Research Article

An *in Vitro* Study in Which New Boron Derivatives Maybe an Option for Breast Cancer Treatment

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Abstract

Objectives: We aimed to investigate the distribution of immunoreactivities of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), and inducible NOS (iNOS) on breast cancer cells in response to treatment with boron derivatives.

Methods: We initially analyzed the cytotoxic effect and IC50 value of boron by MTT assay. For the evaluation of the angiogenesis, expression level of antibodies was detected to following boron derivatives such as boric acid, boron penta (BP), and T-Boron (DPD) in the absence of boron treatment using the indirect immunohistochemical method. The evaluation of these staining was done using the H-scoring system.

Results: It was found that immunoreactivities of VEGF, eNOS, and iNOS increased on control compared to those of the cells of MDA-MB231 human breast cancer cell line. Following boron derivatives treatment, it was observed that they were inhibited the VEGF/NOS labeling in MDA-MB-231 breast cancer cells.

Conclusion: The present data suggest that BP, especially DPD, inhibits the angiogenesis of breast cancer cells through VEGF pathway. From this point, these boron derivatives may provide a novel therapeutic approach for breast cancer treatment.

Keywords: Boron penta, DPD, Immunohistochemistry, iNOS, MDA-MB-231

Breast cancer is the most common cancer among women and is the second leading cause of cancer deaths in women.[1] The agents against breast cancer, the effects of the molecular mechanisms related to the cell proliferation, and survival are tried for the treatment.[2]

Boron compounds are now subject to study due to their possible beneficial effects on human health.[3] There is increasing evidence that leads to the hypothesis that boron has anticarcinogenic properties. In recent studies, it has also been reported that the use of boron-based compounds as anticancer agents has increased, especially in inoperable cancers, and those with high malignancy.[4] However, the

mechanisms that underlie the observed antitumorigenic effects of boron are not clearly known. There are previous studies that promote boron as a chemopreventative agent for prostate cancer,[5] but its effect on MDA-MB-231 human breast cancer cells remains obscure. The boron-soluble forms include boric acid (BA-H_3BO_3), borax pentahydrate or boron penta (BP-$Na_2B_4O_7.5H_2O$), and disodium pentaborate decahydrate or T-Boron (DPD-$Na_2O.5B_2O_3.10H_2O$, BOREN; National Boron Research Institute, Ankara, Turkey). On the other hand, the effect of BA, BP, and T-Boron on angiogenesis and oxidative stress is not known.

Angiogenesis, one of the major hallmarks of cancer, is in-

volved in pathological processes such as tumor growth and metastasis.[6] In angiogenesis, normal recruitment of endothelial progenitor cells is converted to the pathologic sprouting of vessels.[7] There are some cytokines and growth factors secreted by many cancer cells. Among these factors, vascular endothelial growth factor (VEGF), one of the major factors that initiate and regulate angiogenesis[8, 9] and is known as closely associated with increased aggressiveness and metastasis.[10, 11] VEGF promotes endothelial cell migration, proliferation, differentiation,[8, 9] and, in this way, regulate the angiogenesis. Therefore, it has triggered intensive research on antiangiogenic therapeutic modalities.[12] Clinical studies have indicated that low levels of VEGF promote survival and increased response to treatment in advanced breast cancer.[13, 14]

Excluding the VEGF, tumor-associated angiogenesis is in part controlled by NO synthase (NOS) pathway. NO is produced by three different isoforms of NOS: Neuronal, nNOS/NOS1; inducible, iNOS/NOS2; and endothelial, eNOS/NOS3.[15] NO is a bioactive molecule and it may also increase proliferation, chemoresistance, angiogenesis, and immunosuppression and enhance tumor growth.[16, 17] The overexpression of eNOS and especially iNOS is common in breast cancer tumors. More recent data have shown that iNOS is associated with poor outcome in patients with breast cancer by increasing tumor aggressiveness.[17, 18] Due to all these reasons, inhibition of both mechanisms is required for the regression of breast cancer cells.

The aim of our study was to investigate whether boron has anticarcinogenic, antiangiogenic, and antioxidative effects on breast cancer cells. Therefore, the level of antiangiogenic and antioxidant markers for MDA-MB-231 cells was determined under the effect of three boron derivatives.

Methods

Cell Culture and Reagents

MDA-MB-231 breast cancer cells were purchased from European Collection of Authenticated Cell Cultures (Cat No: 92101203 LOT: 08A006 p. 18). Cells were grown in RPMI-1640 (R8758 Sigma Chemical Co., St. Louise, Missouri, USA) medium supplemented with 10% fetal bovine serum (FBS) (F9665, Sigma Chemical Co., St. Louise, Missouri, USA), 1% l-glutamine (G7513-Sigma Chemical Co., St. Louise, Missouri, USA), and, additionally, 1% penicillin/streptomycin (P4333-Sigma Chemical Co., St. Louise, Missouri, USA) under routine cell culture conditions (37°C, 5% CO_2, and 100% humidity) and passaged every 2–3 days.

Sterilized 12 mm diameter circular cover glasses were placed in 24-well plates and 500 µl FBS was added to each well of the plate. After aspiration of the FBS, cells were plated and allowed to grow under standard conditions. The culture system of MDA-MB-231 cells and immunohistochemical method is described by Uluer et al.[19]

Cell Growth Assay

MDA-MB-231 cells (15×103) were seeded in 6-well plate in triplicate and maintained in normal growth medium. The number of cells for experimental and control groups was counted using the trypan blue exclusion method and quantified with a Thoma plate at 24 and 72 h. Doubling time was counted as 24 h. The cells were incubated with BA, BP, and DPD after 24 h.

MTT assay [3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide]

MDA-MB-231 cells (1×103) were seeded in 96-well plate and maintained in normal growth medium. MTT analysis (Cell Proliferation Kit I Roche) was carried out and cell growth at different days in different concentrations of boron derivatives was measured using a Model 680 Microplate Reader S/N 17795[20] by Korkmaz et al.[21] in a previous study. As a result of the analysis, the doses and the time period with the highest significance were determined as 1000 µM and 7 days, respectively. MDA-MB-231 cells were designed as control; BA (1000 µM), BP (1000 µM), and DPD (1000 µM).

Immunohistochemistry

According to MTT results, following experiments were carried out using these doses for 7 days in IHC. After drug treatment, the cells were fixed at 4°C for 30 min for analyses. The fixative solution was 4% paraformaldehyde in a phosphate-buffered saline solution (PBS). Then, washed with PBS and permeabilized with 0.1% Triton X-100 (T8787 - Sigma Chemical Co., St. Louise, Missouri, USA) at 4°C for 15 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min. Subsequent washing with PBS 3 times, cells were incubated with monoclonal primary antibodies: Anti-VEGF (sc-7269, Santa Cruz Biotechnology), anti-eNOS (sc-654, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-iNOS (sc-651, Santa Cruz Biotechnology) dilution of 1:100, at 4°C for overnight in a humidified chamber. Thereafter, removal of the primary antibody and 3 times wash with PBS, the secondary antibodies (85–9043, Invitrogen, Camarillo, CA, USA) were incubated for 30 min. Samples were then incubated with diaminobenzidine/hydrogen peroxide (00–2020, Invitrogen) for 5 min and counterstained with hematoxylin. After cells were washed with tap water, cover glasses were dehydrated through graded alcohols and cleared in xylene prior with mounting medium (clear mount, mounting medium Ref: 008110 Invitrogen, USA), and then, they were evaluated under a light microscope (Olympus BX40, Olympus Corp., Tokyo, Japan).

Statistical Analysis

Experiments were repeated 3 times. Two randomly selected areas were scored and in sections where all the staining appeared intense; one random field was chosen. All cells were scored in a semi-quantitative fashion, by considering the intensity with the percentage of the positive staining. The staining of primary antibodies was graded semi-quantitatively. H-score was calculated using this equation: H-score $= \sum Pi\ (i+1)$, where i = intensity of staining with a value of 0, 1, 2, or 3 (negative [-], weak [+], moderate [++], and strong [+++], respectively) and Pi was taken as the positive percentage, which increased from 0 to 100. The staining scores were evaluated by two observers in a blinded fashion independently. Statistical analyses were determined by evaluation of differences using the ANOVA test and considered statistically significant when <0.05.

Results

Cell Growth Assay

MDA-MB-231 cells (15×103) were seeded in 6-well plate in triplicate and maintained in normal growth medium. Doubling time was counted as 24 h. MDA-MB-231 cells were counted as 30×103 at 24 h. The cells were incubated with BA, BP, and DPD after 24 h.

MTT Results

The cytotoxic effect and IC50 value of boron compounds on MDA-MB-231 cancer cells were evaluated by MTT analysis. Findings from the result of established cell growth analysis have shown that cell line causes a very striking reduction in cell growth for BA and BP. Otherwise, it was determined that DPD caused a substantial decrease in the growth of MDA-MB-231 breast cancer cells after the 72 h at 5 and 7 days after treatment (Fig. 1a-c).

Immunohistochemical Results

In this study, we analyzed the effects of BA, BP, and DPD treatment on the expression of the VEGF, eNOS, and iNOS on MDA-MB-231 cells, using an immunohistochemical staining.

In the boron-treated groups, immune positive-stained cells of all antibodies were less when compared with the control group (group according to H-scores) (Fig. 2). VEGF expression of DPD was lower than BA (p<0.05). eNOS immunoreactivity was most decreased in DPD group while iNOS immunoreactivity was least in BP group. The average values of immunoreactivity H-scores and statistical analysis from groups are shown in Table 1. The control group was accepted 100% and others were calculated according to this.
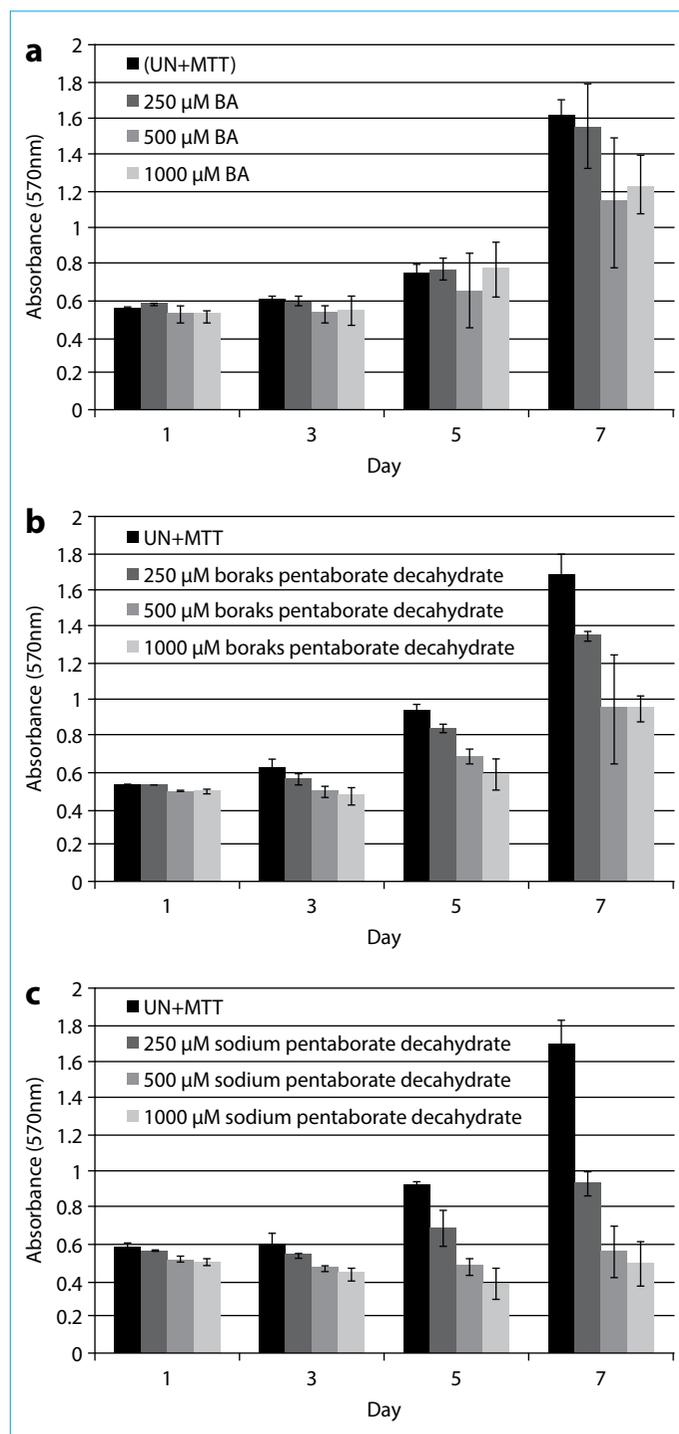

Figure 1 (a–c). Effect of three different boron derivatives on the growth of MDA-MB-231 cells. Different concentrations of three different boron derivatives (boric acid, borax pentahydrate, and disodium pentaborate decahydrate) were treated on MDA-MB-231 breast cancer cell line for 7 days. MTT measurements and standard deviations from the cells implanted in each of the three wells are shown. **(a)** Effect of different concentrations of boric acid on MDA-MB-231 cell line. **(b)** Effect of different concentrations of borax pentahydrate on MDA-MB-231 cell line. **(c)** Effect of different concentrations of disodium pentaborate decahydrate on MDA-MB-231 cell line (UN: Boric acid and its derivatives untreated samples, BA - boric acid).
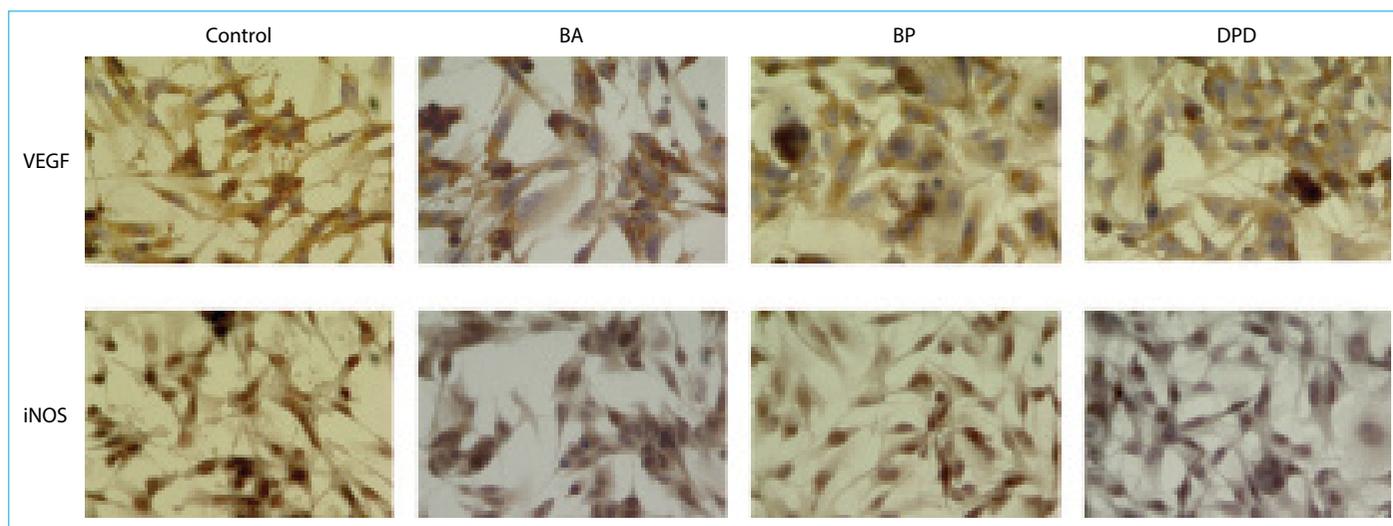
Figure 2. Immunohistochemical staining of vascular endothelial growth factor and inducible nitric oxide synthase primary antibodies in MDA-MB 231 human breast cancer cell line (×200).

Table 1. H-score (%) and P values of VEGF, eNOS, and iNOS immunoreactivities for each group

MDA-MB 231	VEGF (%)	eNOS (%)	iNOS (%)
Control (C)	100[a]	100[a]	100[a]
BA	69.50[b]	83.33[b]	76.59[b]
BP	68.14[bc]	67.17[b]	48.62[c]
T-Boron (DPD)	53.95[c]	47.43[c]	65.48[d]
	p<0.001	p<0.001	p<0.001

a, b, c, and d were shown that differences between the groups. There is no difference in the groups with the same characters; BP: Boron penta; BA: Boric acid.

Discussion

Boron-based compounds are now under study due to their anticarcinogenic properties. Several investigations have supported that boron-enriched environments correlate with lower risks of some cancers such as prostate, breast, cervical, and lung cancers.[22] In the past few years, accumulating evidence shows that the use of boron compounds as anticancer agents has increased, especially in inoperable cancers and those with high malignancy.[4] We examined the impact of boron derivatives on tumor angiogenesis using MDA-MB-231 breast cancer cells. The present data suggest that BA, especially BP and DPD, inhibits the angiogenesis of breast cancer cells due to antioxidant effect.

Several studies have demonstrated the effectiveness of some boron derivatives on MDA-MB-231 breast cancer cells. In a previous study, the different concentrations of CaFB were applied to MDA-MB-231 breast cancer cell line and their results implied that CaFB may have a therapeutic potential in cancer treatment.[23] Scorei et al.[2] suggested that calcium fructoborate (CF) and BA inhibited the prolif-

eration of MDA-MB-231 cells in a dose-dependent manner. It was detected that boron components might be more effective in cancer treatment than BA in some studies.[24, 25] However, BP and DPD from boron derivatives have not been previously studied in breast cancer. In this context, in the present study, anticarcinogenic and antiangiogenic properties related to oxidative stress of three kinds of boron were assayed in MDA-MB-231 metastatic cancer cell with MTT for cytotoxicity.

It was evaluated that the treatment with 1000 µM (1 mM) boron had an antiproliferative effect and especially DPD pronouncedly reduced cell viability. According to MTT results, BA and BP did not affect the cell viability. This could be due to the fact that a low dose of boron was applied with a value of 1 mM. The dosage used in our study was reported to be safe according to another study finding which suggested that up to 1 mg/mL (app. 4 mM) BA and sodium pentaborate pentahydrate (NaB), was found to be non-toxic to proliferating adipogenic cells.[26]

Barranco et al.[27] also demonstrated that 1 mM of BA decreases migration, proliferation, and inhibiting cells attach of the prostate cancer cell line DU-145 in vitro.

Bradke et al.[25] showed that 1 mM exposure of BA or PBA can effectively inhibit the migration of prostate cancer cells.

As known, MDA-MB-231 cells are invasive breast cancer cells and a few published studies have focused on the effect of boron in these cells in vitro. In one of these studies, CF and BA were tried with concentrations of 0.45–11.25 mM on MDA-MB-231 cells, and TUNEL-positive cells were seen in a dose-dependent manner over the concentration of 2.25 mM.[3] One of the importing findings of our study is that DPD reduced both progression of cancer cells and an-

giogenesis with oxidative stress even at low-dose administration such as 1 mM.

Principal et al.[28] suggested that higher levels of iNOS may serve as a marker of poor prognosis and aggressiveness in patients with breast cancer. Wash et al.[29] showed that iNOS has been shown to induce p53 mutation accumulation and activation of the epidermal growth factor receptor; all of which are key components of breast cancer biology. Moreover, iNOS predicts poor outcome in breast cancer, and iNOS inhibitors show efficacy when used in combination with chemotherapy.

In another study, Ranganathan et al.[30] indicated that iNOS expression was positively correlated with tumor-node-metastasis staging of breast cancer and iNOS which cause damage to the cellular DNA, which may have a functional role in cancer progression.

In the current study, in addition to BA, we suggest that it might be useful to reduce the expression of other derivatives for breast cancer such as iNOS of BP and DPD that have not been proposed before.

The relationship between eNOS and angiogenesis has not been fully clarified yet in breast cancer. Kafousi et al. have shown strongly correlation between eNOS and VEGF in MCF-7 cancer cells.[31] In the past, few studies reported that estrogen receptor (ER)-eNOS association in breast cancer patients.[32] Then, it has been demonstrated that ER induces eNOS expression, explaining why estrogens induce eNOS in MCF-7 cell line.[33] However, there is no knowledge about eNOS and MDA-MB-231 cells. Our results indicate that there is VEGF-eNOS association in MDA-MB-231 cell line. Because these two pathways have decreased in similar proportions with boron application.

Despite the latest studies, there are different opinions and it is not yet clear as to the mechanism in which boron compounds inhibit cancer. One potential explanation for anticarcinogenic effects is that some boron composites are histone deacetylase inhibitors (HDIs). HDIs are therapeutic agents for cancer due to their abilities in modifying gene expression, inducing tumor cell apoptosis or cell cycle arrest, preventing metastasis, and stimulating normal cell differentiation.[4] Another one is about action is the inhibition of serine protease (enzyme). Boron components interfere with the physiology and reproduction of cancerous cells through inhibition of serine proteases, mRNA splicing, and cell replication but also receptor binding mimicry and induction of apoptosis.[34] According to some reports, serine protease inhibitors are plasminogen activator inhibitor-1 (PAI-1) and maspin suppresses metastasis, invasion and angiogenesis in breast and prostate cancers.[35, 36]

Our data with VEGF, eNOS, and iNOS obtained by applying boron derivatives in breast cancer cell line seem to support this context. Furthermore, the antiangiogenic effect of boron compounds obtained in our study on breast cancer cells may be through inhibition of serine proteases such as PAI-1 or maspin. However, future studies will be needed to confirm this.

Furthermore, when we were analyzed the elemental composition of boron, boron element's mass percent was 17.4842% in BA (BA - H_3BO_3), 14.8454% in BP (BP - $Na_2B_4O_7.5H_2O$), and 18.3165% in T-boron (DPD - $Na_2O.5B_2O_3.10H_2O$). According to this information, there is no relationship between boron amount and the antiangiogenic effect of these compounds.

Conclusion

To the best of our knowledge, this is the first study investigating the relation of BP and DPD with VEGF and iNOS expression in a breast cancer cell line. Our present findings indicated that BP and DPD have antiangiogenic and antioxidant properties. For this reason, it may be expected that boron can decrease proliferative and metastatic potentials of breast cancer cells and help clinicians for making strategic choices. In conclusion, additional studies will be needed to identify the underlying mechanism responsible for the observed cellular responses to these components and to determine if boron will be suitable for clinical application in breast cancer patients.

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