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ORIGINAL RESEARCH

Cytotoxic and apoptotic effects of boron compounds on leukemia cell line

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Abstract In this study we investigated the effects of boric acid and sodium tetraborate on an acute leukemia cell line and healthy human lymphocytes. We evaluated the effects of boric acid and sodium tetraborate on the HL-60 cell line and healthy human lymphocytes by using the methods of MTT, Neutral Red, AO (flow cytometry) and transmission electron microscope. We found that there were dead cells at a concentration of 500 μM boric acid and sodium tetraborate (50 % and 40 %, respectively). An apoptotic effect was found at a concentration of 1,000 μM concentration in normal lymphocytes and HL-60 (acute leukemia cells) cells (2.5 % and 8.8 % respectively). We observed that boric acid at a concentration of 500 μM caused double nucleus and micronucleus formation in both HL-60 cells and lymphocytes. An expansion in mitochondrial dimension and deformation in cristas also appeared. Our findings suggest that boric acid is more effective than sodium tetraborate on the HL-60, and boric acid in particular showed a cytotoxic effect on HL-60 in comparison to healthy lymphocytes and it also affected the mitochondrial pathway.

Keywords Boron · HL-60 · Flow cytometry · Transmission electron microscopy

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Introduction

Acute myeloid leukemia (AML), a fast-growing cancer of the blood and bone marrow, is a heterogeneous clonal disease characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells, typically leading ultimately to fatal infection, bleeding, or organ infiltration within 1 year of diagnosis in the absence of treatment (Estey and Döhner 2006). Boron compounds are very effective against leukemia cells, breast cancer cells, lung cancer cells, prostate cancer cells and ovarian cancer cells (Yang et al. 2003). As such, interest stems from the tremendous importance of boronic acid in the synthesis of biologically active compounds and the use of boronic acid itself as pharmaceutical agent (Chapin and Ku 1994).

The incorporation of boron in some boron compounds imparts antitumor properties to different cancer cell lines. There are experiments in which boron compounds work as a proteasome inhibitor in cancer cells (Bone and Ashok 1987). The proteasome

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is a eukaryotic cytoplasmic protease complex that has several distinct catalytic sites. It plays a major role in cellular pathways for the breakdown and processing of proteins to peptides and amino acids. Using the same strategy, a series of tri- and dipeptidyl boronic acid analogues were designed based on the replacement of the corresponding aldehyde function of previously reported proteasome inhibitors (Bone and Ashok 1987). Boron is known to be important for animal cell replication and development, but the underlying mechanism remains obscure (Eckhert 1998). Boric acid has also been reported to inhibit (with 64 % less likelihood of developing prostate cancer compared to men who consumed the least amount of boron) the growth of LNCaP prostate tumors in nude mice (Gallardo-Williams et al. 2004). Gallordo-Williams et al. indicated that mice receiving 1.7 or 90 mg/kg/day of boric acid solution orally showed a decrease in tumor size by 38 % and 25 %, respectively (Gallordo-Williams et al. 2003). Two new boron compounds, dihydroxy boron hydrochloride monohydrate and guanidine biboric acid adduct were observed to have an antitumor effect (Murmu et al. 2002). Boric acid has been shown to be nongenotoxic and to support antioxidant enzyme activities in human blood cultures (Turkez et al. 2007). Hepatoprotective effects of boric acid were observed in a study conducted by the same researchers (Turkez et al. 2011). Recently, as the effects on malignancies have been revealed, attention has been focused on boron compounds and is gradually increasing.

In this study, we investigated the effect of boric acid and sodium tetraborate on healthy human lymphocyte cells and the HL-60 (human promyelocytic leukemia cells) cell line via MTT assay, neutral red (NR) assay, flow cytometry analysis with acridine orange (AO) staining, and transmission electron microscope.

Materials and methods

Cell culture

Peripheral mononuclear cells were isolated from heparinized peripheral blood obtained from a healthy donor by density gradient centrifugation (30 min–700G) with ficoll (Sigma-Histopaque 1077). Cells were washed twice with PBS. Lympocytes were cultured at 37 °C in an incubator containing 5 % CO_2 in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA) (pH 7.4) medium supplemented with 10 % heat-inactivated fetal calf serum (Biochrom, Berlin, Germany) and 1 % penicillin–streptomycin (10,000 U/ml and 10 mg/ml, respectively) (Biochrom), 1 ml phytohemaglutinin (Biochrom).

HL-60 cells were obtained from the laboratory of Dr. Ayhan Bilir (Department of Histology and Embryology, Faculty of Medicine, Istanbul University, Istanbul, Turkey), and continuous suspension cultures in RPMI-1640 medium supplemented with 10 % fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in 5 % CO_2 incubator were performed. Viability of cells was determined by using the trypan-blue exclusion. Healthy human lymphocytes and HL-60 cell lines were treated with boric acid and sodium tetraborate for 4 days (Wade and Curtis 2004; Freshney 1994). Mitochondrial and lysosomal activities were evaluated with MTT and NR methods.

MTT assay (Mitochondrial activity)

This is a colorimetric assay that measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,4,diphenyltetrazolium bromide (MTT) by mitochondria (Karp 1999). Boron compounds were dissolved in sterile distilled water, and the concentration-dependent cytotoxic effect was studied (100, 250, 500, 1,000 µM boric acid and sodium tetra borate). 100 µl of cell suspension (lymphocytes and HL-60 cells) and boron compounds were briefly seeded in a 96-well plate. Then, after 24 h of incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and plates were incubated for further 3 h (Mosmann 1983). The formazan crystals produced, which are converted with dye, are solubilized with 1 ml acidic isopropanol (0.04 M HCl). The culture plate was placed on a Biotek (Winooski, VT, USA) Model (Elx808) microplate reader and the absorbance was measured at 570 nm. This process was repeated for a total time of 4 days.

Neutral red assay (Lysosomal Activity)

The assay involves the uptake of a vital stain, NR. Most cells will accumulate NR in lysosomes. The process requires intact membranes and active metabolism of the cell (Holsten-Hansten and Brünner 1998 and Horakova et al. 2001). 100 µl of cell suspension and boron compounds were seeded in a 96-well plate. After 24 h of incubation, 10 µl NR (0.5 % solution)

was added to each well and the plates were incubated for further 3 h. Destaining process was performed using 1 % glacial acetic acid, 49 % dH_20 and 50 % ethanol. The culture plate was placed on a Biotek (Winooski, VT, USA) Model (Elx808) microplate reader and the absorbance was measured at 570 nm. This process was repeated for a total time of 4 days.

Flow cytometry analysis with acridine orange

AO is a nucleic acid selective metachromatic stain useful for cell cycle determination. AO interacts with DNA and RNA by intercalation or electrostatic attraction, respectively. AO may also be useful as a method for measuring apoptosis. For flow cytometric measurements, 3×10^5 lymphocyte and HL-60 cell line were pelleted. 200 µl AO solution A (10 ml 2 N HCl, 4.4 g NaCl, 0.5 ml Triton-X-100, 480 ML distilled water) and solution B (7.77 g citric acide, 17.89 g sodium phosphate, 0.37 g EDTA, 8.7 g NaCl, 1 l distilled water, 0.6 ml AO dye stock solution) were added to the cell suspension. Cells were incubated in an ice pack for 10 min. Then the 3×10^5 cells of each cell line and lymphocyte were analyzed with flow cytometry. The flow cytometric data acquisition was conducted by using FACS Calibur (Becton Dickinson, San Jose, CA, USA). The hypodiploid cells were identified as apoptotic from the histogram of FL-2-height (Darzynkiewicz et al. 1992).

Transmission electron microscopy (TEM)

To examine ultra-structural changes lymphocytes and HL-60 cell lines were collected with centrifugation of 1,200 rpm for 10 min and were immediately fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 4 h. The samples were then taken into sodium phosphate buffer pH 7.4 for 15 min. The cell pellets were post-fixed in 1 % osmium tetroxide in phosphate buffer (pH 7.2) for 1 h. The samples were dehydrated in a series of graded concentrations of ethanol and embedded in pure araldite (Bozola and Russel 1999). Thin sections were cut and stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (Philips BioTwin G2 Spirit) at 300 kV.

Statistical analysis

Proliferation (MTT and NR) values were compared by ANOVA, followed by Tukey's b tests for the determination of statistical differences.

Results

MTT assay (Mitochondrial activity)

It was observed that boric acid and sodium tetraborate caused a decrease in the mitochondrial activity by means of MTT methods applied to healthy human lymphocytes at the highest concentration 1,000 µM (20 % and 25 %, respectively; Fig. 1A, B). There was also a decrease in the mitochondrial activity of HL-60 cells in boric acid and sodium tetraborate (50 % and 20 %, respectively; Fig. 1A, B). Especially, the cell viability of HL-60 cells was significantly decreased at a concentration of 100 µM for an incubation period of 48 h ($p < 0.001$)

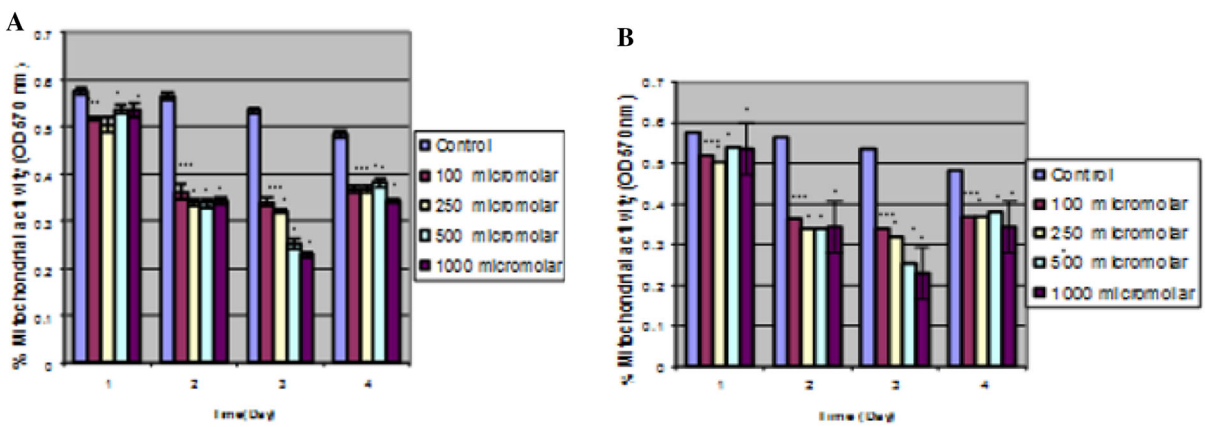

Fig. 1 Effects of boric acid (**A**) and sodium tetraborate (**B**) on mitochondrial activity (MTT-test) (n=8; $p < 0.05$*, $p < 0.01$**, $p < 0.001$***)

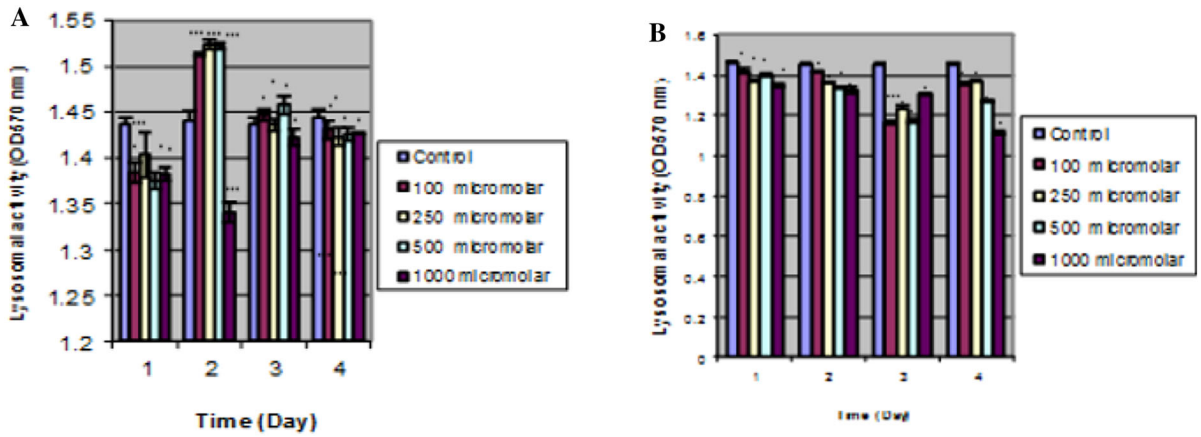

Fig. 2 Effects of boric acid (**A**) and sodium tetraborate (**B**) on lysosomal activity (Neutral Red-test) (n=8, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***)

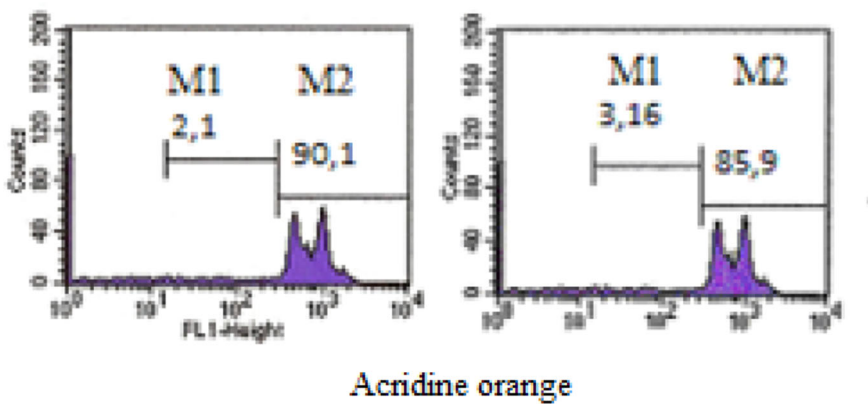

Fig. 3 The apoptotic effects of boric acid (left diagram) and sodium tetraborate (right diagram) on healthy human lymphocytes when using acridine orange dye staining (M: Apoptotic cell; M2: Viable cell)

Neutral red assay (Lysosomal Activity)

When boric acid was applied to the healthy human lymphocytes with the NR method related to the increase of the concentration, a decrease occurred in the lysosomal activity. On the other hand, there was hardly any difference between the controls and different concentrations of sodium tetraborate for 24, 48 h. Using the Neutral Red method it was observed that boric acid applied at concentrations of 100, 250 and 500 µM on HL-60 cells brought about an increase in the lysosomal activity; however, a 4% decrease was noticed when using sodium tetraborate for 24, 48 h ($p < 0.001$) (Fig. 2A, B).

Flow cytometry analysis with acridine orange

Healthy human lymphocytes and HL-60 cells were cultured for 24 h with boric acid and sodium tetraborate at concentrations 1000 µM. By using acridine orange dye in the flow cytometry, the apoptotic effects of boric acid and sodium tetraborate on healthy human lymphocytes were 2.1 % and 3.16 %, respectively. With respect to HL-60 cells the apoptotic effects of boric acid and sodium tetraborate were 8.8 % and 4.9 %, respectively (Figs. 3, 4). According to this comparative test boric acid had a four times higher apoptotic effect on HL-60 cells than on human lymphocytes.

Transmission electron microscopy

According to the results obtained by the application of boric acid on healthy human lymphocyte and HL-60 cells, as well as obtained via transmission electron microscopy (TEM), it was observed that a concentration of boric acid beyond 500 µM induced the formation of micronuclei and double nucleus in healthy human lymphocyte and leukemia cells. In

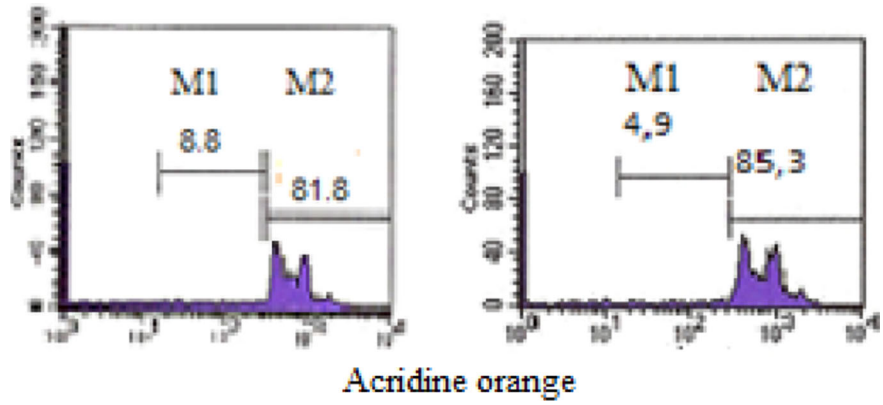

Fig. 4 The apoptotic effects of boric acid (left diagram) and sodium tetraborate (right diagram) on HL-60 cells when using acridine orange dye staining (M1: Apoptotic cell; M2: Viable cell)

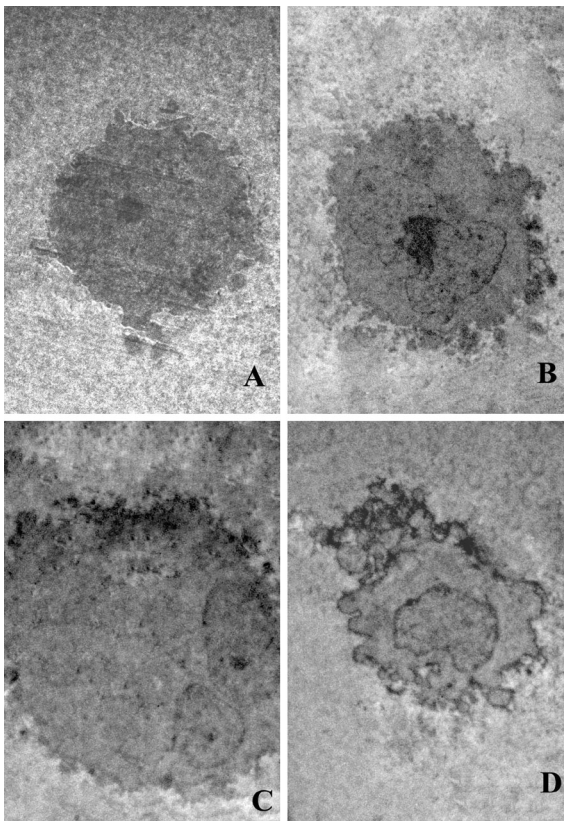

Fig. 5 Effects of boric acid on HL-60 cell line. **A** Untreated group (×2550) **B** addition of 250 µM boric acid (×6000) **C** addition of 500 µM boric acid (×2500) **D** addition of 1000 µM boric acid (×2500)

addition, it was found by applying sodium tetraborate that mitocondria were the most damaged among all organelles, and also that the endoplasmic reticulum lost its integrity. In the literature there is no study describing a similar case (Figs. 5, 6).

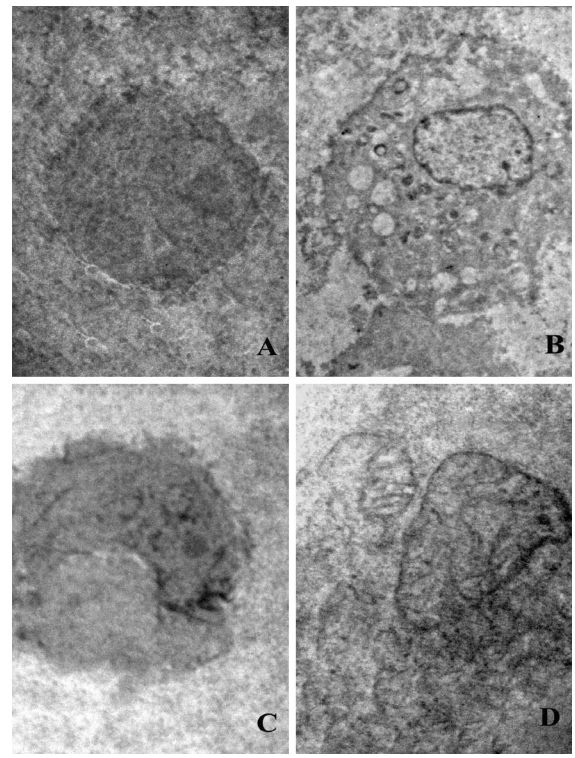

Fig. 6 Effects of sodium tetraborate on HL-60 cell line. **A** Untreated group (×2550) **B** addition of 250 µM sodium tetraborate (×6000) **C** addition of 500 µM sodium tetraborate (×2500) **D** addition of 1000 µM sodium tetraborate (×2500)

Discussion

Some boron compounds have antitumor properties against different cancer cell lines. The cytotoxic agents used in this study were chosen for their usefulness in anticancer effects. Boron compounds are very effective against leukemia cells, breast cancer

cells, lung cancer cells, prostate cancer cells and ovarian cancer cells (Yang et al. 2003). There is no article that considers the evaluation of boric acid and sodium tetraborate on healthy lymphocytes in terms of the mitochondrial and lysosomal activity.

However, the same concentrations were used in the study on prostate cancer cells. It was detected that there was a decrease in the mitochondrial activity at a rate of 29 % at 500 μM; 25 % at 1,000 μM, and therefore, there is a close similarity between this study and our results (Fig. 2; Zhang et al. 2001). It is possible to find studies done using different boron compounds and different cell groups in the literature. Using guanidine biboric acid, dihydroxy boron hydrochloride monohydrate and hydroxo salicyl hydroxomate boron derived from new boron compounds, an antineoplastic effect was found on HL-60 and U-937 cell lines in that study. In another study on HL-60 cell line using guanidine biboric acid, an IC50 value of 2 mg/ml was obtained (Murmu et al. 2001). In a study using B16F10 murine melanoma phenyl boronic acid nanoparticles have been shown to have cytotoxic effects on cells (Deshayes et al. 2013). In a study on the effects of Borato-1,2-diaminocyclohexane platinum (II) on murine L1210 leukemia cells, DU 145 cells, A549 and MCF-7 cells (Dibas et al. 2000) observed a cytotoxic effect of 97 % cytotoxic effect at a concentration of 15 μM. There have not been many studies on the effects of boron compounds on HL-60 cell lines and healthy human lymphocytes. Boron compounds have an apoptotic effect on cancer cells that proliferate randomly and that have a damaged apoptosis mechanism. Apoptotic cells are also characterized by cell shrinkage, cytoskeletal disruption, membrane blebbing, chromatin condensation and ordered cleavage of DNA (Bursh et al. 1992).

The present study shows that boric acid especially has a cytotoxic effect on HL-60 cells compared with normal lymphocytes and this effect occurs via the mitochondrial pathway. We have shown that boron compounds have effects on HL-60 cell lines. There is hope that the use of drug Bortezomib, made from a boric acid polymers, will be initiated, in particular, for the treatment of multiple myeloma cells. Our study is essential for the use of new treatment options, those deploying boron compounds, for the treatment of acute leukemia. Further studies on different leukemia cell lines and "leukemic patients" cells are necessary.

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