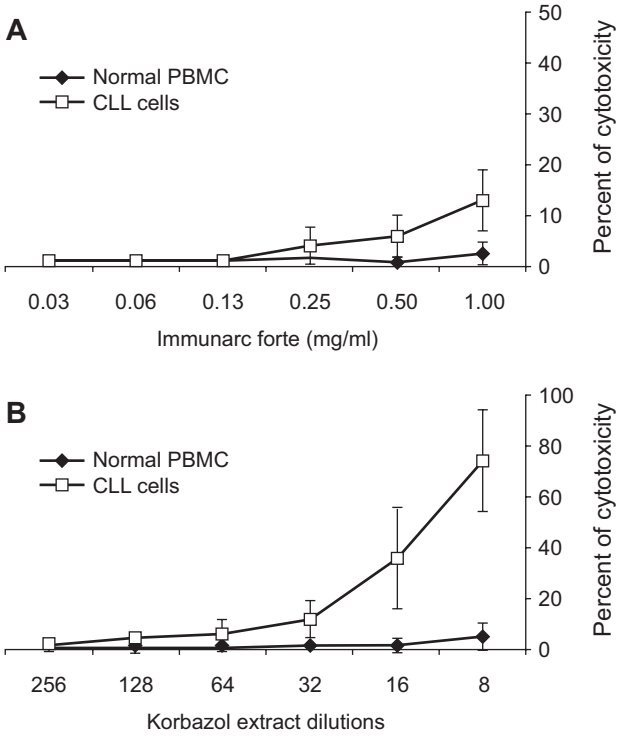


**Figure 2.** Selective toxicity of Immunarc forte and Korbazol toward CLL cells. CLL cells and normal PBMC were incubated for 48h with the indicated concentration of Immunarc forte **(A)** or dilutions of Korbazol extract **(B)**. Cell death was determined by MTT assay. Data are shown as percentages of cytotoxicity. Values represent the mean (+SD) of triplicate samples from 20 different experiments.

*Immunarc forte and Korbazol are synergistic in B-CLL apoptosis*

To examine whether the tested compounds have synergistic effect, B-CLL cells were incubated with optimal and suboptimal concentrations of Immunarc forte and Korbazol and apoptosis was quantified. The synergistic action of these two compounds was constantly demonstrated within a broad range of concentrations (Figure 3). The ability of Immunarc forte to exert great impact on the cytotoxic activity of Korbazol was most clearly evident in a situation when low doses of Immunarc forte, inefficient to induce apoptosis in B-CLL cells, were combined with different doses of Korbazol. Very high levels of B-CLL cells apoptosis (~ 70%) was reached when low dose Immunarc forte (0.12 mg/ml) were used in combination with Korbazol at extract-dilution 1:16, while the same results were obtained with Korbazol alone at 2-fold higher concentration. The difference between the combined vs. single use was statistically significant (One way ANOVA, $p=0.006$). Moreover, the same combinations had little effect on apoptosis of normal PBMC in identical culture conditions (data not shown).

*Effect of synthesis inhibitor on Korbazol-induced B-CLL apoptosis*

To assess whether the apoptotic activity of Korbazol is under the control of active metabolism, B-CLL cells were incubated with cycloheximide at concentrations previously shown to be inhibitory to protein synthesis in the human mononuclear cells. Preincubation for 1h or even 48h coincubation with cycloheximide failed to prevent Korbazol-induced apoptosis in B-CLL cells. Moreover, using drug-free cultures we demonstrated that cycloheximide in fact enhanced/induced rather than abrogated spontaneous apoptosis in B-CLL cells (Figure 4). These

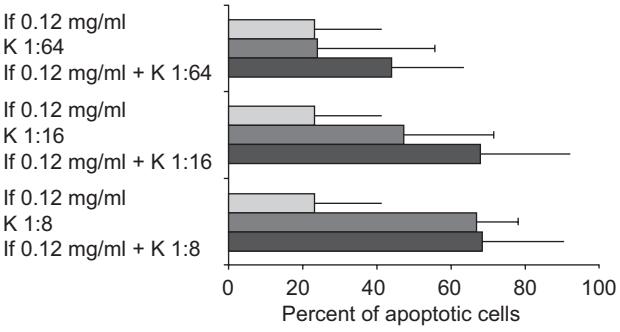


**Figure 3.** Synergism of Immunarc forte and Korbazol in CLL apoptosis. CLL cells were incubated for 48h with Immunarc forte (If) (0.12 mg/ml) and increasing doses of Korbazol (K) (extract dilutions 1:64, 1:16 and 1:8) as indicated in the Figure. Percentages of apoptotic cells are the mean (+SD) from 6 different experiments.

results clearly demonstrate that induction of apoptosis by Korbazol does not require *de novo* protein synthesis, raising the possibility that this compound uses some kind of pre-existing death machinery.

*Korbazol, but not Immunarc forte, decreases spontaneous apoptosis in cultured polymorphonuclear leukocytes*

Contrary to mononuclear cells, peripheral blood-derived polymorphonuclear leukocytes (PMN) do not survive more than 24-48 h *in vitro* without the addition of survival-promoting cytokines. This physiological apoptosis is markedly expressed in cultured PMN which are rapidly undergoing apoptosis, as soon as after 24 h of incubation *in vitro,* when more than 80% of cultured PMN show morphological signs of apoptosis. Thereafter, we investigated the effects of Immunarc forte and Korbazol on spontaneous apoptosis of cultured normal PMN leukocytes.

The percent of apoptotic PMN cultured in medium without the tested products was more than 90% of the total cells. Immunarc forte did not alter spontaneous apoptosis of PMN. Treatment with this compound failed to either protect or induce PMN apoptosis (Figure 5A). On the contrary, the response of PMN to Korbazol was quite different, since this compound decreased spontaneous apoptosis in cultured PMN, demonstrating mild antiapoptotic effect. Whereas viability of PMN cultured for 48 h without the tested compounds was only 6.2%, in the presence of Korbazol viability increased up to 3-fold. The highest increase in viability of PMN was observed at Korbazol extract dilution 1:64 and 1:32 (18.6 and 19.9%, respectively) (Figure 5B). The protective effect was dose-dependent. However, at the highest concentration (dilution 1:16 and 1:8), this effect decreased.

## Discussion

In the present work we examined the apoptotic effects of two different natural products using B-CLL cells and normal mononuclear and polymorphonuclear cells as targets. B-CLL is the most common leukemia in adults and is characterized by accumulation of lymphocytes, most likely as a consequence of their longer survival compared with normal mononuclear leukocytes [45]. CLL is a typical example of malignancy caused by a failure in cell death mechanisms rather than escape from the proliferative control mechanisms. This may be explained, at least in part, by the high levels of the anti-apoptotic protein Bcl-2 found in most B-CLL cells and an associated low expression of the proapoptotic protein

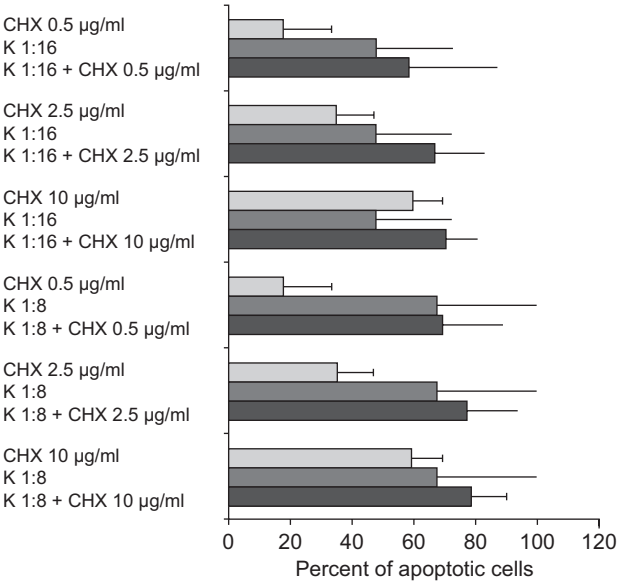


**Figure 4.** Effect of protein synthesis inhibitor on Korbazol-induced CLL apoptosis. Suspensions of CLL cells were incubated for 48h with Korbazol extract (K) dilutions 1:16 and 1:8 in the presence or absence of increasing doses of cycloheximide (CHX) (0.5, 2.5 and 10 µg/ml). Data are shown as percentages of apoptotic cells detected in cultures. Results represent means (+SD) from triplicate samples from 4 experiments.

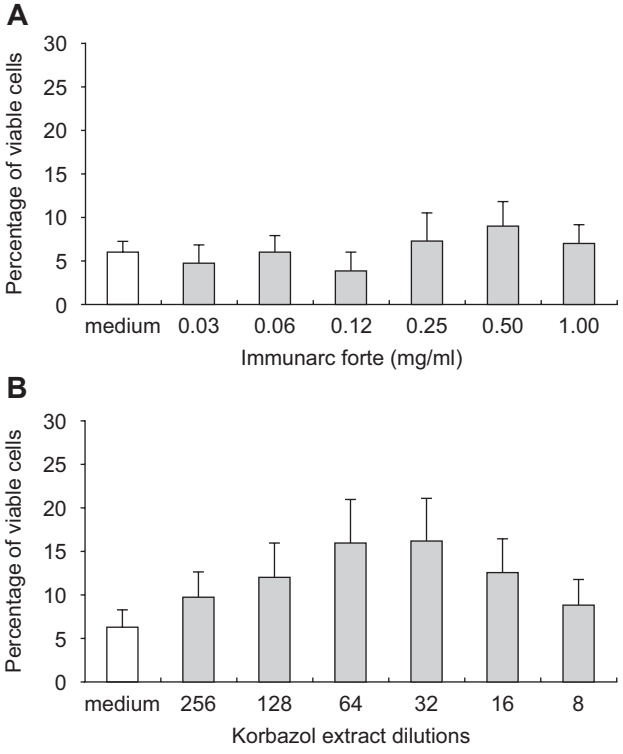


**Figure 5.** Effects of Immunarc forte and Korbazol on spontaneous apoptosis in polymorphonuclear leukocytes. Normal polymorphonuclear leukocytes were cultured in medium without the tested products, or with different doses of Immunarc forte **(A)** or Korbazol **(B)**. Data are shown as percentages of viable cells. Values represent the mean (+SD) of duplicate samples from 10 CLL cases.

Bax, loss of functional p53, mitochondrial defects and prosurvival cytokines in the microenvironment [46,47]. Moreover, accumulation of mutations in apoptosis programs may result in limited benefit of chemotherapeutic drugs and the development of multidrug resistance [48]. As such, novel therapeutic interventions and novel molecular targets for the induction of apoptosis are required. There is an increasing interest in the possible use of natural products for malignant diseases since recent studies have shown an impressive array of tumor inhibitory compounds of plant origin [49].

The results of the present study show that the natural product Korbazol strongly and selectively induces apoptotic killing of CLL cells. The dose-response of apoptosis induction illustrates the marked sensitivity of CLL cells to this agent. However, whereas CLL cells were sensitive to Korbazol, normal mononuclear cells were significantly less sensitive. On the contrary, Korbazol decreased spontaneous apoptosis in cultured normal polymorphonuclear cells and in that way demonstrated protective, antiapoptotic effect.

Immunarc forte induced lower apoptotic response in B-CLL cells compared with Korbazol, but these two compounds synergized in inducing the killing of a significant proportion of B-CLL isolates. However, higher degrees of synergy were observed using low dose Immunarc forte with Korbazol. For example, whereas dilutions 1:8 and 1:16 of Korbazol extract were required to achieve ~70% and ~30% killing of the B-CLL cells, the same level of death was obtained with Korbazol extract dilutions 1:32 and 1:64 when combined with 0.12 mg/ml Immunarc forte, giving a dose reduction index of 2 and 4, respectively. Therefore, co-administration of Immunarc forte may allow substantial reductions in the doses of the cytotoxic drugs required to achieve substantial cell killing. The combined use of several chemotherapeutic drugs is advantageous, because each can have a somewhat different range of toxic side effects, and several relatively minor side effects are more tolerable than a single major toxicity.

Furthermore, our study showed that Korbazol-induced apoptosis is not dependent on *de novo* protein expression. However, the primary mechanism of action and the cellular targets of this agent are under investigation. Some unpublished data suggest that free radicals may be responsible for the observed cytotoxic activity of the tested compounds. Still, in this study we couldn't uncover any changes in nitrogen reactive species production by B-CLL cells after treatment with Immunarc forte or Korbazol (data not shown). In relation to drug composition and preliminary findings, we hypothesized that the probable mechanism of action may be associated with elevations in cytosolic $Ca^{2+}$ and opening of mitochondrial permeability transition (PT) pores leading to release of cytochrome-C and other proapoptotic molecules [50-52].

In conclusion, the studies reported here identify Korbazol as potential therapeutic agent that induces apoptosis of B-CLL cells. The resistance of normal mononuclear cells and antiapoptotic effects on normal polymorphonuclear cells, as well as its ability to synergize with Immunarc forte, suggest that further evaluation may result in therapeutic application of Korbazol in the treatment of B-CLL. Furthermore, the selectivity of Korbazol may provide the basis for new treatment programs against a broad spectrum of human malignancies.

## Acknowledgements

This work was partially supported by the grant 1637 from the Ministry of Science, Republic of Serbia. We thank Dr. Biljana Milicic for statistical expertise. We also thank Milan Milojevic for administrative, technical and logistic assistance.

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