Boric acid inhibits human prostate cancer cell proliferation

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Abstract

The role of boron in biology includes coordinated regulation of gene expression in mixed bacterial populations and the growth and proliferation of higher plants and lower animals. Here we report that boric acid, the dominant form of boron in plasma, inhibits the proliferation of prostate cancer cell lines, DU-145 and LNCaP, in a dose-dependent manner. Non-tumorigenic prostate cell lines, PWR-1E and RWPE-1, and the cancer line PC-3 were also inhibited, but required concentrations higher than observed human blood levels. Studies using DU-145 cells showed that boric acid induced a cell death-independent proliferative inhibition, with little effect on cell cycle stage distribution and mitochondrial function.

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1. Introduction

Boron has been shown to be beneficial for many species, but its cellular processing in animals remains obscure. The boron atom has a high affinity for oxygen and in nature is present in the form of borates [1]. Soluble forms include boric acid B(OH)₃ and the monovalent anion B(OH)₄⁻, with the presence of the dominant form dependent upon solvent pH. In plasma, boric acid predominates and its concentration reflects dietary intake and respiratory exposure [2].

Boron is known to be important for animal cell replication and development, but the underlying mechanisms remain obscure. Boric acid stimulates embryonic growth in trout [3] and is essential during the pre-blastula cleavage stage of zebrafish [4]. In frogs (Xenopus), boron deficiencies interfere with normal oocyte maturation, embryonic growth and morphogenesis [5]. Deficiencies in Xenopus also lead to inhibition of oocyte germinal vesicle breakdown, possibly due to an alteration in progesterone receptor binding [6].

Evidence leading to the hypothesis that boric acid may be anti-carcinogenic was derived from epidemiological screening, where the risk of prostate cancer was observed to be inversely proportional to dietary intake of boron in a dose responsive manner [7,8]. Boric acid has also been reported to inhibit the growth of LNCaP prostate tumors in nude mice [9]. In the present paper, we report that boric acid...
inhibits the proliferation of human prostate cancer cell lines in a dose-dependent manner. The mechanism of inhibition was investigated by examining the impact of boric acid on the cell cycle, apoptosis, and mitochondrial activity of the DU-145 cell line.

2. Materials and methods

2.1. Cell culture

Cell lines were cultured in media that support maximum proliferation. Prostate cancer cells (DU-145, LNCaP, and PC-3) were obtained from Dr Allan Pantuck and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml; 100 μg/ml), and L-glutamine (200 mM) (Gemini Bioproducts, Woodlands, CA). Non-tumorigenic prostate cell lines (RWPE-1 and PWR-1E) were acquired from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Keratinocyte-SFM supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor, and 1 × antibiotic/antimycotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone) (Invitrogen). All cells were cultured at 37 °C, 5% CO2 and were removed from culture plates via trypsinization (0.53 mM EDTA, 0.05% trypsin).

2.2. Culture media

Boron-depleted media was prepared by treatment with the boron-specific ionic exchange resin, Amberlite IRA-743 (Sigma). The resin was added to supplemented media and agitated by rotation on a Koala-Ty rotator at 80 rpm, overnight, at 4 °C. Resin-treated media was then transferred to sterile 250 ml centrifuge tubes (Corning). Boric acid solutions were prepared using ultra-pure boric acid in ion exchange treated water [10]. Experimental media was prepared by the addition of concentrated boric acid solutions to provide a range of concentrations (0, 100, 250, 500 and 1000 μM B). Low boron exposures were also performed using concentrations from 15 to 100 μM B. Boron was quantified by inductively coupled plasma mass spectrometry (ICPMS) as previously described [10].

2.3. Experimental culture

Cells were plated, using non-resin treated media, on culture plates (Fisher) at densities to prevent confluence and to maintain a proliferative state throughout the duration of exposure. LNCaP, DU-145, and PC-3 cells were plated onto six-well culture plates at densities of 300, 1000 and 2000 cells/cm2. RWPE-1 and PWR-1E cells were plated onto 24-well culture plates at a density of 4100 cells/cm2. Because of the expense associated with media preparations, non-tumorigenic lines were cultured on smaller plates to reduce the volume of media used during exposures. Following an overnight incubation, non-treated media was removed and replaced daily with boric acid-supplemented media. Cells were cultured, in the presence of boric acid supplemented media, for 8 days and were counted using a hemocytometer with the aid of Trypan Blue for exclusion of non-viable cells (Invitrogen).

2.4. Cell cycle analysis

DU-145 cells were seeded on 100 × 20 mm tissue culture plates at 178 cells/cm2 and were cultured for 8 days in the presence of boric acid (0–1000 μM B), with daily media replacement. After 8 days of exposure, and at 50% confluence, adherent cells were washed once with cold PBS and scraped from the plates with a rubber policeman. Cells were suspended in PBS and centrifuged at 1200 rpm for 5 min. Following centrifugation, PBS was aspirated and the cell pellets were re-suspended in a hypotonic propidium iodide DNA staining buffer (1 mg/ml sodium citrate, 0.3% Triton-X100, 100 μg/ml propidium iodide, 20 μg/ml ribonuclease A) and allowed to incubate for 30 min at 4 °C, protected from light. Cell cycle analysis was then performed with a FACS Calibur flow cytometer, while Modfit LT 3.0 software was used for data analysis. Analysis of each sample was performed on greater than 10,000 events and coefficients of variation never exceeded 5%.

2.5. Treatment of G0 arrested cells with boron

DU-145 cells were cultured on 75 cm2 culture plates for 3 days in RPMI 1640 serum-free media, supplemented with penicillin/streptomycin...
(100 U/ml; 100 μg/ml) and L-glutamine (2 mM), to induce G₀ arrest. Arrested cells were trypsinized and plated onto six-well culture plates, at a density of 3 × 10⁵ cells/well, and treated daily with serum-free media, supplemented with boric acid (0–1000 μM B). Following 8 days of exposure, cells were trypsinized and counted using a hemacytometer, with the aid of Trypan Blue to exclude non-viable cells (Invitrogen).

2.6. Caspase-3 analysis via Western blot and PhiPhiLux assay

DU-145 cells were cultured in the presence of boric acid (0–1000 μM B), for 8 days, on 100×20 mm tissue culture plates, with daily media replacement. Cell monolayers were washed with PBS, scraped off with a rubber policeman, and centrifuged at 1200 rpm for 5 min. Protein was extracted from pellets with lysis buffer (250 mM NaCl, 0.1% NP40, 50 mM HEPES (pH 7.0), 5 mM EDTA, 1 mM dithiothreitol (DTT), 10% protease inhibitor mixture (Sigma #P8340)), sonication, and a 40 min incubation at 4 °C. Two hundred microgram of protein from each sample was separated on 12% polyacrylamide-sodium dodecyl sulfate gels (SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblotting was performed with polyclonal antibodies to caspase-3 (sc-7148, Santa Cruz) using actin (sc-1616, Santa Cruz) as a control. Secondary antibodies were anti-goat (sc-2033, Santa Cruz) and anti-rabbit (sc-2317, Santa Cruz). Protein expression was detected with the ECL detection kit (Amersham Int., Arlington Heights, IL).

PhiPhiLux (G₁D₂), a cell-permeable fluorogenic substrate containing the caspase-3 cleavage site GDEVDG, was utilized to monitor caspase-3 activity, according to the manufacturer’s instructions (OncoImmunin, Inc., Kensington MD). Briefly, DU-145 cells were cultured on six-well culture plates for 8 days, using a range of boric acid concentrations (0–1000 μM B), with daily media replacement. Cells were removed from the plates, with the aid of a rubber policeman in the presence of PBS, and suspended in 50 μl of substrate solution and 5 μl of FBS. Suspensions were allowed to incubate for 60 min at 37 °C, in the absence of light. Following incubation, cells were washed once with flow cytometry dilution buffer (1 ml), centrifuged, aspirated, and then re-suspended in dilution buffer (1 ml). Fluorescence emission was measured on a FACS Calibur flow cytometer, utilizing an FL1 channel, with greater than 8000 events per sample. Cell Quest 3.3 software was used for data analysis.

2.7. DNA fragmentation assay

For DNA fragmentation detection, DU-145 cells were cultured for 8 days, in the presence of boric acid (0–1000 μM B) on 100×20 mm tissue culture plates, with daily media replacement. Positive controls of DNA fragmentation were cultured for 24 h in the presence of 5 and 10 μM sodium selenite [11]. Following treatment, cells were trypsinized from plates and washed with PBS, transferred to 1.5 ml microcentrifuge tubes and centrifuged at 1200 rpm for 5 min. Pellets were digested in lysis buffer (10 mM Tris–HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) and allowed to incubate overnight at 37 °C. DNA was extracted from digest with an equal volume of phenol:chloroform (1:1), centrifuged at 14,000 rpm for 15 min until phases resolved, and then the supernatant was transferred to a fresh tube for precipitation. DNA-containing elution was precipitated with 7.5 M ammonium acetate (1/2 vol) and 100% ethanol (2 vol) overnight. The precipitate was centrifuged at 14,000 rpm for 15 min the following day, and the DNA was solubilized in TE buffer (10 mM Tris–Cl (pH 7.5)). Ten microgram of DNA was loaded per well in a 1.8% agarose gel containing ethidium bromide (1 μg/ml). The gel was run in 1 X TAE buffer for 2.5 h at 75 V, visualized under UV light, and photographed.

2.8. Assessment of mitochondrial activity and cell proliferation with the MTT assay

To assess mitochondrial activity, DU-145 cells were plated onto 96-well culture plates, at a density of 10⁴ cells/well, and allowed to incubate overnight. Cells were then exposed to 200 μl of boric acid supplemented media (0–1000 μM B) for 2.5 or 7.5 h. (The 7.5-h exposure involved supplementing the MTT solution with boric acid in the subsequent 5-h step.) After 2.5 h, boric acid supplemented media...
were removed and replaced with 200 μl of MTT-supplemented media (1.25 mg/ml) and allowed to incubate for 5 h. Following incubation, the MTT solution was removed and replaced with 200 μl of DMSO and 25 μl of Sorenson’s buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) and mixed thoroughly to dissolve the formazan crystals. Optical density of the MTT formazan product was read on a microplate reader at 590 nm (Table 1).

To assess cell proliferative activity, DU-145 cells were plated onto 96-well culture plates at a density of 100 cells/well, and allowed to incubate overnight. Cells were then exposed to 200 μl of boric acid supplemented media (0–1000 μM B) every 24 h, for 8 days. MTT analysis was performed identically as in the mitochondrial activity assay (with a boron-free MTT solution) and the number of cells quantified.

2.9. Statistical evaluation of proliferation values

Proliferation values were compared by ANOVA, followed by Bonferroni and Holme tests for determination of statistical differences.

3. Results

3.1. Proliferative inhibition

A dose-dependent, anti-proliferative relationship was observed in all cancer cell lines exposed to boric acid, with the hormone-independent DU-145 line showing the highest sensitivity to boric acid. At day 8, proliferation was depressed 32, 62, 87 and 98% at concentrations of 100, 250, 500, and 1000 μM B, respectively (Fig. 1A). This inhibition, in the DU-145 cell line, was also observed with the aid of MTT conversion analysis (Fig. 1B). In addition, these doses significantly depressed proliferation in the hormone-dependent, LNCaP cell line by 40, 51, 59 and 77% and the PC-3 line by 29% at 500 μM, and 52% at 1000 μM B (Fig. 1). Sub-100 μM B concentrations were evaluated in the DU-145 cell line and statistically significant proliferative inhibition occurred as low as 60 μM B (data not shown).

Non-tumorigenic prostate cells displayed reduced sensitivity to boron relative to DU-145 and LNCaP, but not PC-3. After 8 days of exposure, the HPV-18 immortalized prostate epithelial cell line, RWPE-1, displayed statistically significant reductions in proliferation of 26 and 76% at 500 and 1000 μM B exposure levels (Fig. 1). At 8 days, PWR-1E prostate epithelial cells, immortalized with Ad12-SV40 adenovirus, exhibited a 41% reduction in proliferation only at 1000 μM B level of exposure (Fig. 1).

3.2. Cell cycle analysis with propidium iodide

The impact of boron acid on the cell cycle was examined in the most sensitive cell line, DU-145. Propidium iodide is an intercalating dye with a specific affinity for DNA. Its quantitation, in permeabilized cells, measures DNA content and is used to estimate the cell cycle distribution in populations of cells. A small, but significant dose-dependent shift, in cell cycle stage distribution, was detected in cells exposed for 8 days to 500 and 1000 μM B, with a 2.2 and 4.5% shift from G0/G1 to G2. A 50% reduction in proliferation, observed at 250 μM B for DU-145 cells, occurred with no significant cycle stage shift (Fig. 2).

3.3. Boron toxicity in G0 arrested cells

Cells enter a state of growth arrest, accompanied by a shift to the G0 cell cycle stage, when serum starved. An exposure study was performed, on growth arrested DU-145 cells, in order to evaluate boron induced proliferative inhibition in non-cycling cells. Cycling DU-145 cells have a population doubling time of 24 h, while manual cell counts indicated that only one cell division occurred over the 8-day

Table 1

<table>
<thead>
<tr>
<th>BA (μM)</th>
<th>Exposure time (h)</th>
<th>2.5</th>
<th>7.5</th>
</tr>
</thead>
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<tr>
<td>0</td>
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<td>0.441±0.009</td>
<td>0.555±0.029</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>0.409±0.014</td>
<td>0.571±0.015</td>
</tr>
<tr>
<td>250</td>
<td></td>
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<td>0.479±0.027</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>0.557±0.011</td>
<td>0.563±0.016</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>0.411±0.021</td>
<td>0.493±0.024</td>
</tr>
</tbody>
</table>

Following 2.5 and 7.5 h exposures to boric acid (0–1000 μM), mitochondrial activity of DU-145 cells was detected via the MTT assay, indicative of mitochondrial conversion of MTT to formazan. Absorbance values measured at 590 nm, mean ± SEM, n=6.
exposure period, in the serum-free, zero-boron treated control cells. It is often the case that apoptotic and necrotic cells detach from culture plate surfaces, yet the strongly growth inhibiting 1000 μM B concentration had no effect on cell adherence, and exposed cells maintained their initial seeding value of 30 × 10^4 cells, throughout the 8 day exposure (Fig. 3).

3.4. Apoptosis analysis with PhiPhiLux, Western blot, and DNA fragmentation assay

Caspase-3 is an aspartate-specific, cysteine protease expressed during apoptotic cell death and was used as an indicator of apoptosis. The absence of a population shift into the R2 (Region 2) gated area showed that boric acid did not increase caspase-3 activity relative to the 0 μM B exposed DU-145 cells (Fig. 4A). This was reinforced by the Western blot analysis showing the absence of caspase-3 protein in exposed cell extracts (Fig. 4B).

During apoptosis, DNA is degraded into fragments of unique lengths, known as a ladder, in contrast to necrosis, where a wide range of fragment sizes are apparent in electrophoresis gels. A dose-dependent fragmentation was not detected in the genomic DNA isolated from DU-145 cells, relative to the 8 day boric acid exposure concentrations, yet was present in the positive controls (Fig. 5).
3.5. Mitochondrial activity analysis via MTT assay

Mitochondrial viability can be assessed through detection of formazan, at 590 nm, derived from the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase. No differences occurred in formazan concentrations, in DU-145 cells exposed to boric acid (0–1000 \( \mu \text{M} \) B) at 2.5 and 7.5 h (Table 1).

3.6. Boron analysis

Boron concentrations were determined by ICPMS. The detection limit of the ICPMS for boron was 0.001 mg B/l and ultra-pure water below this limit was used to perform all dilutions. Analysis of cell culture media treated with the ionic exchange resin Amberlite IRA-743 and supplemented with boric acid showed boron concentrations slightly higher than their theoretical level of supplementation. The concentrations of boric acid in the 0, 100, 250, 500 and 1000 \( \mu \text{M} \) supplemented media were determined to be 4.1±0.4, 109.0±0.9, 276±1.2, 565.6±4.5 and 1149±2.2 \( \mu \text{M} \) B, respectively. Since a molecule of boric acid contains one atom of boron the molar...
concentrations of boron are equivalent to those of boric acid.

4. Discussion

In this paper we examined the impact of boric acid on cell proliferation using three prostate cancer and two non-tumorigenic prostate cell lines. These were LNCaP cells derived from a lymph node metastasis that retained the androgen-responsive phenotype and two cell lines displaying the androgen-independent phenotype: DU-145, derived from a brain metastasis, and PC-3, derived from a lower lumber skeletal metastasis [12]. Proliferation of both the hormone-dependent (LNCaP) and independent (DU-145) cancer lines was reduced by boric acid in a dose-dependent manner, thus providing evidence that the mechanism responsible for the inhibition did not require the androgen receptor. Fourfold higher concentrations of boric acid were required to achieve 50% growth inhibition in PC-3 and the non-tumorigenic cell lines, PWR-1E and RWPE-1. This suggests that the sensitivity to boric acid was dependent on cellular mechanism(s) that are more highly expressed in LNCaP and DU-145 than the other cell lines.

Cell cycle analysis of DU-145 cells using propidium iodide showed that boric acid induced proliferative

Fig. 4. Flow cytometry analysis of caspase-3 activity through PhiPhiLux-G1D2 probe cleavage, among DU-145 cells exposed to boric acid (100–1000 μM B), relative to 0 μM exposure. The y-axis represents fluorescence detected with the FL1 channel (PhiPhiLux) while the x-axis represents FL-2 channel-detected fluorescence. Region 1 (R1) represents baseline fluorescence among non-exposed cells, while region 2 (R2), set in the 0 μM exposure output, encompasses a gated area that displays cells expressing higher PhiPhiLux fluorescence. Values of cell population percentages in both regions are indicated, along with the boric acid exposure concentration. Values represented as mean (%) ± SEM, n = 6 (A). Western blot analysis of caspase-3 (32 kDa) presence in DU-145 cells exposed to boric acid (0–1000 μM B) for 8 days, while actin (43 kDa) was used as a control (B).
inhibition did not cause a pronounced cell cycle stage shift. Since apoptosis is often accompanied by a cycle stage shift, this suggested that the inhibition of cell proliferation occurred in the absence of cell death. Further, study showed that boric acid did not effect the adherence of G0 arrested cells, nor did it induce detectable caspase-3 expression or activity, both indicators of apoptosis. Support for a death-independent, proliferative inhibition was also obtained from fragmentation analysis that showed intact, non-degraded DNA, regardless of exposure concentration. The absence of MTT reduction also indicated that mitochondrial activity was not affected during acute boric acid exposures. Together, these results support the interpretation that boric acid reduced growth by proliferative inhibition, rather than through the induction of cell death.

In the present study, proliferative inhibition by boric acid occurred at levels as low as 60 μM B in the DU-145 cell line. Although this concentration is high relative to pharmacological anti-cancer drugs, boron blood levels in this range have been reported in human dietary and pharmacological studies. Human blood levels reflect dietary intake and have been reported to vary from 13 to 70 μM B [13]. Drinking water boron concentrations of 2683 μM B in Turkey have not been shown to cause deleterious effects in humans populations exposed over many generations [14]. Serum levels of 0.9 M and 2.4 M B were reported in human infants accidentally fed infant formula prepared with medicinal boric acid that provided 30.4 and 94.7 mg B/kg BW, respectively [15]. These very high levels produced vomiting, diarrhea and temporary skin rashes, but no permanent damage. However, chronic toxicological oral doses of 26 mg B/kg BW causes atrophy of the seminiferous epithelium and testes in the rat [16].

The best characterized interaction of boric acid with cellular constituents is with nucleotides. Boric acid binds to cis-diol on the ribose moiety of nucleotides forming nucleotide–borate complexes including NAD\(^+\) and S-adenosylmethione [17,18]. Direct measurements using mass spectrometry gave relative affinities for boric acid that ranked: NAD\(^+\)>AMP≈CMP≈GMP≈UMP with binding affinities greatly reduced by phosphorylation [18,19]. The affinity of boric acid for hydroxyl groups underlies its ability to inhibit serine proteases, including prostate specific antigen (PSA), a marker for prostate cancer [20]. However, this affect on PSA cannot explain the inhibition of proliferation in the DU-145 cell line because it does not express PSA [21]. Given the available information, the most probable explanation for boric acid’s anti-proliferative effect is through the formation of nucleotide–borate complexes that changes the function or utilization of the nucleotides.

Diet rich in fruits and vegetables, and low in fats, are widely recommended for cancer prevention [22]. Vegetables, nuts, fruits and their beverage by-products are also the primary source of boron exposure in humans. In men 31–70 years, the mean US intake is 1.42 mg/d with a wide range from 0.57 to 3.01 mg/d between the first and 99th percentiles of intake [23]. This information, taken together with the epidemiological evidence [7,8], mouse tumor suppression study [9], and the present cell culture study suggest that boron is an unrecognized natural anti-cancer agent present in the human diet. Future investigations are needed to elucidate the mechanism of boron’s effect on proliferation and the relative importance of boron compared to dietary selenium, vitamin E and lycopene in the prevention and control of prostate cancer [24].
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