THE CYTOTOXICITY OF KORBAZOL AGAINST MURINE CANCER CELL LINES

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CITOTOKSICNO DEJSTVO KORBAZOLA NA ĆELIJE MIŠJIH TUMORSKIH LINIJA

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ABSTRACT

Background/aim: *Korbazol is a natural product that has been shown to exert cytotoxic effects on leukemic cells in vitro. The cytotoxicity and biochemical effects induced by Korbazol were investigated in the murine cell lines 4T1, B16 and BCL1.*

Methods: *The cytotoxic activity of the tested compound was assessed using a colorimetric MTT assay. The concentration of the superoxide anion radical ($O_2^{\cdot-}$) and the activity of superoxide dismutase (SOD) in the samples were determined using spectrophotometric methods. The MDA content was determined using a TBA assay.*

Results: *We found that Korbazol induced cell toxicity, an increased the concentration of the lipid peroxidation end product MDA, decreased superoxide dismutase activity and increased superoxide anion formation.*

Conclusions: *Altogether, these results suggest that oxidative stress is involved in Korbazol-induced cytotoxicity in the investigated cell lines.*

Keywords: *Korbazol; 4T1; B16; BCL1; apoptosis; oxidative stress*

SAŽETAK

Uvod. *Korbazol je prirodni proizvod koji u in vitro uslovima ispoljava selektivno citotoksično dejstvo na ćelije izolovane iz krvi obolelih od hronične limfocitne leukemije.*

Cilj. *Ciljevi ovog istraživanja su bili da se ispita dejstvo Korbazola na ćelije mišjih tumorskih linija 4T1, B16 i BCL1 i da se utvrde mehanizmi njegovog dejstva.*

Metode. *Za utvrđivanje citotoksičnog dejstva Korbazola korišćen je MTT test. Koncentracija superoksid anjona i aktivnost superoksid dizmutaze utvrđena je spektrofotometrijskim metodama. Koncentracija MDA, krajnjeg produkta lipidne peroksidacije, određivana je pomoću TBA testa.*

Rezultati. *Korbazol na ispitivane ćelijske linije deluje citotoksično, povećava koncentraciju MDA, smanjuje aktivnost superoksid dizmutaze i povećava stvaranje superoksid anjon radikala.*

Zaključak. *Korbazol u ispitivanim tumorskim linijama izaziva ćelijsku smrt indukujući oksidativni stres.*

Ključne reči. *Korbazol; 4T1; B16; BCL1; apoptoza; oksidativni stres*

INTRODUCTION

Our previous studies have demonstrated that Korbazol selectively kills human leukaemia cells (1) by causing endoplasmic reticulum stress (2), most likely through the inhibition of SOD and GPx activity and the accumulation of reactive oxygen species (ROS) (unpublished data).

Free radicals, including ROS, are produced in the body primarily as a result of aerobic metabolism. At physiologic levels, they serve as signalling and regulatory molecules in diverse cellular processes (3, 4), but when in excess, these highly reactive radicals can damage intracellular macromolecules (i.e., DNA, RNA, proteins, and lipids). Under normal circumstances, the level of ROS is controlled by antioxidant enzymes, and a disturbance of this control results in oxidative stress, which may lead to cell death (5). The major intracellular source of ROS is the mitochondria (6), but the endoplasmic reticulum also contributes to the production of ROS (7). A variety of intrinsic and extrinsic insults can cause ER stress, and persistent or excessive ER stress induces an increase in ROS that promotes lipid peroxidation, the perturbation of calcium homeostasis and the activation of several apoptotic pathways (8, 12). Nevertheless, oxidative stress is not always disadvantageous. Many new therapeutic strategies act through the generation of free radicals or by inhibiting cellular antioxidative defences (9, 10, 11, 13).

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Because we found that Korbazol exerts a selective cytotoxic effect on B-cell chronic lymphocytic leukaemia (B-CLL) cells, the objective of the present study was to evaluate the magnitude and mechanism of Korbazol cytotoxicity against solid tumours, specifically murine metastatic breast cancer (4T1), murine melanoma (B16) and murine B cell lymphoma (BCL1). Based on our previous results, the redox status in Korbazol-treated cells was examined.

MATERIALS AND METHODS

Cell culture

The cell lines used in this study were 4T1 (murine metastatic breast cancer), B16 (murine melanoma) and BCL1 (murine B cell lymphoma), all of which were obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium containing 10% foetal calf serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. The culture medium for BCLl was supplemented with 5×10^{-5} mmol/L 2-mercaptoethanol. Cells were incubated in humidified atmosphere containing 5% CO_2 at 37°C and were in logarithmic growth phase at the time of analysis. Exponentially growing cells were cultured with or without Korbazol extract for up to 24 hours. To measure MDA concentration and superoxide dismutase activity, cell suspensions were frozen at -70°C and lysed by three freeze-thaw cycles just prior to analysis.

Tested compound

The natural product Korbazol (Biofarm Group, Serbia, SCG), which has been registered as dietary supplement (Department of Preventive Medicine, MMA, Belgrade, SCG: Korbazol HA108/05), contains plant extracts (*Echinacea purpurea, Paullinia cupana*), micronised zeolite, pollen, and propolis, preserved in honey (Table 1). To produce the extract, 3.2 g of Korbazol was dissolved in 10 ml of water/5% DMSO (Merck), and the water soluble fraction of Korbazol was filtered through a nitrocellulose filter (Millipore, USA). The sterile extract was stored at -20 °C .

MTT cell viability assay

The viability of cultured cells was determined by assaying the reduction of MTT to formazan. In brief, cells were plated at a density of 5×10^3 cells/well into 96-well plates and allowed to grow overnight. Then, cells were treated with different dilutions of Korbazol extract (1:8, 1:16, 1:32 and 1:64) or cultivated in medium alone (control). After 24 h of incubation at 37°C in an atmosphere containing 5% CO_2 and absolute humidity, the culture medium was removed, and MTT (0.5 mg/1 ml of PBS) was added to each well. The cells were then incubated at 37 °C for 4 h, and DMSO (100 µl/well) was added to dissolve the formazan crystals. Absorbance was measured at 590 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria).

KORBAZOL (250G)
Guarana (liquid extract) 1,19ml
Propolis (dry extract) 595,24mg
Pollen P2 1071,43mg
Echinaceapurpurea 1190,48mg
Honey ad 250g

Table 1. Composition of Korbazol.

MDA determination

The MDA content was determined by the TBA assay as described by Ohkawa et al. (14). Briefly, 4T1, B16 or BCL1 cells (10^6) were incubated with or without Korbazol extract (dilution 1:8) at 37°C in an atmosphere containing 5% CO_2 and absolute humidity. After 24 h, cells were harvested, and the concentration of the lipid peroxidation end product MDA in the cell lysates was measured. The thiobarbituric acid reactants (TBARs) concentration was measured using a spectrophotometer (LKB Biochrom Ultrospec 4050, Cambridge, UK) and expressed as nM/10^6 cells (14).

Determination of superoxide anion production

The concentration of the superoxide anion radical ($O_2^{\cdot-}$) in the sample was determined spectrophotometrically based on the reduction of nitroblue tetrazolium (NBT) to nitroblue-formazan in the presence of $O_2^{\cdot-}$ (15). Briefly, cells were plated at a density of 10^5 cells/well into 96-well plates. After overnight growth, cells were treated with 100 µl of Korbazol extract (dilution 1:8) or cultivated in medium only (control) for 24 h. At the end of the treatment, 100 µl of 0.01% NBT was added to each well and incubated for 1 hour at 37°C in culture hood. Then, 50 µl of 2 M KOH and 50 µl of DMSO were added, and absorbance (A) was read at 590 nm. The superoxide anion concentration was calculated according to relevant mathematic formulas and expressed as nM/ml (15).

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was estimated in cell lysates using Ransod and Ransel kits, supplied by Randox Laboratories, Ardmore, Northern Ireland, UK, according to the manufacturer's instructions. This assay uses xanthine and xanthine oxidase to generate superoxide anions that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction. For this assay, 4T1, B16 or BCL1 cells (10^6 cells) were incubated with or without a 1:8 dilution of Korbazol extract for 24 h at 37°C in an atmosphere containing 5% CO_2 and absolute humidity. The activity of SOD was determined in cell lysates and expressed as U/mg of proteins.

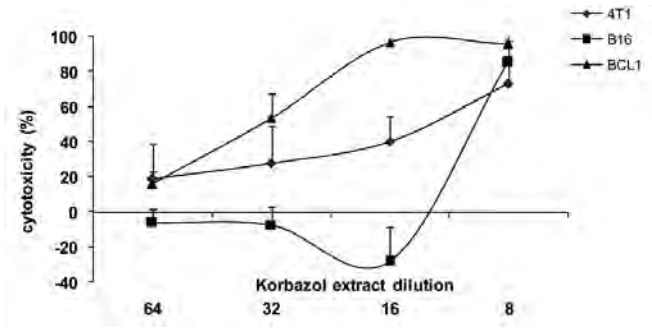

Figure 1. Korbazol-induced cytotoxicity in cancer cell lines. Cells (5×103) were incubated for 24 h with the indicated dilution of Korbazol extract. Cell death was determined by MTT assay. The data are expressed as percentages of cytotoxicity. The values indicated represent the mean (±SD) of triplicate samples from five different experiments.

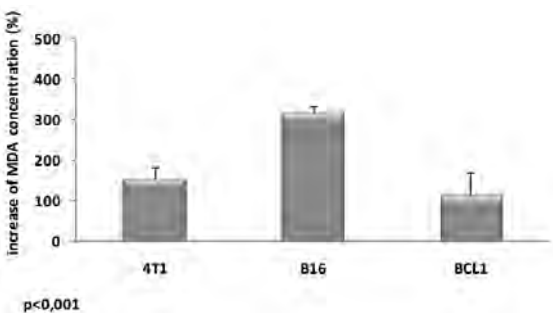

Figure 2. Lipid peroxidation induced by Korbazol. 4T1, B16 and BCL1 cells (106) were incubated with or without Korbazol extract (1:8 dilution) and harvested after 24 h. Cells were lysed by three freeze-thaw cycles. The concentration of the lipid peroxidation end product MDA in cell lysates was determined by thiobarbituric acid assay. The data are expressed as per cent increase in MDA concentration in treated cells relative to the controls. The values represent the mean (±SD) of three different experiments.

Data analysis and statistics

The data were expressed as the mean ± SD. The distributions of data were evaluated for normality using the Kolmogorov-Smirnov test. Quantitative parametric data were compared between two study groups using the unpaired t-test. The Mann-Whitney test was used to compare nonparametric data between two groups. The Kruskal-Wallis test was used for comparisons of nonparametric data between more than two groups. One-way ANOVA was performed to compare parametric data between more than two groups. When ANOVA indicated significant differences, the Bonferroni test was used to identify intergroup differences. All statistical analyses were carried out with commercial statistical software (SPSS version 13.0; SPSS Inc., Chicago, IL). P values less than 0.05 were considered significant.

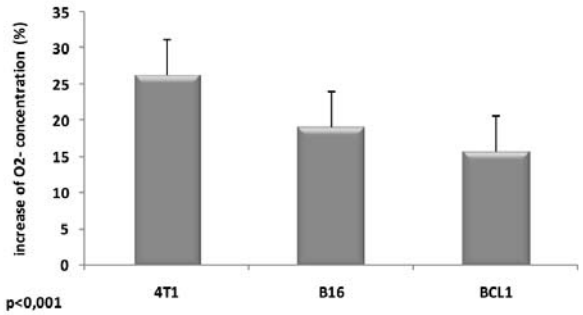

Figure 3. Korbazol induces superoxide anion accumulation. Murine 4T1, B16 and BCL1 cells (105) were incubated with or without Korbazol extract (1:8 dilution) for 24 h. The concentration of the superoxide anion radical (O2.-) in the sample was determined by spectrophotometric method based on the reduction of nitroblue tetrazolium (NBT). The data are expressed as the per cent increase in O2.- concentration in treated cells relative to the controls. The values represent the mean (±SD) of six different experiments.

RESULTS

Korbazol induces cytotoxicity in tumour cell lines

Cell lines were incubated with decreasing dilutions of Korbazol extract (1:64, 1:32, 1:16 and 1:8) or medium alone (control) for 24 h and analysed by the MTT assay. As shown in Figure 1, Korbazol demonstrated a noticeable cytotoxic effect on all three cell lines, enhancing apoptosis in a dose-dependent manner. In the cell line 4T1, all dilutions of Korbazol extracts showed notable cytotoxic effects (73% at 1:8, 40% at 1:16, 28% at 1:32 and 18% at 1:64). Korbazol showed an even stronger effect on BCL1 cells: 96% cytotoxicity at 1:8, 97% at 1:16, 53% at 1:32 and 16% at 1:64. In contrast, only the highest concentration of Korbazol tested (1:8 dilution) induced cell death in cell line B16 (85%); lower concentrations had no cytotoxic effect on this cell line.

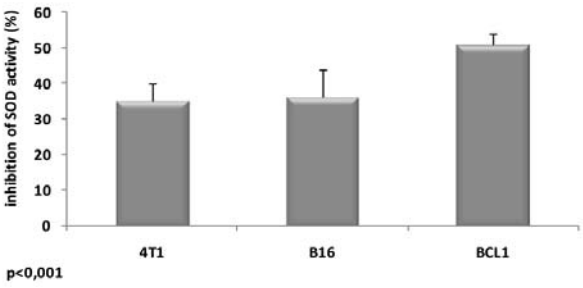

Figure 4. SOD activity is decreased in Korbazol treated cells. Cells (106) were treated with Korbazol extract (1:8 dilution) or cultivated in medium alone (control) for 24 h. SOD activity was estimated in cell lysates using Ransod and Ransel kits. The data are expressed as per cent inhibition of SOD activity in treated cells relative to controls. The values represent the mean (±SD) of three different experiments.

Korbazol treatment induces oxidative stress

To test whether the Korbazol treatment may have caused oxidative stress, the lipid peroxidation end product

malondialdehyde (MDA) was measured. As shown in Fig. 2, a two-fold increase of MDA concentration was noted in Korbazol-treated 4T1 and BCL1 cells, and a three-fold higher MDA concentration was measured in B16 cells after treatment. Specifically, the concentration of MDA (expressed per million cells) increased from 11,3 nM to 28,8 nM in 4T1 cells (155% increase), from 13,3 nM to 28,4 nM in BCL1 cells (114% increase) and from 11,8 nM to 49,1 nM in B16 cells (317% increase). The increase was statistically significant in all cell lines ($p<0,001$).

Korbazol induces superoxide anion production

To investigate the cause of the observed oxidative stress, cellular $O_2^{\cdot-}$ content was determined in untreated control cells and after incubation with Korbazol, and the per cent increase in superoxide anion concentration was calculated. As shown in Figure 3, treatment with Korbazol induced a statistically significant increase ($p<0,001$) in $O_2^{\cdot-}$ formation: 26% in 4T1 cells (from 226,2 nM/ml to 285,4 nM/ml), 19% in B16 cells (from 214,7 to 255,6 nM/ml) and 16% in BCL1 cells (from 219,5 to 237,1 nM/ml).

SOD activity is affected by Korbazol treatment

Because SOD is the key enzyme involved in the metabolic elimination of $O_2^{\cdot-}$, we measured superoxide dismutase activity after treatment with Korbazol and in untreated cells. We noted a 35% inhibition in 4T1 breast cancer cells (activity of SOD declined from 4,720 U/mg proteins in untreated cells to 3,055 U/mg protein after treatment), 36% in B16 melanoma cells (from 5,450 to 4,028 U/mg protein) and 51% in BCL1 cells (from 4,921 to 2,416 U/mg protein).

DISCUSSION

The interest in anticancer agents from natural sources has increased in recent years. Some active constituents have been isolated and used to treat human tumours (16-20). The study of cytotoxic properties of new compounds and selective action is required as a first step. The natural product Korbazol has been shown to strongly and selectively induce apoptosis in CLL cells (1). Therefore, the aim of the present study was to elucidate the effect of Korbazol on other types of cancer.

In this report, we describe the *in vitro* cytotoxic activity of Korbazol against three murine cell lines. Our data showed that Korbazol exerts cytotoxic effects in all three cell lines. The increase in the concentration of MDA markedly increased the percentage of dead cells. The precise mechanism responsible for the cytotoxic effect of Korbazol is not still thoroughly understood, although our most recent results point to oxidative stress as the main mechanism in inducing apoptosis in CLL-lymphocytes (unpublished data). Therefore, we wished to determine whether the same mechanism was engaged in other types of cancer cells. We first measured the concentration of MDA, as the end product of lipid peroxidation and an indicator of oxidative stress. We found a significant Korbazol-induced increase in MDA production for all cell lines ($p<0.001$). Because an increase in reactive oxygen species was found in our previous study on human CLL cells, we also determined the superoxide anion concentration. $O_2^{\cdot-}$ was elevated in Korbazol-treated 4T1, B16 and BCL1 cells ($p<0,001$). In both plant and animal cells, the first line of defence against oxygen-derived free radicals is superoxide dismutase, which catalyses the dismutation of the $O_2^{\cdot-}$, thus contributing to a decrease in oxidative reactions. The inhibition of SOD leads to the accumulation of $O_2^{\cdot-}$. Indeed, we found that treatment with Korbazol significantly ($p<0.001$) inhibited SOD activity in all examined cell lines.

Changes in cellular ROS status have been shown to play an important role in apoptotic cell death. These observations also suggest that it may be possible to damage cancer cells by increasing their free radical contents through the inhibition of antioxidative enzyme activity. The results of our research on different types of cancer cells reveal oxidative stress as a candidate mechanism by which Korbazol exerts its cytotoxic potential. Its activity on other metabolic pathways remains to be elucidated.

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