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Comparative Effects of Boric Acid and Calcium Fructoborate on Breast Cancer Cells

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Abstract Recent studies suggested that boron has a chemo-preventive role in prostate cancer. In the present report, we investigated the effects of calcium fructoborate (CF) and boric acid (BA) on activation of the apoptotic pathway in MDA-MB-231 human breast cancer cells. Exposure to BA and CF inhibited the proliferation of breast cancer cells in a dose-dependent manner. Treatment with CF but not BA resulted in a decrease in p53 and bcl-2 protein levels. Furthermore, after the treatment with CF, augmentation of pro-caspase-3 protein expression, cytosolic cytochrome *c* level, and caspase-3 activity were observed, indicating apoptotic cell death induction. This was also demonstrated by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end-labeling assay. In conclusion, our data provide arguments to the fact that both BA and CF inhibited the growth of breast cancer cells, while only CF induced apoptosis. Additional studies will be needed to identify the underlying mechanism responsible for the observed cellular responses to these compounds and to determine if BA and CF may be further evaluated as chemotherapeutic agents for human cancer.

Keywords Boric acid (BA) · Calcium fructoborate (CF) · Breast cancer · Apoptosis · MDA-MB-231

Introduction

Boron compounds are now under study because of their possible beneficial effects on human health. Several investigations have reported that in amounts typically found in human diet, boron improves bone health (independent of vitamin D status), interacts with specific steroid

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hormones, and increases circulating concentrations of 25-hydroxycholecalciferol [1] and 17β-estradiol [2, 3]. Borate or borate analogues can inhibit the in vitro activities of several enzymes in the eicosanoid pathway related to inflammation and immune function [4–7]. There are also evidences that boron has a chemo-preventive effect against prostate cancer. Epidemiologic data support the premise that the risk of prostate cancer is inversely proportional to dietary intake of boron [8]. In addition, the development of tumors induced by injection of prostate cancer cells has been reduced in animals by treatment with BA [9].

The mechanisms that underlie the observed ant-itumorigenic effects of boron are not precisely known. Apoptosis is one of the most potent defenses against cancer, as this process eliminates potentially deleterious mutated cells [10]. Accumulating evidence show that the efficiency of anti-tumor agents is connected to the intrinsic property of the tumor target cells to respond to these agents by apoptosis.

The present investigation was initiated to evaluate the proapoptotic effects of boric acid (BA), the naturally occurring form of boron circulating in human plasma, and calcium fructoborate (CF), a boron-based nutritional supplement that has a chemical structure similar to the natural forms of boron found in edible plants, which might provide an explanation of boron's cancer chemo-preventive activity. We examined the effects of CF and BA on human breast tumor cells MDA-MB-231. The level of cell proliferation and apoptosis after cell treatments with CF and BA and also the molecular markers of apoptosis (p53, bcl-2, caspase-3, cytochrome *c*) were studied in connection with the possible use of these compounds as anticancer agents.

Materials and Methods

Materials

CF was synthesized according to Miljkovic's patent [11]. BA was acquired from Sigma Chemicals.

Cell Line and Culture Conditions

MDA-MB-231, a highly invasive breast cancer cell line (kindly donated by Dr. Beatrice Bachmeier, Klinische Chemie und Kninische Biochemie der Ludwig Maximilian Universitat, Munchen, Germany) is a prototype for the study of hormone-independent breast cancer, as it is estrogen receptor negative. The cells were maintained in minimum essential medium (MEM) with Earle's salts, supplemented with L-glutamine, (2 mM), sodium pyruvate (1 mM), nonessential amino acids and vitamins, and 5% (v/v) heat-inactivated fetal calf serum (all media components from Gibco Invitrogen, Burlington, ON, Canada). Cells were grown at 37°C in an atmosphere containing 5% CO_2, at 98% relative humidity. 24 h after the plating, the MDA-MB-231 cells were incubated in fresh MEM plus different CF or BA concentrations for an additional 24 h.

Viability Test

Cell viability was measured by assessment of the ability of mitochondrial dehydrogenases to reduce 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to the corresponding purple formazan. After the treatment, the cells were washed twice with PBS and incubated with 1 mg/ml MTT for 4 h at 37°C. After incubation, the MTT solution

was removed by rinsing with PBS, and the formazan was released from the cells by incubation with isopropanol. Absorbance of the supernatant was measured at 550 nm.

Examination of Apoptotic Cell Death

Deoxyribonucleic acid cleavage, which commonly occurs in apoptosis, was measured by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end-labeling (TUNEL) test using a kit (Dead™ Colorimetric TUNEL System; Promega).

Western Blot Analyses

Western blot assay for p53, Bcl-2, or pro-caspase-3 was carried out. Trysinized cells were rinsed twice with PBS and then lysed by sonication. Total cell lysates containing 50 μg protein, as determined by the Bradford method [12], were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins in the gels were transferred to a nitrocellulose membrane (Invitrogen 0.2-μm pore nitrocellulose/filter paper sandwich; XCell II™ Blot Module), blocked with 5% nonfat milk, and probed with antibodies against p53, Bcl-2, or caspase-3 overnight at 4°C. Monoclonal mouse anti-human p53, Bcl-2, or caspase-3 antibodies were used. To detect the antibodies that have bound, the membranes were incubated with goat anti-human IgG–alkaline phosphatase for 1 h and developed with *p*-nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt solution. The blots were reprobed with anti-β-actin to confirm equal protein loading. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology.

Cytochrome c ELISA Assay

Cells exposed to the conditions described in the text were harvested, washed three times with ice-cold phosphate-buffered saline, resuspended in cold buffer containing 10 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 10 μM aprotinin A, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride and homogenized five to ten times with an homogenizer on ice. The samples were centrifuged at 10,000×*g* for 60 min at 4°C, and the supernatant was collected and used for measurement of cytosolic cytochrome *c* by an enzyme-linked immunosorbent assay (ELISA) kit from CHEMICON (cat. no APT 200). The protein content was determined by the Bradford method [12].

Caspase-3 Assay

We measured the activity of caspase-3 by using a Chemicon's Colorimetric Caspase-3 Assay Kit that is based on enzyme's ability to recognize and cleave a substrate containing the DEVD motif. Practically, the activated caspase-3 in the lysed cells cleaves the *para*-nitro aniline (pNA)-DEVD substrate. pNA is subsequently determined by measuring absorbance at 405 nm and compared to a standard curve obtained by serial dilutions of the pNA standard provided in the kit. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity. One unit is the amount of enzyme that cleaves 1.0 nmol of colorimetric substrate pNA per hour at 37°C under saturated substrate concentrations.

The changes in the protein level of pro-caspase 3 after CF or BA treatments of tumor cells were determined by Western blot analysis.

Statistical Methods

Each experimental condition was performed at least in triplicate. Data are expressed as the mean±SEM. Statistical differences were analyzed using Student's *t* test or analysis of variance when suitable.

Results

To verify breast cancer cells viability after 24 h of CF and BA exposure, the MTT assay was carried out. The results presented in Fig. 1 indicated that CF and BA induced a concentration-dependent cytotoxicity in MDA-MB-231 cells. Specifically, CF and BA concentrations of 0.45, 2.25, 5, 11.25, and 22.5 mM induced 98.2, 86.1, 78.2, 71.5, and 52.6% and 94.9, 86, 75, 66, and 60% relative survival of MDA-MB-231 cells, respectively.

Anticancer treatment using some cytotoxic drugs is considered to mediate cell death by activating key elements of the apoptosis program and the cellular stress response. In our investigation on apoptosis and sensitivity of MDA-MB-231 cells to CF and BA treatments, TUNEL assay was performed. BA treatment had little effect on apoptosis, whereas CF treatment increased the number of TUNEL-positive cells in a dose-dependent manner over the concentration of 2.25 mM (Fig. 2).

Mammalian Bcl-2 protein family of apoptosis-associated proteins consists of members that inhibit apoptosis (Bcl-2, Bcl-xl) and others that induce apoptosis (Bax, Bak, etc.). We investigated the changes of the level of Bcl-2 in MDA-MB-231 cells treated with CF and BA because it is well known that Bcl-2 protects cells from various stresses such as ionizing radiation, chemotherapeutic agent treatment, and reactive oxygen species [13]. As shown in Fig. 3, CF in concentrations higher than 2.25 mM downregulated Bcl-2 protein expression in MDA-MB 231 cells, while BA had no effect at this level.

A major checkpoint in the mammalian cell death pathway is the ratio of proapoptotic (Bax) to antiapoptotic (Bcl-2) members. Downstream to this checkpoint are two major execution programs: mitochondria dysfunction and caspase pathway [14]. Mitochondrial alteration includes a change of the mitochondrial membrane potential, production of reactive oxygen species, opening of the permeability transition pore, and the release of the intermembrane space protein, cytochrome *c*. Released cytochrome *c* activates Apaf-1, which in turn activates a downstream caspase program. To confirm the finding that CF induces apoptosis in MDA-MB-231, the level of cytosolic cytochrome *c* protein was examined by ELISA. Analysis (Fig. 4) confirms the induction of the apoptotic process by

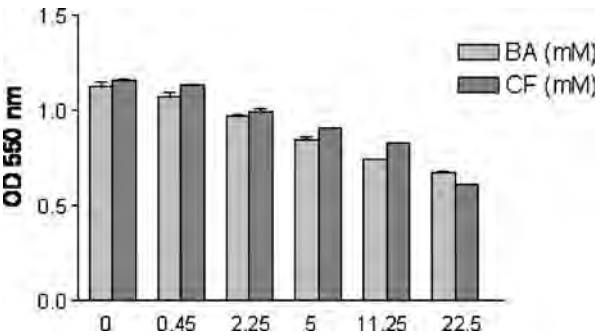

Fig. 1 Survival rate following the MDA-MB-231 cell exposure for 24 h to different concentrations of CF and BA, as indicated by the MTT assay. Data shown are means±SEM (n=3)

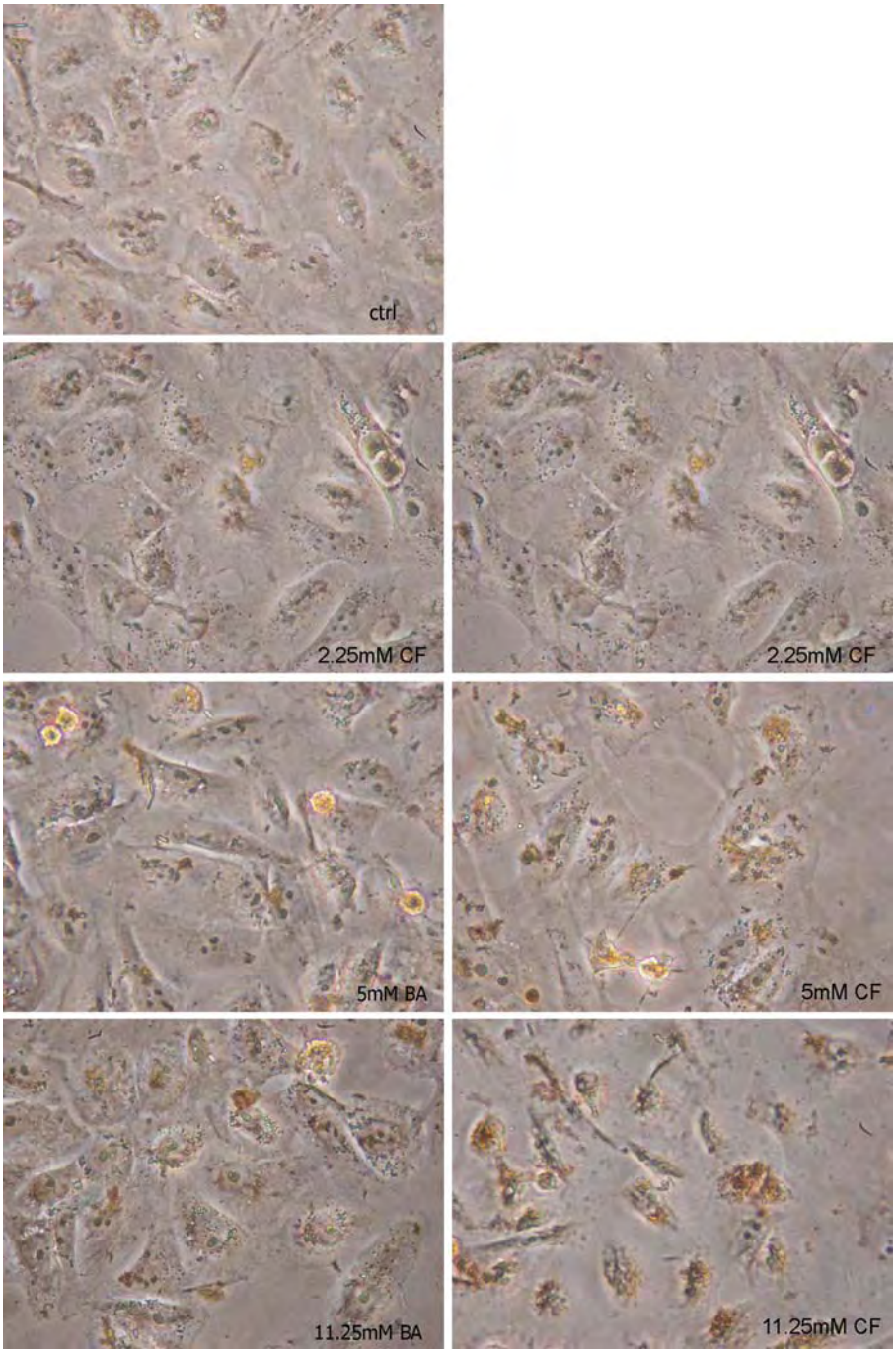

Fig. 2 Examination of cell death induction by CF and BA treatment - TUNEL staining showing the cells that undergo apoptosis, as indicated by nuclear intense (*brown*) staining

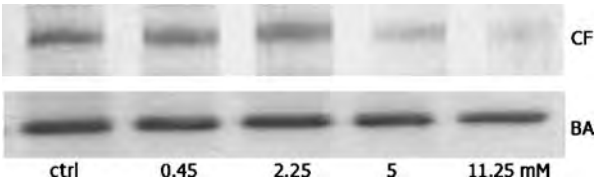

Fig. 3 Effect of CF and BA on Bcl-2 protein level in breast cancer cells, analyzed by Western blotting after 24 h exposure. MDA-MB-231 cells were treated with indicated concentrations of CF/BA. The lysates containing equal amounts of protein (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with specific Bcl-2 antibodies. A representative study is shown; two additional experiments yielded similar results

CF in MDA-MB-231 cells. Thus, treatment with concentrations of 0.45, 2.25, 5, and 11.25 mM CF resulted in an increase of 11, 46, 99, and 1,075% of cytochrome *c* level. However, BA treatment of cancer cells did not change the cytosolic level of cytochrome *c*.

We also studied caspase-3 activation as an indicator of apoptosis induction because different upstream pathways leading to apoptosis depend on caspase-3 induction for final apoptotic execution. Figure 5a shows the effects of CF and BA on the extent of caspase-3 induction in MDA-MB-231 cells. A change of caspase-3 activity in both cases was noticed. However, while CF treatment with 0.45, 2.25, 5, and 11.25 mM resulted in a 12.2, 30, 70.1, and 149.1% increase in caspase-3 activity, the same concentrations of BA augmented caspase-3 activity with only 7.1, 18, 23.21, and 41% compared to the control. Western blot analysis showed a dose-dependent increase in the protein expression of pro-caspase-3 (Fig. 5b) after the treatment with CF. On the other hand, BA exerted a little effect on pro-caspase-3 protein level.

In its normal form, the p53 gene is a tumor suppressor, but when mutated, it loses its protective function; this allows mutations to accumulate in other genes and leads to more than 50% of all human cancers, including breast cancers. Some p53 lesions result in malignancies that are resistant to the most commonly used treatments. Despite the complexity of mechanisms regulating its function, p53 activity has been associated with prognosis and prediction of tumor response to various therapies and deserves further investigations with the perspective of developing more targeted treatments [15]. A strong link between the apoptotic function of p53 and tumor suppression has been demonstrated [16]. In our study, we examined the impact of the MDA-MB-231 treatment with CF and BA upon the level of the p53 protein (Fig. 6).

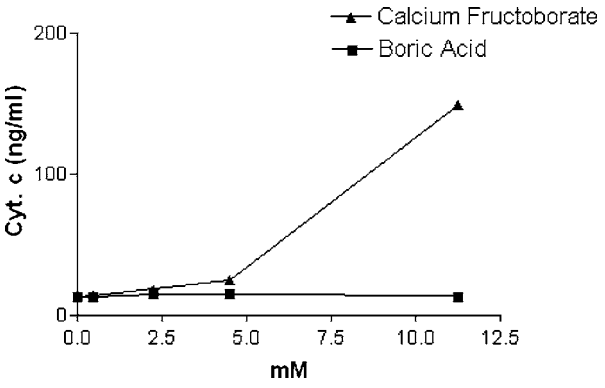

Fig. 4 Cytosolic levels of cytochrome c in MDA-MB-231 cells treated with CF or BA for 24 h. Data shown are means±SEM (n=3)

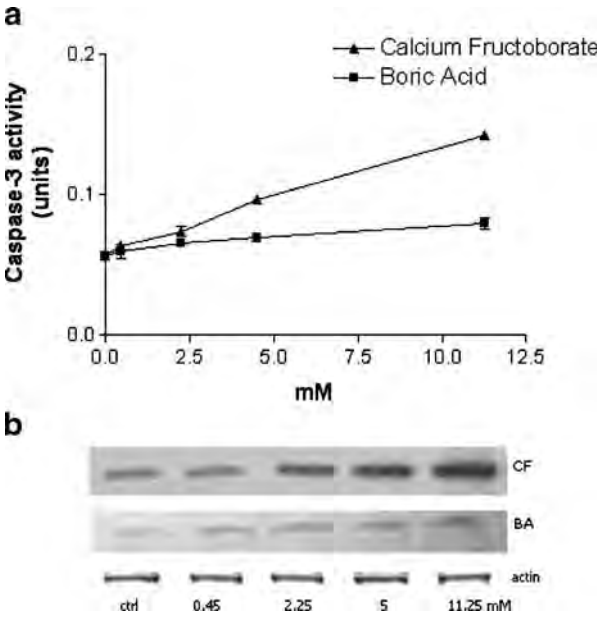

Fig. 5 Effects of CF and BA on caspase-3 activity and pro-caspase-3 protein expression. **a** MDA-MB-231 cells were incubated with indicated concentrations of CF/BA for 24 h. Caspase-3 activity was determined as described in "Materials and Methods." Data shown are means±SEM (n=3). **b** The cell lysates containing 50 μg protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed with specific anti-pro-caspase-3 antibodies and with anti-β-actin antibody to serve as control for the equal protein loading

No effect on p53 protein expression was observed when MDA-MB-231 cells were incubated with CF doses of 0.45 and 2.25 mM. At CF concentrations higher than 2.25 mM, we found a decrease in the level of p53 protein expression. In the case of BA treatment, no significant variations in the p53 protein level were noticed.

Discussion and Conclusions

In the present study, we demonstrated that CF and BA inhibited the proliferation of breast cancer cells MDA-MB-231 in a dose-dependent manner. As revealed by different experiments (TUNEL, Bcl-2 and pro-caspase-3 protein expression, and cytochrome *c* caspase-3 activities), it appeared that the anti-proliferative effect of CF in breast cancer cells MDA-MB-231 is mediated by induction of apoptosis. On the other hand, BA induced a cell death-independent proliferative inhibition of breast cancer cells. In a previous study, Barranco and Eckhert [17], using DU-145 prostate cancer cells, showed that BA inhibited cell proliferation without inducing apoptosis. They demonstrated that BA induces conversion to a senescent-like cellular phenotype and also causes a dose-dependent reduction in cyclins A–E, as well as mitogen-activated protein kinase proteins, suggesting their contribution to proliferative inhibition [18]. Unlike BA, the mechanism underlying the

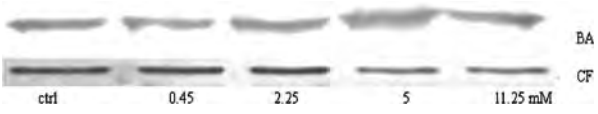

Fig. 6 The effects of CF and BA on p53 protein expression in MDA-MB-231 cells. The lysates containing equal amounts of protein (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with specific p53 antibodies. A representative study is shown; two additional experiments yielded similar results

antiproliferative activity of CF has not been elucidated. When MDA-MB-231 cells were treated with 0.45 mM CF, for 24 h, no effects on cell viability, Bcl-2, caspase-3, and p53 protein expression were observed. Cytotoxic effects and an increase in caspase-3 activity were seen in MDA-MB-231 cells treated with CF doses higher than 2.25 mM. Therefore, the treatment of tumor cells with CF resulted in a rapid release of cytochrome *c* from the mitochondria, which preceded caspase 3 activation. At CF concentrations greater than or equal to 5 mM CF, the cytotoxic effects became stronger, and repression of Bcl-2 protein expression was seen. The mechanism of the noticed downregulation of Bcl-2 by CF remains to be delineated. This may occur at the transcription level and/or post-transcription level and may also involve reduced Bcl-2 messenger ribonucleic acid stability, leading to a decrease in Bcl-2 expression.

Taking into account the central role of p53 in the regulation of apoptosis, it is of great interest to study p53 involvement in the apoptosis induction by different potential chemotherapeutic agents. In this context, we investigated the relationship between p53 tumor suppressor protein immunoreactivity and CF action upon MDA-MB-231 cells and found out a downregulation of p53 protein expression at doses greater than or equal to 5 mM. An explanation of this finding might be that higher doses of CF stimulated degradation of p53 mediated by cytoplasmic 26S proteasomes [19, 20] and that in MDA-MB-231 cells, the apoptotic process is p53 independent.

Based on these results, we conclude that while both BA and CF inhibit the growth of breast cancer cells, only CF induces apoptosis. Further studies will be needed to determine if BA and CF will be suitable for clinical application in breast cancer patients.

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