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Specific Blockage of Caspase-1 Activation by Purple Bamboo-Salt Prevents Apoptosis of Auditory Cell Line, HEI-OC1

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ABSTRACT Cisplatin is a highly effective chemotherapeutic agent but with significant ototoxic side effects. Apoptosis is an important mechanism of cochlear hair cell loss following exposure to an ototoxic level of cisplatin. The present study investigated the effects of purple bamboo-salt (BS) on cisplatin-induced apoptosis. We demonstrated that apoptosis induced by cisplatin was inhibited by treatment with BS in a dose-dependent manner. Activation of caspase-3, caspase-8, and caspase-9 was observed within cisplatin-treated HEI-OC1 cells. BS inhibited activation of caspase-3, caspase-8, and caspase-9. BS also inhibited release of cytochrome *c* and translocation of apoptosis-inducing factor. BS inhibited cisplatin-induced reactive oxygen species production. Lastly, BS suppressed cisplatin-induced caspase-1 activation. In conclusion, these findings show that BS blockage of a critical step in apoptosis may be a useful strategy to prevent harmful side effects of cisplatin ototoxicity in patients undergoing chemotherapy.

Key Words: • *apoptosis* • *caspase-1* • *cisplatin* • *HEI-OC1 cells* • *ototoxicity* • *purple bamboo-salt*

INTRODUCTION

CISPLATIN IS A HIGHLY EFFECTIVE and widely used anticancer agent.[1] The risk of ototoxic and nephrotoxic side effects commonly hinders the use of higher doses that could maximize its antineoplastic effects.[2] Cisplatin has been shown to induce auditory sensory cell apoptosis *in vitro*[3,4] and *in vivo*.[5] Devarajan *et al*.[6] recently have reported cisplatin-induced apoptosis in an immortalized cochlear cell line. The cytotoxic effects of cisplatin may occur via several putative pathways. One well-studied mechanism is the formation of DNA adducts, which block progression through the cell cycle. Another mechanism involves the production of reactive oxygen species (ROS).[7,8] Cisplatin toxicity was also associated with an increase in caspase-3, caspase-8, and caspase-9 activity, Bid truncation, cytochrome *c* release, and activation of mitogen-activated protein kinase, including extracellular signal-regulated kinase.[9]

Apoptosis-inducing factor (AIF), a mitochondrial intermembrane flavoprotein, has been found to translocate from mitochondria to nuclei in a caspase-independent fashion. When added to purified nuclei, recombinant AIF causes caspase-independent large-scale (~50-kb) DNA fragmentation and a type of peripheral chromatin condensation that resembles the first stage of nuclear apoptosis (stage I) observed in intact cells undergoing apoptosis.[10,11]

Caspase-1 is a member of the cysteine–aspartic acid protease (caspase) family.[12] Caspase-1 is characterized by its ability to activate the inactive precursors of interleukin-1β and interleukin-18, cytokines that are involved in inflammation. It contains an N-terminal caspase recruitment domain, which promotes proteolytic activation of recruited caspases in apoptosis and inflammation.[13]

Purple bamboo-salt (BS) is a specially processed salt according to a traditional recipe using normal salt, bamboo, pine tree firewood, pine resin, and yellow earth. It contains abundant minerals such as natural sodium, potassium, calcium, chloride, and magnesium.[14] It is known to have therapeutic effects for diseases such as viral diseases, dental plaque, gastropathy, diabetes, circulatory organ disorders, cancer, and anti-inflammatory disorders.[14–17]

Because hair cells do not regenerate in the mammalian cochlea, cell loss, *e.g*., because of noise, hypoxia, or cisplatin, is irreversible and cumulative.[4,18] Cisplatin primarily damages the outer hair cells of the organ of Corti, which are the specific effectors of mammalian cochlear amplification and frequency determination. Larger doses of cisplatin are associated with additional damage to auditory neurons, stria vascularis, and supporting cells near the outer hair cells.[19] Therefore, selective inhibition of ROS generation, AIF expression, and activation of various caspases may provide a strategy to minimize cisplatin-induced ototoxicity. The

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present study investigated the beneficial effects of BS on cisplatin-induced apoptosis.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco/BRL (Grand Island, NY, USA). Cisplatin, dimethyl sulfoxide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Caspase-3, cytochrome *c*, and AIF antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase assay kit was purchased from R & D Systems Inc. (Minneapolis, MN, USA). The cytotoxicity detection kit (lactate dehydrogenase [LDH]) was purchased from Promega (Madison, WI, USA).

Cell culture

The HEI-OC1 cell line was a kind gift from Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA, USA). Establishment of immortal cell lines has been facilitated by the development of a transgenic mouse, Immortomouse™ (Charles River Laboratories, Wilmington, MA, USA), which harbors a temperature-sensitive mutant of the *SV40 large T-antigen* gene under the control of an interferon-γ-inducible promoter element. Cochlear half turns from Immortomice at postnatal day 7 were cultured on uncoated plastic culture dishes under permissive conditions (33°C) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50 U/mL interferon-γ without antibiotics. Cochlear explants were moved to nonpermissive conditions (39°C) at different periods and allowed to differentiate for up to 180 days. Cultures were monitored on a daily basis by phase-contrast and video microscopy. Cells growing in a region formerly associated with the organ of Corti were isolated by lifting them with a micropipette after a 2–5-minute incubation of the explants with trypsin-EDTA. A cell line, termed HEI-OC1, was cloned using the limiting dilution method and characterized by phase-contrast light microscopy, western blotting, and immunofluorescence. No antibiotics were used at any step of the cloning. HEI-OC1 cells, which express several molecular markers characteristic of organ of Corti sensory cells (α-thyroid hormone, brain-derived neurotrophic factor, calbindin, calmodulin, connexin 26, Math 1, Myosin 7a, organ of Corti protein 2, tyrosin kinase receptor B and C, platelet-derived growth factor receptor, and prestin), are extremely sensitive to ototoxic drugs.[20] The cells used in this study were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 33°C in air with 5% CO_2.

Preparation of BS

BS was provided by Tae-sung Food Inc. (Jeonbuk, Republic of Korea). It was processed by a special technique: nine times processing at extremely high temperature reaching about 1,500°C with bay salt, bamboo, pine tree firewood, pine resin, and yellow earth, etc. It contains mostly sodium chloride (about 91.7% of BS) along with potassium, calcium, iron, copper, manganese, sulfur, zinc, and magnesium in elementary quantities. Powdered BS was dissolved in distilled water to reach appropriate concentrations (0.01–1 mg/mL; Dulbecco's modified Eagle's medium + 1 mg/mL = 353.28 mOsm), filtered through a syringe filter (pore size, 0.22 μm), and kept at 4°C.

MTT assay

Cells were pretreated with various concentrations of BS (0.01–1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. The cell survival fraction was determined with the MTT dye-reduction assay. In brief, after incubation with cisplatin, MTT solution (5 mg/mL BS) was added (50 μL per well). Plates were further incubated for 4 hours at 37°C, the formazan crystals formed were centrifuged, and the pellets were dissolved by addition of dimethyl sulfoxide. Absorption was measured with a spectrometer at 540 nm.

Cell death assessment by DNA fragmentation assays

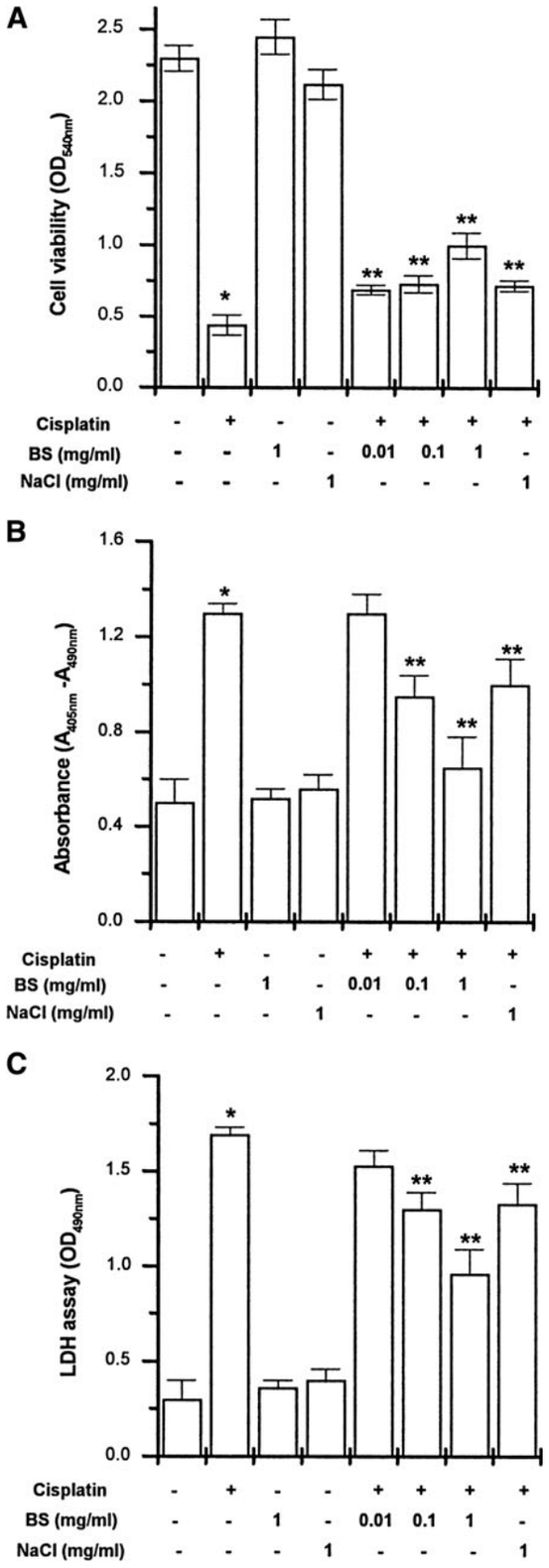
Internucleosomal DNA fragmentation was quantitatively assayed according to the manufacturer's specifications using the Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals, Mannheim, Germany) that measured accumulated DNA fragments in dying HEI-OC1 cells with intact cell membranes. Absorbance at 405 nm and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 5 minutes, was determined with a microplate reader. Signals in the wells containing the substrate only were subtracted as background.

LDH-release assay

Cells were pretreated with various concentrations of BS (0.01–1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. The plate was centrifuged at 1,000 *g* for 4 minutes. The supernatant containing the released LDH from the damaged cells was set aside. The cells remaining in the plate were lysed to release all intracellular LDH. The LDH released from the damaged cells and that from the lysed cells were separately subjected to LDH assay. In brief, 50 μL of the mixture of LDH assay substrate, cofactor, and dye solution (1:1:1 by volume) was added to each well, and the plate was incubated at room temperature for 30 minutes, followed by adding 15 μL of 1 *N* HCl to each well. The absorbance at 490 nm was measured on an enzyme-linked immunosorbent assay reader with the reference wavelength of 690 nm.

Annexin V–fluorescein isothiocyanate assay

HEI-OC1 cells were cultured in six-well plates. The culture medium was replaced with new medium when the cells were 60% confluent. Cells were pretreated with various concentrations of BS (0.01–1 mg/mL) for 2 hours and then



stimulated with cisplatin (20 μM) for 24 hours. Apoptosis was determined by staining with an annexin V–fluorescein isothiocyanate staining kit (Invitrogen, Eugene, OR, USA). The percentage of apoptotic cells was calculated using CellQuest™ software (BD Biosciences, Franklin Lakes, NJ, USA).

Caspase assay

Caspase activity was measured according to the manufacturer's specifications using a caspase assay kit (R & D Systems). Recombinant caspase-1, −3, −8, and −9 enzymes are available for use as a positive control.

Preparation of cytosol fraction

After treatment, cells were harvested by centrifugation at 600 *g* for 10 minutes at 4°C. Cytosolic fractions were obtained by selective plasma membrane permeabilization with digitonin. In brief, 1×10^6 cells were lysed for 1–2 minutes in lysis buffer (75 m*M* NaCl, 8 m*M* Na_2HPO_4, 1 m*M* NaH_2PO_4, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride, and 350 mg/mL digitonin). The lysates were centrifuged at 12,000 *g* for 1 minute at 4°C, and the supernatant was collected.

Preparation of nuclear fraction

The cells were washed with ice-cold phosphate-buffered saline, left on ice for 10 minutes, and then resuspended in isotonic homogenization buffer (250 m*M* sucrose, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* disodium EDTA, 1 m*M* disodium EGTA, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, and 10 m*M* Tris-HCl [pH 7.4]) containing a proteinase inhibitor mixture. After 80 strokes in a homogenizer, the unbroken cells were spun down at 30 *g* for 5 minutes. The nuclei fractions were fractionated at 750 *g* for 10 minutes from supernatant. The nuclei fraction was washed three times with homogenization buffer containing 0.01% Nonidet P40.

Western blot analysis

Cell extracts were prepared by the detergent lysis procedure. Samples were heated at 95°C for 5 minutes and briefly cooled on ice. Following the centrifugation at 15,000 *g* for 5 minutes, 50-μg aliquots were resolved by

FIG. 1. Effect of bamboo-salt (BS) on cisplatin-induced apoptosis. (**A**) Cells (1×10^5 per well) were treated with various concentrations of BS (0.01–1 mg/mL) or NaCl (1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. Cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. OD, optical density. (**B**) Internucleosomal DNA fragmentation was quantitatively determined by assaying for cytoplasmic mononucleosome- and oligonucleosome-associated histone accumulated in membrane-intact cells at the indicated time points. A, absorbance. (**C**) Lactate dehydrogenase (LDH) levels were assayed by a cytotoxic assay kit. Data are mean ± SD values of three independent experiments performed in duplicate. $*P < .05$, significantly different from unstimulated cells; $**P < .05$ compared with cisplatin alone.

10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 m*M* Tris (pH 8.5), 200 m*M* glycerin, and 20% methanol at 25 V. Blots were blocked for at least 2 hours with 1×phosphate-buffered saline containing 0.05% Tween 20 containing 5% nonfat dry milk and then incubated with primary antibodies for 1 hour at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Bioseciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Measurement of intracellular ROS generation

The intracellular ROS level was measured using a fluorescent dye, 2′,7′-dichlorofluorescein (DCF) diacetate (DCFH-DA) (Eastman Kodak, Rochester, NY, USA). In the presence of an oxidant, DCFH is converted into the highly fluorescent DCF. For assay, HEI-OC1 cells were cultured for overnight and then treated with 20 μM cisplatin in the presence or absence of BS. Cells were washed twice with serum-free medium without phenol red and incubated with 5 μM DCFH-DA in serum-free medium without phenol for 10 minutes. After two washes with serum-free medium without phenol, the fluorescent intensity was measured with excitation at 485 nm and emission at 538 nm in a spectrofluorometer.

Statistical analysis

The experiments shown are a summary of the data from at least three experiments and are presented as mean ± SD values. Statistical evaluation of the results was performed by analysis of variance with Tukey's *post hoc* test. The results were considered significant at a value of $P < .05$.

RESULTS

Effect of BS on cisplatin-induced apoptosis

To determine the effects of BS on cisplatin-induced apoptosis, HEI-OC1 cells were exposed to 20 μM cisplatin in combination with increasing concentrations of BS (0.01–1 mg/mL). We first assessed the effect of BS, NaCl, $MgCl_2$, $ZnSO_4$, or KCl on viability of HEI-OC1 cells using the MTT assay. As shown in Figure 1A, when the cells were treated for 48 hours with cisplatin, the cell viability significantly decreased compared with the medium control ($P < .05$). BS or NaCl significantly inhibited cisplatin-induced cell death of the HEI-OC1 cells ($P < .05$). BS or NaCl also inhibited the nucleosome-sized DNA fragmentation and LDH increase by cisplatin (Fig. 1B and C). However, $MgCl_2$, $ZnSO_4$, or KCl did not inhibit cisplatin-induced cell death (data not shown). This indicates that NaCl is an active component of BS. However, the protective effect of BS was greater than that of NaCl. Cell death induced by cisplatin as determined by morphology was decreased by pretreatment with BS (1 mg/mL) (Fig. 2). ◀F1 ◀F2

Effect of BS on cisplatin-induced apoptotic cells

To determine whether BS inhibited apoptosis, the early translocation of phosphatidylserine from the internal to external leaflet, a hallmark of early apoptosis, was investigated by incubating HEI-OC1 cells with BS (1 mg/mL). Dot-plots were obtained by staining with annexin V and propidium

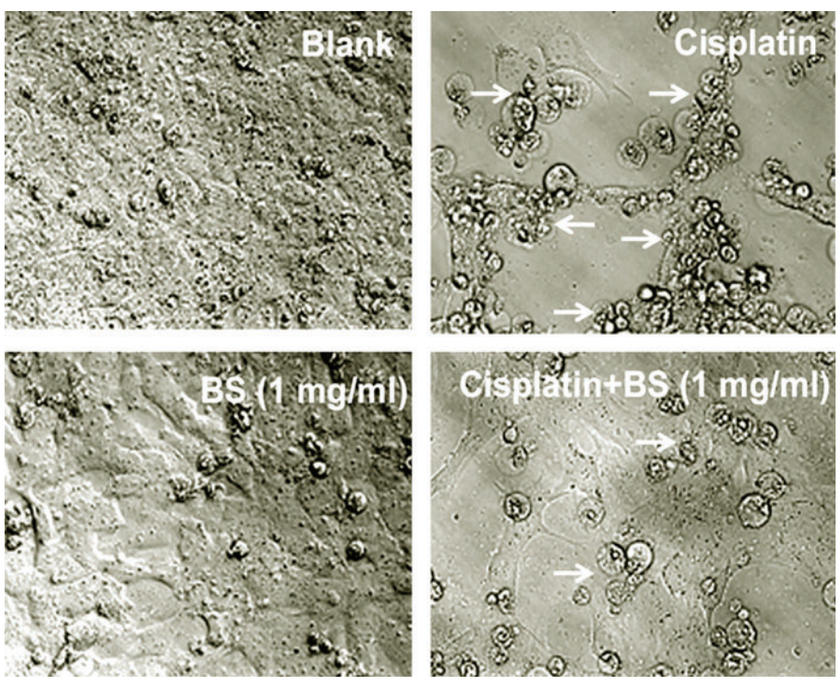


FIG. 2. Effect of BS on cisplatin-induced apoptotic bodies. Cells (1×10^5 per well) were treated with BS (1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. The apoptotic body was photographed by microscopy. Data are representative of three independent experiments. Original magnification, ×400. ◀4C

iodide (PI) as a measure of early apoptotic (lower right quadrant, annexin V^+/PI^- cells), late apoptotic (upper right, annexin V^+/PI^+ cells), and necrotic (upper left, annexin V^-/PI^+ cells) cell populations; the lower left quadrant (annexin V^-/PI^- cells) depicts live cells.

F3 ▶ As shown in Figure 3, treatment of HEI-OC1 cells with cisplatin (20 μM) resulted in about 74.52% of the cells being positive for annexin V binding (annexin V^+/PI^- staining cells) (unstimulated cells, 16.66%). Prior treatment of HEI-OC1 cells with BS or NaCl blocked annexin V binding 24 hours after cisplatin treatment (by 57.61% for BS and 69.98% for NaCl, respectively).

Effect of BS on cisplatin-induced caspase-3, caspase-8, and caspase-9 activation

Caspase-3, caspase-8, and caspase-9 activities are known to increase in cisplatin-induced apoptosis in HEI-OC1 cells. To determine if BS inhibits caspase activation induced by cisplatin, cells were exposed to 20 μM cisplatin in the presence or absence of various concentrations of BS (0.01–1 mg/mL). Extracts prepared from HEI-OC1 cells exposed to cisplatin contained strong caspase-3, caspase-8, and caspase-9 activity (0.52 ± 0.05 for caspase-3, 0.26 ± 0.50 for caspase-8, and 0.15 ± 0.01 for caspase-9, respectively) compared with unstimulated cells (0.25 ± 0.01 for caspase-3, 0.08 ± 0.00 for caspase-8, and 0.05 ± 0.00 for caspase-9, respectively). As shown in Figure 4, increased caspase-3, caspase-8, and caspase-9 activities were significantly inhibited by treatment with BS (0.20 ± 0.04 for caspase-3, 0.12 ± 0.01 for caspase-8, and 0.05 ± 0.00 for caspase-9 at 1 mg/mL, respectively, $P < .05$). However, NaCl significantly inhibited cisplatin-induced caspase-3 and −8 activation but not caspase-9. Inhibitory effects of BS were greater than that of NaCl on cisplatin-induced apoptosis. ◀F4

Effect of BS on cisplatin-induced cytochrome c *release*

The mitochondrial apoptotic cascade requires the release of intermitochondrial membrane cytochrome *c* to the cytosol. To determine if BS inhibits cytochrome *c* release induced by cisplatin, cells were exposed to 20 μM cisplatin in the presence or absence of various concentrations of BS (0.01–1 mg/ml). Cytochrome *c* release from mitochondria into cytosol was evaluated by western blot analysis, which indicated that cisplatin treatment induced cytochrome *c* release, which was inhibited by BS (Fig. 5A). ◀F5

Effect of BS on cisplatin-induced AIF translocation

BS did not completely inhibit the cisplatin-induced caspase activation (Fig. 4). Because AIF is known to be involved in apoptosis through a caspase-independent pathway, we next examined whether BS inhibits cisplatin-induced AIF translocation into the nucleus. As shown in Figure 5B,

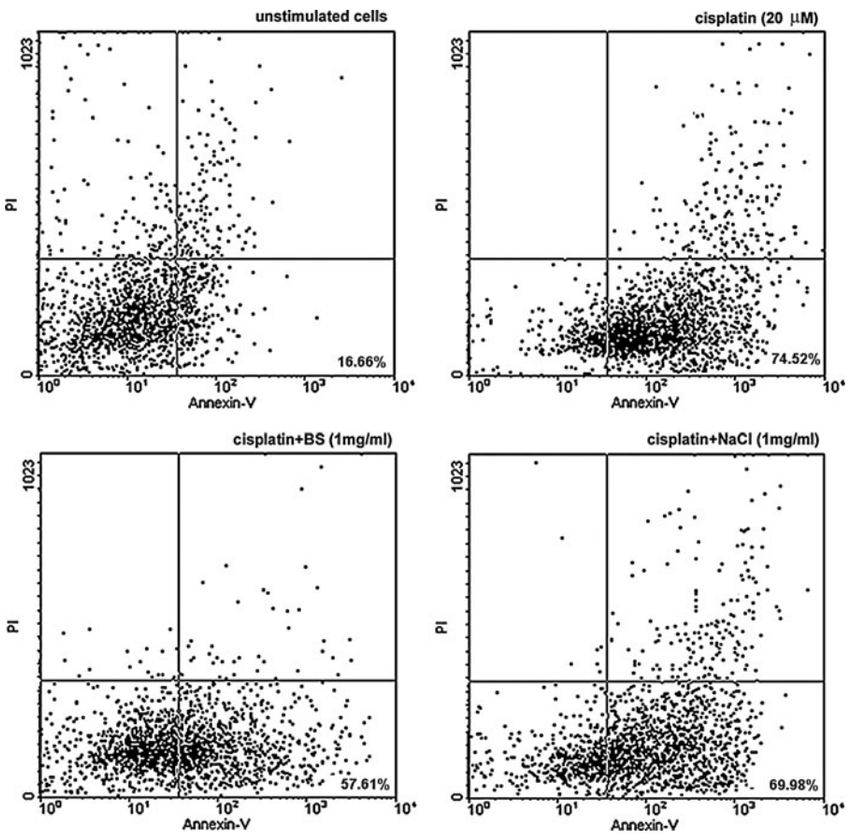
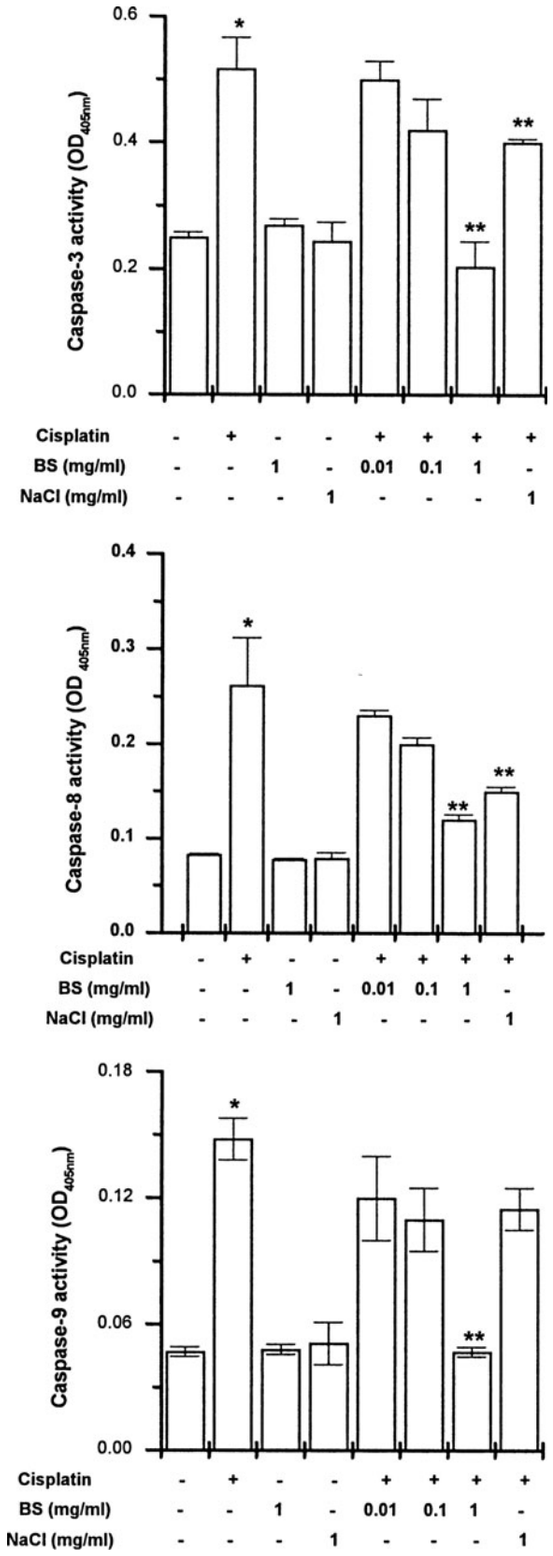


FIG. 3. Effect of BS on cisplatin-induced apoptosis by flow cytometry analysis. Cells (1×10^5 per well) were treated with various concentrations of BS (0.01–1 mg/mL) or NaCl (1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 24 hours. Cell apoptosis and necrosis were assessed by flow cytometry using annexin V and propidium iodine staining (PI), respectively. Data are representative of three independent experiments. Lower left quadrant, annexin V^-/PI^- cells (normal); lower right quadrant, annexin V^+/PI^- cells (early apoptosis); upper right quadrant, annexin V^+/PI^+ cells (late apoptosis); upper left quadrant, annexin V^-/PI^+ cells (necrosis).



cisplatin induces mitochondrial AIF release and translocation to the nucleus. BS (1 mg/mL) prevents cisplatin-induced AIF translocation.

Effect of BS on cisplatin-induced ROS generation

Next, we investigated the effect of cisplatin on intracellular ROS generation. Cells were treated with 20 μM cisplatin for 24 hours. Then, the level of intracellular ROS was monitored by spectrofluorometry using a peroxide-sensitive fluorescent probe, DCFH-DA. As shown in Figure 6, treatment of cisplatin significantly increased the generation of intracellular ROS. We found that BS significantly inhibited the intracellular ROS generation by cisplatin (about 50% at 1 mg/mL, $P < .05$). ◀F6

Effect of BS on cisplatin-induced caspase-1 activation

Caspase-1 is activated in a variety of cell death paradigms, and it can be activated by treatment with cisplatin.[21] To determine if BS inhibits caspase-1 activation induced by cisplatin, cells were exposed to cisplatin in the presence or absence of various concentrations of BS. Extracts prepared from HEI-OC1 cells exposed to cisplatin contained strong caspase-1 activity compared with unstimulated cells. As shown in Figure 7A, increased caspase-1 activity was significantly inhibited by treatment with BS in HEI-OC1 cells. Next, we investigated the effect of BS on caspase-1 activation induced by cisplatin. Western blot analysis for caspase-1 was performed. Caspase-1 activation was significantly decreased by the treatment with BS (Fig. 7B). ◀F7

DISCUSSION

The findings herein show for the first time that BS inhibits the cisplatin-induced apoptosis of auditory cells. Furthermore, BS suppressed the activation of caspase-1 in HEI-OC1 cells.

The nature of apoptotic signals from mitochondria has been well documented.[22] Cytochrome *c* is well known as a pro-apoptotic molecule released from the mitochondria.[23] Cytochrome *c* usually is released from the mitochondrial intermembrane space into the cytosol as a consequence of the mitochondrial membrane potential loss.[23] Cytochrome *c* released from the mitochondria forms a complex with procaspase-9 and apoptotic protease-activating factor-1, resulting in activation of procaspase-9. In this study, cytochrome *c* release and caspase activation were prevented by BS

FIG. 4. Effect of BS on cisplatin-induced caspase-3, caspase-8, and caspase-9 activation. Cells (1×10^6 per well) were treated with various concentrations of BS (0.01–1 mg/mL) or NaCl (1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. Caspase-3, caspase-8, and caspase-9 activities were determined by a colorimetric kit using substrates. Data are mean ± SD values of three independent experiments performed in duplicate. $*P < .05$, significantly different from unstimulated cells; $**P < .05$ compared with cisplatin alone.

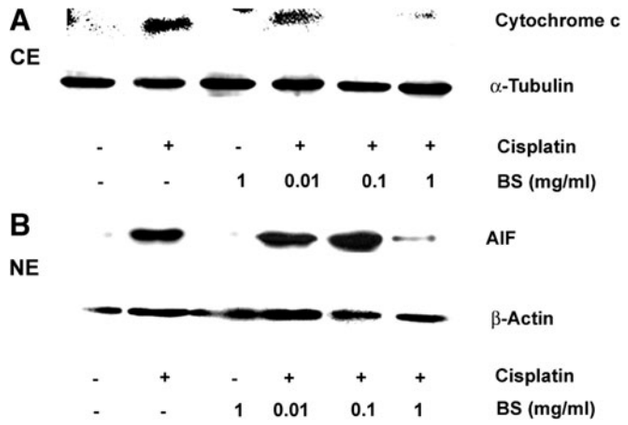


FIG. 5. Effect of BS on cisplatin-induced (**A**) cytochrome *c* release and (**B**) apoptosis-inducing factor (AIF) translocation to the nucleus. Cells (1×10^6 per well) were treated with various concentrations of BS (0.01–1 mg/mL) for 2 hours and then stimulated with cisplatin (20 μM) for 48 hours. Cytochrome *c* release into cytosol and AIF translocation were determined by western blot analysis. Results are representative of three independent experiments. CE, cytosol extract; NE, nuclear extract.

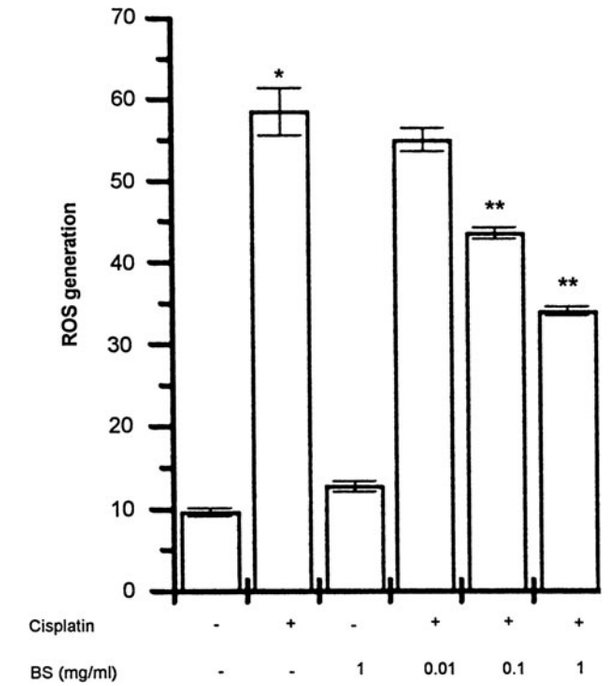


FIG. 6. Effect of BS on cisplatin-induced reactive oxygen species (ROS) generation. Cells (1×10^4 per well) were treated with various concentrations of BS (0.01–1 mg/mL) for 2 hours. ROS generation was increased 24 hours after cisplatin treatment. Data are mean ± SD values of three independent experiments performed in duplicate. $*P<.05$, significantly different from unstimulated cells; $**P<.05$ compared with cisplatin alone.

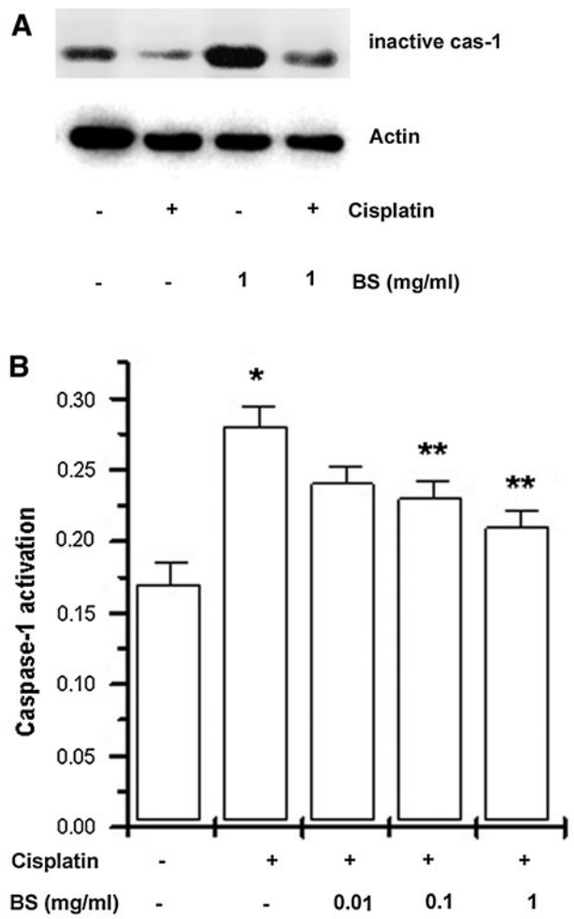


FIG. 7. Effect of BS on cisplatin-induced caspase-1 activation. Cells (1×10^4 per well) were treated with various concentrations of BS for 24 hours. (**A**) The level of caspase-1 was assayed by western blot analysis. (**B**) The enzymatic activity of caspase-1 was tested by a caspase colorimetric assay. Data are mean ± SD values of three independent experiments. Inactive cas-1, inactive form of caspase-1.

◀AU1

treatment. These results indicate that BS prevents caspase activation through inhibition of cytochrome *c* release.

AIF is a protein that is normally located in the intermembrane space of mitochondria. When the cell received a stimulus, AIF is released from the mitochondria and then translocated to the nucleus and bound to DNA, which triggers the destruction of the DNA and cell death. AIF then shuttles from mitochondria to the nucleus and induces chromatin condensation, large-scale fragmentation of DNA, and, ultimately, apoptosis.[24] AIF is an important factor for caspase-independent apoptosis. In our study, BS also inhibited the expression of AIF in cisplatin-treated HEI-OC1 cells. From this, we speculate that BS also inhibited the apoptosis via inhibition of a caspase-independent pathway.

ROS play important roles in the regulation of several normal physiological processes, including cell proliferation,

survival, senescence, and apoptotic cell death.[25] In some circumstances, increased ROS levels represent the first step in a series of events from which result in cell death. The elucidation of ROS generation in cisplatin ototoxicity is very important for understanding the pathogenesis of cisplatin ototoxicity. In the present study, ROS generation was initiated after cisplatin stimulation, and BS blocked the increase of ROS produced by cisplatin. Therefore, ROS generation may be critical for cisplatin-induced cell death in HEI-OC1 cells, and blocking ROS may be responsible for the protective effect of BS.

Caspase proteases constitute a family of proteases that normally exist as inactive enzymes. These are cysteine-dependent, aspartate-specific proteases that function to mediate apoptotic destruction of the cell.[13,26] Caspases are activated by extrinsic and intrinsic apoptotic pathways.[27] Caspase-1 is an apical caspase in neuronal cell death pathways, mediating Bid cleavage, release of mitochondrial apoptogenic factor (cytochrome *c*, Smac/Diablo, and AIF), and activation of caspase-3 and caspase-9.[28] Recent research has shown that ovarian cancer cell death is associated with the activation of caspase-1, caspase-3, and caspase-9.[29] In our study, BS inhibited cisplatin-induced caspase-3, −8, and −9 activities. These results suggest that BS inhibits cisplatin-induced apoptosis via a blockade of the caspase-1 pathway.

BS contains abundant minerals such as natural sodium, potassium, calcium, chloride, magnesium, manganese, iron, zinc, copper etc. Maintenance of ion concentration gradients is essential for the function of many organs, including the kidney, the cornea, and the inner ear. In the inner ear, the stria vascularis is responsible for generating proper ion concentrations in the endolymph, which is essential for hearing. Mutations of *SLC4A11* (sodium transporter) in humans lead to syndromes associated with deafness.[30] The efficacy of magnesium, administered either to prevent or to treat noise-induced hearing loss, has been demonstrated in several studies in animals and in humans.[31] Various mineral ions also play a crucial role in many cell functions such as cell proliferation, energy metabolism, protein and DNA syntheses, cytoskeleton activation, and ROS scavenging activity. Cisplatin enhances urinary sodium excretion in male rats.[32] Cisplatin treatment caused a significant decrease in magnesium. Bodnar *et al.*[33] reported the protective effect of magnesium salt supplementation in cancer patients. Cisplatin results in hearing loss via inducing an ion imbalance. BS and NaCl, but not $MgCl_2$, $ZnSO_4$, or KCl, inhibited the cisplatin-induced apoptosis. The protective effect was greater with BS than with pure NaCl. Therefore, these results suggest that the combination of NaCl and various other mineral ions has a synergistic effect. However, other active components of BS should be identified in further studies to clarify whether the components may also be effective in hearing loss.

In conclusion, we have demonstrated that treatment with BS inhibited the cisplatin-induced apoptosis via inhibition of membrane and mitochondrial apoptotic pathways. Further investigation is necessary to determine other possible anti-apoptotic mechanisms of BS and to apply it clinically in ototoxicity environments.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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AU1: '*' and '**' are found in figure but not explained in legend. Please check.