



Boric acid's cytotoxic effect on CD133⁺ cancer stem cells in H460 cell line

✉ Muradiye Acar

Istinye University, Faculty of Medicine, Department of Medical Genetics, Istanbul, Türkiye

Abstract

Aim: Cancer stem cells (CSCs) are found in a wide range of cancers, and CD133 is one of the most commonly used markers for detecting CSCs. Compared to CD133⁻ populations in lung cancer, CD133⁺ cells are more capable of self-renewal, drug resistance and tumor initiation and demonstrate increased CSC capabilities. Though the anti-cancer activity of boric acid (BA) has been reported in relation to various cancers, there is no study showing its effect on CSCs. The aim of this study is to investigate the anti-cancer effects of BA in CD133⁺ CSCs and CD133⁻ cells from lung cancer cells.

Materials and Methods: The H460 lung carcinoma cell strain was obtained from the American Type Culture Collection. CD133⁺ CSCs and CD133⁻ cells were separated using CD133 antibody conjugated to magnetic beads. The sphere formation assay (coated with SpheroMake) was used to assess the ability to maintain stemness. Cells were grown in serum-free RPMI1640 medium (with B27, N2 supplement, EGF and bFGF) to observe the development of spheres. To determine CD133 mRNA expression, total RNA was extracted and RT-qPCR was performed. The xCELLigence Real-Time Cellular Analysis (RTCA) system was used to determine the cytotoxic effects of BA. 25 mM, 12 mM, 6 mM, 3 mM, and 1 mM BA solutions were added to CD133⁺ CSCs and CD133⁻ cells.

Results: The CD133⁺ CSCs composed approximately 1% of H460 cells. The mRNA expression of CD133 was 16 times higher in CD133⁺ CSCs cells than in CD133⁻ cells. CD133⁻ cells failed to form spheres but CD133⁺ CSCs did so, demonstrating CD133⁺ CSCs stemness characteristics. The xCELLigence RTCA system determined the IC₅₀ doses of BA to be 6.7 mM and 5 mM for CD133⁺ CSCs and CD133⁻ cells.

Conclusion: BA exhibited anti-cancer activity against the CD133⁺ CSCs and CD133⁻ cells. This study is the first to demonstrate BA's anti-cancer effect on CSCs.

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Introduction

Lung cancer is a predominant cause of cancer-related deaths worldwide [1]. The vast majority of lung cancers are non-small cell lung cancers (NSCLC), while a small proportion are small cell lung cancers (SCLC) [2]. Lung cancer treatments include surgery, radiotherapy, and chemotherapy, but resistance to therapy and high relapse rates has always presented challenges to the success of treatment. Resistance is thought to be caused by cancer stem cells (CSCs) [3].

First identified in 1977 [4], CSCs have become a hot topic in cancer research. CSCs are a rare subset of the cancer cell population (1% in most solid tumors). They have a high tumorigenic potential [5]; their self-renewal capacity, high

tumorigenicity, invasion-migration tendencies, differentiation, and resistance to therapy are all important drivers of cancer's aggressiveness [6]. Current treatment methods and targeted therapy have not succeeded in abolishing the CSC population, which is the primary reason for relapse [7].

It is thought that CSCs maintain cancer cell progenitors and control carcinogenesis; if true, targeting CSCs may offer a strategy for cancer therapy [8]. Therapeutic investigations currently focus on cell surface proteins that act as surface markers for CSCs [9]; these include CD44, CD24, and CD133 [10].

A cell-surface glycoprotein with five transmembrane domains and two large glycosylated extracellular loops, CD133 is one of the most widely used markers to detect CSCs [11]. CD133 can be used for identifying CSCs in a range of cancers that includes lung [12], prostate [13], renal [14], hepatocellular [15], and colon [16] cancers. CD133⁺

*Corresponding author:

Email address: acarmuradiye@yahoo.com (✉ Muradiye Acar)

cells are more capable of self-renewal, drug resistance, and tumor initiation and demonstrate increased CSCs capabilities compared to the CD133⁻ populations in lung cancer [17].

Natural products that directly target CSCs may lead to more accurate and effective methods of abolishing lung cancer [7]. An essential trace element for humans, Boron is found in plants and drinking water [18]. It is absorbed completely from the human gastrointestinal tract and resides in body as boric acid (BA) [19]. BA is used widely for medical and nonmedical purposes [20]. It is one of the most studied Boron-containing molecules and has been shown to control the proliferation of some types of cancer cells [21-30].

BA's anticancer effect has been shown on melanoma, glioblastoma, liver, prostate, breast cancer, and lung cancer cells. BA inhibits human prostate cancer cell proliferation *in vitro* and *in vivo* [21-26] and reduces breast cancer cell proliferation in a dose-dependent manner [27]. High concentrations of BA have an anti-proliferative effect and promote apoptosis in melanoma cells [28]. Limited studies of lung cancer have shown BA significantly reduces cell number and induces apoptosis [29, 30]. Studies of the anticancer effect of BA in CSCs have not been published. This research aimed to analyze the anti-cancer activity of BA and to show its effect on CSCs.

Materials and Methods

Cell culture

The H460 (ATCC® HTB-177™) lung carcinoma cell strain was obtained from the American Type Culture Collection (Manassas, USA). Prior to beginning cell culture studies, DMEM medium (Lonza, USA) was prepared with 10% Foetal Calf Serum (Lonza, USA), 1% penicillin/streptomycin (Lonza, USA), and 1% L-glutamine (Lonza, USA) in sterile conditions. Cells were cultured at 37 °C with 5% CO₂.

Magnetic activated cell sorting (MACS)

The CSCs were isolated by the MACS system. The MACS assay was carried out with a MiniMACS Cell Sorter (Miltenyi Biotec, Germany). The H460 cell line was magnetically marked with CD133 (Miltenyi Biotec, Germany); MACS MS column (Miltenyi Biotec, Germany) was used to separate the cells. All stages of the experiment were carried out according to the manufacturer's guidelines.

Initially, the H460 cell suspension was centrifuged to completely remove the supernatant and count the 5x10⁷ cells. The cell pellet was resuspended in 300 µl of buffer and the cells were incubated at 4 °C for 30 min in the dark with a CD133 antibody conjugated to MicroBeads. The CD133⁺ cells were marked and enriched by MiniMACS magnet and MS columns.

Sphere formation assay

Sphere formation is a property of CSCs. Lung cancer stem cells can produce floating spheres enriched with CSCs [31]. The conditions described below achieved this. After magnetic sorting, CD133⁺ CSCs and CD133⁻ single-cell suspensions were seeded in 12-well culture plates (Eppendorf, Germany) coated with SpheroMake (HD Bioink

Corp., Turkey) and cultured in tumor sphere medium consisting of serum-free RPMI 1640 medium (Lonza, USA) supplemented with B27 (1:100, Gibco, USA), N2 (1:200, Gibco, USA), 200 ng/mL EGF (Abcam, USA), 30 ng/mL FGF2 (Abcam, USA). CD133⁺ CSCs and CD133⁻ cells were grown at 37 °C in 5% CO₂ for 14 days, with new medium supplied every three days. The spheres were captured with Axio Observer Z1 microscope (Zeiss, Germany). The stemness ability was evaluated by the quantity of spheres.

Total RNA isolation

Total RNA was isolated from CD133⁺ CSCs and CD133⁻ cells. Cells were dissolved in RiboEx reagent. The Hybrid-R (GeneAll, Korea) kit was used according to the manufacturer's guidelines. RNA concentration and purity was measured using NanoDrop system (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was generated with Transcriptor High Fidelity cDNA Synthesis Kit (Roche, USA). The cDNAs were kept at -80 °C.

Quantitative Reverse Transcriptase PCR (RT-qPCR)

RT-qPCR measured CD133 gene mRNA expression using the qPCR master mix (Solis BioDyne, Estonia) according to the manufacturer's guidelines on a CFX96™ Real-Time PCR Detection System (Biorad, USA). Table 1 presents the primer sequences used. Initial activation at 95 °C for 12 min, after 40 PCR cycles at 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s followed denaturation at 95 °C for 120 s. Melting curve analysis was performed. CD133 gene was normalized to *β-actin*. The 2^{-ΔΔCT} method was used to calculate the CD133 mRNA expression.

Preparation of BA

BA was acquired from Sigma Chemicals. A 1M BA stock was prepared at room temperature in complete cell culture medium and filtered with a 0.22 µm filter. The stock solution was then diluted to the desired BA concentrations (25, 12, 6, 3, 1 mM). According to the literature, these concentrations were selected at which BA inhibited cancer cell proliferation and caused cytotoxicity [23-30, 40, 41].

Cytotoxicity assay

xCELLigence real-time cellular analysis (RTCA)

The toxic concentration of BA was measured using the xCELLigence system (ACEA Biosciences, USA). Through electrical impedance, this technology enables real-time measurement of cell attachment, cell number, and cell spreading changes in response to the addition of BA, indicating the vitality of the cells. Cells in contact with the golden microelectrode on the underside of E-plates generate impedance and the system detects changes in electrode impedance resulting from contact between test cells and electrodes. Impedance is called Cell Index (CI). Using the xCELLigence RTCA program, cytotoxicity was assessed by measuring the vitality of cells treated with BA and untreated control cells. Sigmoidal dose-response analysis determined the half-maximal inhibitory concentration (IC₅₀).

Table 1. Primer sequences used in RT-qPCR.

Gene	Sense primer (from 5'to 3')	Antisense primer (from 5'to 3')	Product Length
β -Actin	TTCCTGGGCATGGAGTCCT	AGGAGGAGCAATGATCTTGATC	204 bp
CD133	ACACTACCAAGGACAAGGCG	TCCTTGATCGCTGTTGCCATG	136 bp

For background measurements, 100 μ L of culture medium was added to a 16-well E-plate (Acea Biosciences, USA) that was placed in the xCELLigence incubation system for 10 min; the medium's CI background value was calculated. Next 100 μ L of CD133⁺ CSCs and CD133⁻ cell suspensions (2×10^4 cells/well) were plated into 16-well E-plates of the xCELLigence system. The cells were allowed to adhere for 40 min under a sterile hood before the E-plate was installed in the xCELLigence system to begin the testing. The machine was programmed to take a reading every 15 min. For cell integration and growth, the plate was perused for about 24 h. After 24 h when the cells had begun their growth phase, the 100 μ L of culture medium were removed. 100 μ L of different concentrations of BA were added; the control groups received the same amount of culture medium. After 72 h of incubation with BA, the typical CI-time profile was used to plot cell state and cytotoxic effect. The CI was adjusted to the BA treatment time point. The RTCA 2.0 software was used for the evaluations. Cells were examined with an inverted Axio Observer Z1 microscope (Zeiss, Germany).

Statistical analysis

Statistical calculations were carried out with SPSS v.26 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant when p values were less than 0.05 ($p < 0.05$), shown by asterisks in the graphs. The significance of group differences were assessed with Student's two tailed t-test and one-way ANOVA, followed by a Tukey HSD post-hoc test. All values are expressed as the mean \pm standard deviation.

Results

Isolation of CD133⁺ CSCs by MACS

The expression of the surface marker CD133 permitted the isolation and identification of H460 CSCs. Counting cells after MACS showed approximately 1% of the CSCs population overexpressing CD133 in H460 cancer cells. After CD133⁺ CSCs isolation by MACS, purity of isolated CSCs and CD133 mRNA expression were assessed by RT-qPCR. CD133 expression was 16 times higher in CD133⁺ CSCs cells than in CD133⁻ cells (Figure 1).

Stem cell properties of CD133⁺ CSCs and CD133⁻ cells

Only CSCs can live and grow in serum-free, non-adherent conditions, so cancer cells are cultured there to increase CSCs populations. CSCs' capacity to produce floating spheres is an indication of their capacity for self-renewal.

Sphere formation was observed for 14 days. Spheres began to form on the 4th day. While CD133⁻ cells failed to form spheres, CD133⁺ CSCs formed spheres, demonstrating CD133⁺ CSCs stemness characteristics (Figure 2).

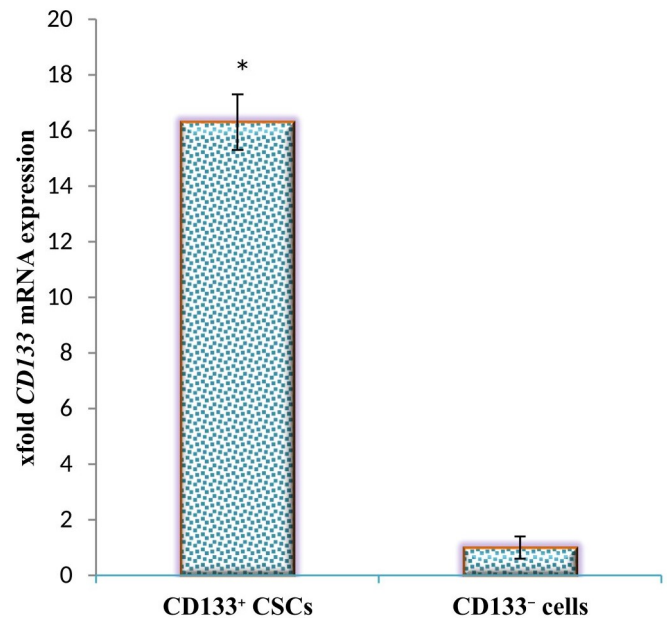


Figure 1. CD133 mRNA expression in CD133⁺ CSCs and CD133⁻ cells ($p < 0.05$).

Cytotoxic effects of BA on CD133⁺ CSCs and CD133⁻ cells

CD133⁺ CSCs and CD133⁻ cells were treated with 1, 3, 6, 12, and 25 mM BA concentrations. To determine the cytotoxic activity of BA, live cell proliferation was monitored for 100 h with the xCELLigence RTCA system.

About 24 h after the seeding, the CI was normalized at the point of treatment. The normalized CI reflects the amount of adhesion and thus relates to cell viability. Sigmoidal dose-response curves were generated by the RTCA 2.0 software's data analysis tool and used to calculate the IC₅₀ values for the cells. Analysis determined that BA was most effective 62 h after treatment. The xCELLigence system determined the IC₅₀ doses of BA to be 6.7 mM and 5 mM for CD133⁺ CSCs and CD133⁻ cells. Figure 3 illustrate the CI of the CD133⁺ CSCs, and CD133⁻ cells were treated with varying concentrations of BA (1-25 mM) compared to the untreated control groups.

CD133⁺ CSCs treatments with 3, 6, 12 and 25 mM BA were found statistically significantly decrease in cell viability compared to controls (85, 60, 37, and 11%). Treatments with 1 mM of BA were found to statistically insignificantly reduce cell viability compared to controls (94%), and above 3 mM, increasing BA concentrations generally decreased cell viability percentages, demonstrating a dose-dependent cytotoxic effect (Figure 4a).

It was detected that CD133⁻ cell treatments with 1, 3, 6, 12, and 25 mM of BA statistically significantly decreased

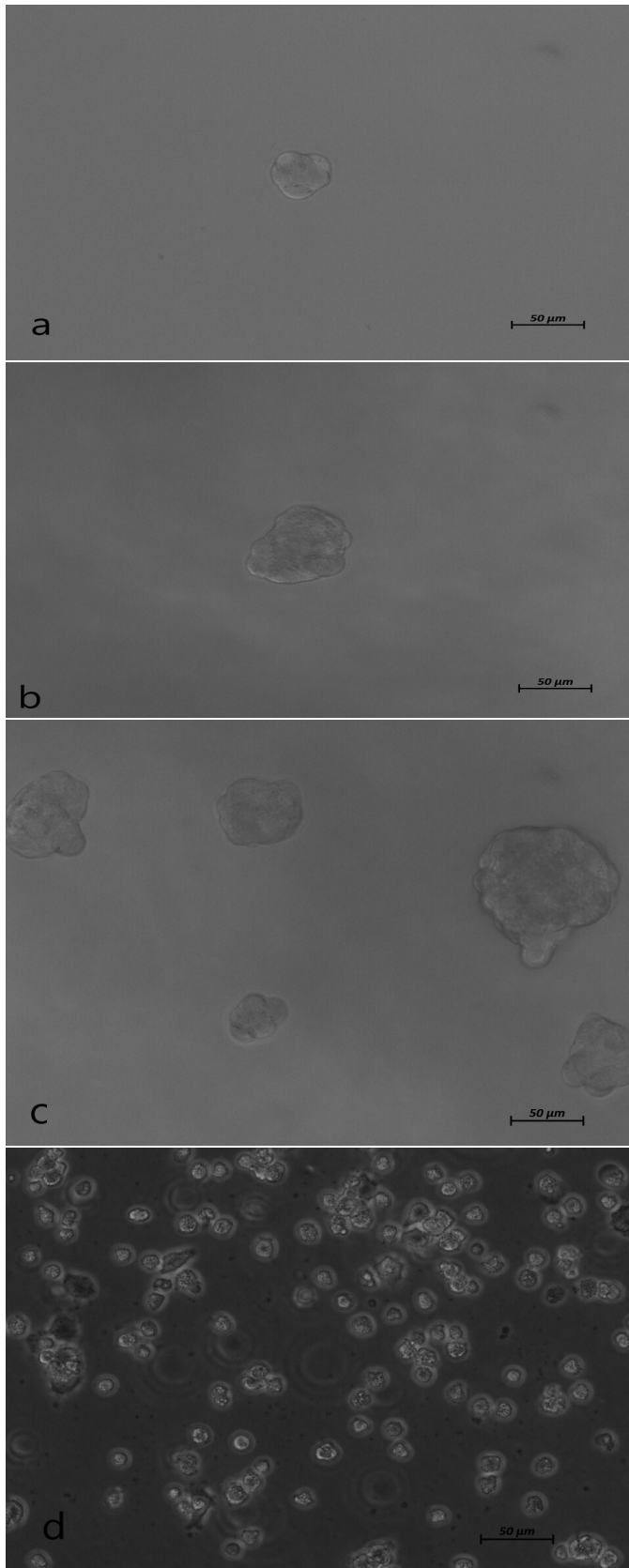


Figure 2. Characterization of cells with CSCs characteristics from H460 cell. CD133⁺ CSCs and CD133⁻ cells were grown in serum-free medium under nonadhesive conditions for 14 days. CD133⁺ CSC spheres from day 3 to day 9 (a, b, c), and CD133⁻ cells (d); magnification is 200X. Pictures were taken using a Zeiss Axio Observer Z1 microscope. The bar represents a distance of 50 μm .

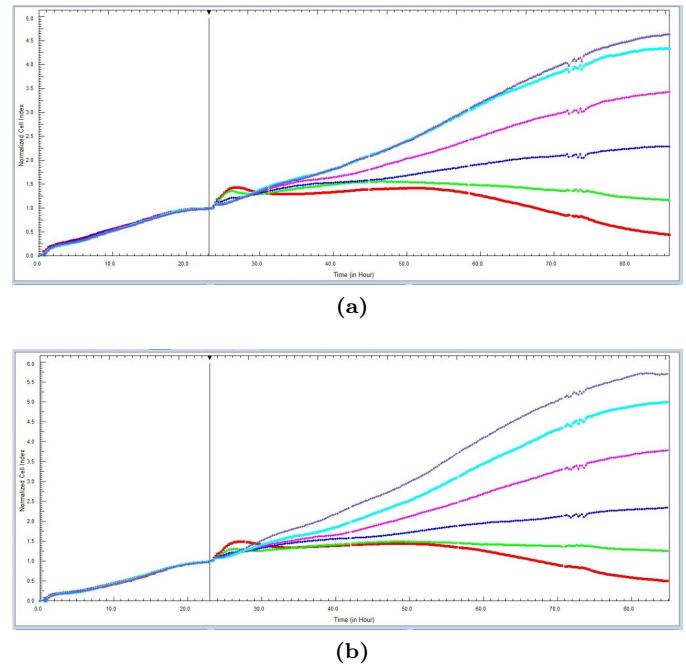


Figure 3. xCELLigence system analysis of CD133⁺ CSCs and CD133⁻ cells treated with BA. CI of the CD133⁺ (a) and CD133⁻ (b) was measured using the xCELLigence RTCA. Cells were treated 1, 3, 6, 12, and 25 mM BA. The controls were untreated cells. Black vertical line indicates the time that the BA was added. The IC₅₀ value is 6.7 mM (R₂=1) for CD133⁺ CSCs and 5 mM (R₂=1) for CD133⁻ cells.

cell viability compared to untreated cells (89, 74, 49, 25, and 12%), as indicated in Figure 4b. The RTCA system showed BA exerts a dose-dependent cytotoxic effect on CD133⁻ cells.

Discussion

Lung cancer is the most common cancer type globally [3]. CSCs are a distinct subset of tumor cells that sustain the growth of a cancerous cell population. They have the capacity to produce new tumors because of their stem cell characteristics and their ability to develop into various cell lineages. Recently, CSCs have drawn increasing attention in cancer research [10].

CD133 is one of the most commonly used markers for detecting CSCs. Previous research has discovered CD133⁺ CSCs in human lung cancer; these have the capacity to regenerate themselves and are highly tumorigenic [32]. CD133 expression is related to the prognosis, lymph node metastasis, and differentiation in NSCLC [33]. Some studies also show that lung cancer CD133⁺ cells are resistant to chemotherapy [34, 35, 36]. Several investigations have been conducted to define CD133⁺ cells in NSCLCs. In NSCLCs, CD133 expression occurs in just 1% of cells in varying but minimal proportions [32].

Sphere formation is used to evaluate the fraction of CSCs in a carcinoma cell population in addition to the CSCs' self-renewal capacity. To enhance the number of CSCs, cancer cells are cultured within serum-free, non-adherent

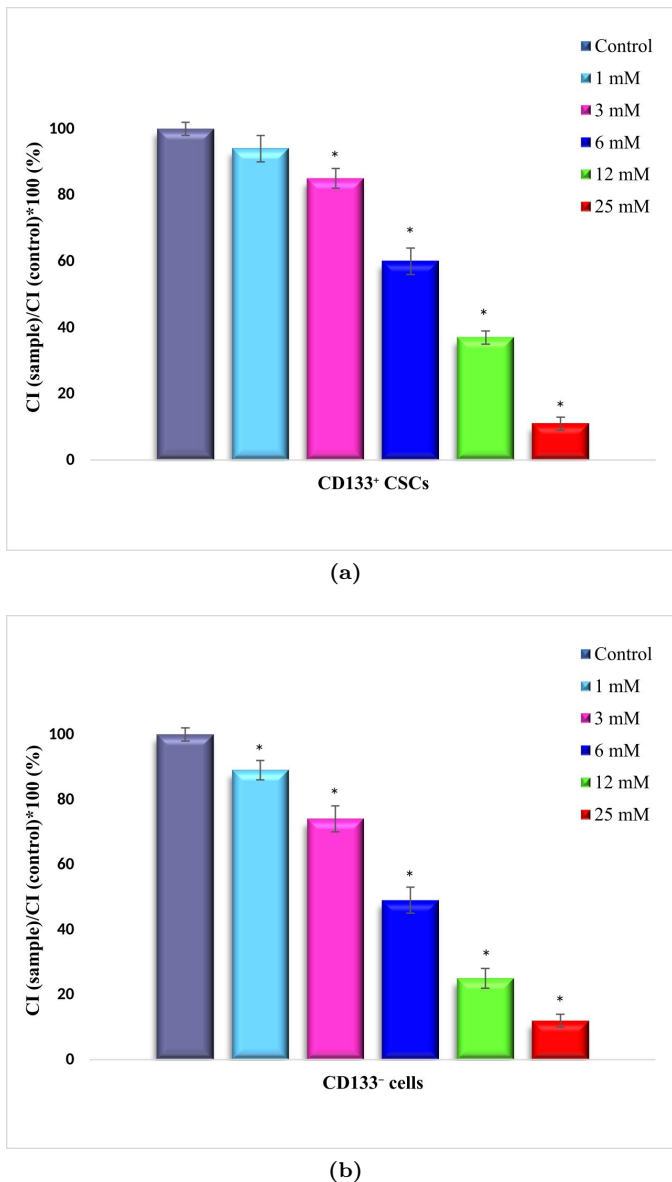


Figure 4. BA inhibits the cell growth in CD133⁺ CSCs and CD133⁻ cells. The graphs show the dynamics of the CI at the 62 h time points of BA treatment. The treated cells' CI was normalized to the CI of untreated controls and multiplied by 100. Graphs of CD133⁺ CSCs (a) and CD133⁻ cells (b) were generated in Excel; asterisks indicate BA concentrations causing statistically significant ($p < 0.05$) changes in the cell index, as assessed by one-way ANOVA analysis.

environment because only CSCs can live and proliferate there. As a result, a tumor sphere formed from a single cancer stem cell [37]. Several lung cancer studies have revealed that CD133⁺ cells produce spheroids and possess cancer stem cell properties [32, 38].

The anticancer effect of BA has been studied in many cancers, especially prostate cancer. Studies have demonstrated that BA inhibits prostate cancer cell proliferation in vitro and in vivo [21-26]. BA has reportedly inhibited cell proliferation at concentrations of 100-1000 μM in DU-145 and LNCaP prostate cancer cells [22]. 1 mM BA sig-

nificantly reduced proliferation and migration of DU-145 cells [23]. Baranco et al. found BA inhibits cell proliferation by 30% at 100 μM , 60% at 250 μM , and 97% at 1000 μM BA [24]. Likewise, McAuley et al. suggested that 1 mM of BA arrests growth and prevents migration of DU145 cells [25]. Meanwhile, DU-145 cells treated with 0.19-12.5 mM BA concentrations showed insignificantly reduced cell viability at doses below 6.25 mM. The IC_{50} of BA were defined at 10.77 mM, and BA treatment caused oxidative stress [26]. Daily BA dietary supplements of 1.7 and 9.0 mgB/kg decreased the proliferation and development of carcinoma cells in an LNCaP animal model [21]. Increased underground water boron concentration levels in Texas were associated with a lower prostate cancer incidence and mortality [39].

BA caused cytotoxicity in MDA-MB-231 human breast cancer cells in a dose-dependent manner. BA concentrations of 0.45, 2.25, 5, 11.25, and 22.5 mM resulted in relative cell survival rates of 94.9, 86, 75, 66, and 60% [27]. Skin melanoma cells cultured with BA concentrations between 0 to 50 mM showed BA concentrations of 5, 12.5, 25, and 50 mM decreased cell viability to 40%, 29%, 14%, and 3%. Together, these results suggest that 12.5, 25 and 50 mM BA concentrations affect proliferation and promote apoptosis [28]. After 24 h of BA treatment in HepG2 cells, the IC_{50} value was determined as 24 mM [40]. U87-MG glioblastoma cells cultured with 2.5 mM, 25 mM, and 50 mM boron for 48 h defined the IC_{50} value as 17 mM, and determined that high-dose boron exerts a cytotoxic effect [41].

Boron from food sources has been shown to be protective against lung cancer in women. Women who consume boron have a lower risk of lung cancer than women who consume little boron and do not use HRT [M42]. A study evaluating the anti-cancer effect of BA in SCLC cells determined the IC_{50} value to be 1000 $\mu\text{g}/\text{mL}$ after 72 h of treatment and that BA induced apoptosis by disrupting the expression of pro- and anti-apoptotic genes [29]. Treatment with 5 mM BA was found to reduce cell growth by less than 25% in NSCLC cell lines such as H1299 and COR-L23. Furthermore, short-term treatments with BA concentrations over 25 mM significantly reduce cell number and death and affect autophagy [30].

Conclusion

In this study, CD133⁺ CSCs were successfully isolated by MACS method. While the CD133⁻ cells did not form spheres, the CD133⁺ CSCs did. This is significant because CD133⁺ lung cancer stem cells exhibit stemness characteristics through sphere formation. The literature shows the anti-cancer activity of BA in various diseases but contains no study investigating CSCs. This study is the first to demonstrate BA's anti-cancer effect on CSCs. To determine BA's cytotoxic activity, live cell proliferation was monitored using xCELLigence RTCA system. Treatments with 3, 6, 12 and 25 mM BA statistically significantly decreased cell viability in both CD133⁺ CSCs and CD133⁻ cells. Additionally, BA showed a cytotoxic effect in a dose-dependent manner, with IC_{50} concentrations of 6.7 mM and 5 mM for CD133⁺ CSCs and CD133⁻ cells after 62 h treatment. Studies in the literature have ob-

served that high dose BA concentrations inhibit cell proliferation in melanoma, glioblastoma, liver, prostate, breast cancer, and lung cancer cells. Our results are consistent with the literature. Additional research could contribute to our understanding of the concentration-dependent effects and processes of BA.

Ethical approval

Commercial cell (cell line) was used in this study. Ethics committee approval is not required for such studies.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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