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Research Article

Protective Effect of Metformin Against Cisplatin-Induced Ototoxicity in an Auditory Cell Line

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ABSTRACT

Metformin, an antidiabetic drug with potent anticancer activity, is known to prevent oxidative stress-induced cell death in several cell types through a mechanism dependent on the mitochondria. In the present study, we investigated the influence of metformin on cisplatin ototoxicity in an auditory cell line. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (Sigma, St. Louis, MO, USA) cell proliferation assay. Oxidative stress and apoptosis were assessed by flow cytometry analysis, Hoechst 33258 staining, reactive oxygen species (ROS) measurement, and western blotting. Intracellular calcium concentration changes were detected using calcium imaging. Pretreatment with 1 mM metformin prior to the application of 20 μM cisplatin significantly decreased the frequency of late apoptosis in HEI-OC1 cells and also significantly attenuated the cisplatin-induced increase in ROS. In addition, metformin inhibited the activation of caspase-3 and levels of poly-ADP-ribose polymerase (PARP). Pretreatment with metformin prevented the cisplatin-induced elevation in intracellular calcium concentrations. We propose that metformin protects against cisplatin-induced ototoxicity by inhibiting the increase in intracellular calcium levels, preventing apoptosis, and limiting ROS production.

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INTRODUCTION

Cisplatin is an important chemotherapeutic agent used in the treatment of solid tumors such as ovarian, cervical, testicular, lung, and head and neck cancers. However, treatment is accompanied by dose-limiting adverse effects such as nephrotoxicity and ototoxicity. While nephrotoxicity can to some extent be reversed by increasing saline hydration, there is currently no way to cure or prevent ototoxicity. Ototoxicity is a medication-induced auditory or vestibular functional loss that results in hearing loss or disequilibrium (Roland and Cohen 1998). Cisplatin induces bilateral and irreversible hearing loss, and the elevation of hearing threshold has been reported in 75–100 % of patients treated with cisplatin (McKeage 1995).

Though the mechanism mediating cisplatin-induced ototoxicity is not completely understood, it is believed to be different from that involved in the drug's anti-tumor activity. Cisplatin-induced ototoxicity is related to the drug's interaction with cochlear tissues such as outer hair cells, the stria vascularis, the spiral ligament, and spiral ganglionic cells. These interactions generate reactive oxygen species (ROS) (Clerici et al. 1995; Dehne et al. 2001; Banfi et al. 2004) while depleting the antioxidant enzyme system that would scavenge and neutralize this increase in superoxides (Rybak et al. 2000) The cochlea, because of its unique anatomical position as a closed system, is unable to rapidly flush out accumulated toxin. This

leads to an ROS overload, which in combination with reduced antioxidant activity leads to cell injury and apoptosis. Furthermore, ROS-induced mitochondrial dysfunction has been implicated in the pathogenesis of cisplatin-induced ototoxicity (Davis et al. 2001). Other reports showed that cisplatin induces apoptosis in auditory hair cells by triggering the mitochondrial apoptotic pathway (Boulikas and Vougiouka 2003).

Apoptosis is primarily regulated by the activation of caspases through either internal or external pathways (Rybak and Kelly 2003). The internal pathway is initiated by free radicals or stress signals; mitochondria then release apoptogenic factors through the mitochondrial permeability transition pore (PTP) (Jacotot et al. 1999) into the cytoplasm to initiate apoptosis in a caspase-dependent or caspase-independent manner. The molecular characteristics of PTP are not well known, but calcium is known to play a role (Bernardi et al. 2006). One study demonstrated that highly toxic aldehyde 4-hydroxynonenal (4-HNE) generated by the interaction between free hydroxyl radicals and the cell membrane has been associated with increased calcium influx into the outer hair cell and apoptosis (Lee et al. 2004a, b).

Recent investigations of metformin, an antidiabetic drug, have shown that this agent prevents oxidative stress-induced death in several cell types through a mechanism that attenuates PTP opening and cytochrome c release (Guigas et al. 2004; El-Mir et al. 2008). Over the past decade, it has gained significant attention as a potent anticancer drug (Ben Sahra et al. 2010; Gonzalez-Angulo and Meric-Bernstam 2010).

The aim of this study was to examine the potential protective properties of metformin against cisplatin-induced ototoxicity in an auditory cell line.

METHODS

HEI-OC1 cell culture

The HEI-OC1 cell line was provided by F. Kalinec (House Ear Institute, Los Angeles, CA, USA). Establishment of the HEI-OC1 cell line was facilitated by the development of a transgenic mouse, Immortomouse, which harbored a temperature-sensitive mutant of the SV40 large T antigen gene. Cochlear half-turns from Immortomice at postnatal day 7 were cultured under permissive conditions (33.8 °C) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) and 50 U/mL interferon-γ without antibiotics. Extremely sensitive to ototoxic drugs, HEI-OC1 cells express several molecular markers which are characteristic of organ of Corti sensory cells (Kalinec et al. 2003). The HEI-OC1 cell line is therefore a useful experimental model for the study of ototoxic drugs. The cells used in this study were maintained in DMEM with 10 % FBS at 33 °C under 10 % CO_2 in air.

Calcium imaging

The intracellular Ca^{2+} concentration in HEI-OC1 cells was measured with Fura-2AM (5 μM) using digital microscope (Universal; Carl Zeiss, Inc., Thornwood, NY, USA). The cells were divided into four groups: a control group, a metformin (1 mM) group, a cisplatin (6 mM) group, and a cisplatin (6 mM) group of cells that were pretreated with metformin (1 mM). In the metformin pretreatment group, the cells were incubated with 1 mM metformin for 24 h in advance. Then, a field of cells was monitored by sequential dual excitation at 340 and 380 nm, and the ratio was analyzed as described elsewhere (MacGlashan 1989). The ratio images were acquired every 3 s (1 cycle), and agents were applied at the 30th cycle (90 s). Cells were finally exposed to ionomycin (10 μM) which was used to obtain the maximum obtainable response (positive control).

MTT assay

The uptake and conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma, St Louis, MO, USA) to crystals of dark violet formazan provides an index of cell viability. Thus, HEI-OC1 cells (10^5 cells/well of 24-well plate) were incubated with 20 μM of cisplatin (a concentration which is known to result in 48 (±6.9)% cell viability after 48 h; Fig. 1) for 48 h, and the effects of cisplatin were measured using an MTT assay. In order to examine the effects of metformin on cisplatin ototoxicity in the auditory cell line, the cells were pretreated with metformin (1 mM) for 24 h, and then exposed to cisplatin (20 μM, 48 h). For the MTT assay, 50 μL of MTT solution (0.25 mg) were added to 0.5 mL of the cell suspension, and the plates were then incubated for 4 h at 33 °C in 10 % CO_2. The insoluble formazan crystals were centrifuged, and the pellets were dissolved by the addition of DMSO (500 μL/well). Optical density was measured using a microplate reader at 570 nm (Spectra Max, Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry analysis

The rate of apoptosis among HEI-OC1 auditory cells treated with 20 μM cisplatin for 48 h (in the group pretreated with metformin as well as the nonpretreated group) was determined using Annexin V-FITC (Ezway Annexin V-FITC Apoptosis Detection Kit, Komabiotech, Seoul, Korea). The

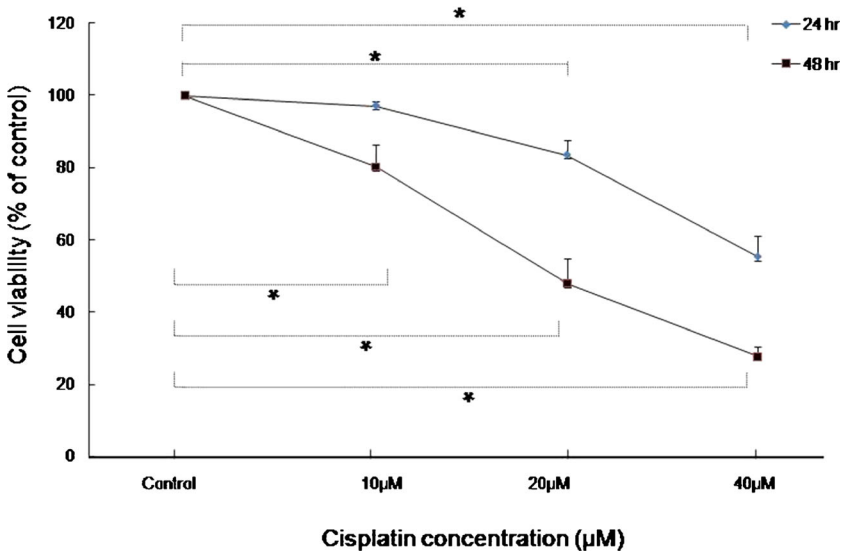

FIG. 1. Cytotoxic effect of cisplatin on HEI-OC1 cells. Viable cells were counted using the MTT assay. This figure is expressed as a percentage of the untreated control cells in a time- and dose-dependent manner. Cells were treated with 10, 20, or 40 μM of cisplatin for 24 and 48 h. When cells were treated with 10, 20, and 40 μM cisplatin for 24 h, the cell viability was 97.2 (±1.2), 83.6 (±3.7), and 55.3 (±5.2)%, respectively. In addition, the cell viability for 10, 20, and 40 μM cisplatin for 48 h were 80.2(±6.1), 48.0 (±6.99), and 27.6 (±2.9)%, respectively. Twenty micomoles of cisplatin for 48 h was selected for further experiments ($P<0.001$, for comparison between the control and 20 μM).

specific binding of Annexin V-FITC was performed by incubation of the cells for 15 min at room temperature in binding buffer containing a saturating concentration of Annexin V-FITC and PI (propidium iodide). After washing with binding buffer, the cells were resuspended with 500 μL binding buffer containing 10 μL PI. Cells that had undergone the late apoptosis were identified by PI staining. The results were acquired and analyzed with CellQuest Pro software. The high mean fluorescence of Annexin V-FITC in a flow cytometric histogram was used to characterize the apoptotic cell population. The entire procedure was repeated three times.

Measurement of intracellular ROS production and Hoechst 33258 staining

Intracellular ROS levels were measured using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY, USA). In the presence of an oxidant, DCFH is converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF). For the assay, HEI-OC1 cells were cultured on cover slips, and then treated with 20 μM cisplatin for 48 h in the presence or absence of metformin (1 mM, 24 h pretreatment). Cells were washed twice with serum-free medium without phenol red and incubated with 5 mM DCFH-DA in serum-free medium without phenol red for 15 min at 33 °C. After three washings with serum free medium without phenol red, cells were fixed with 3.7 % glutaraldehyde for 10 min at room temperature. Cells were incubated with 10 μg/mL Hoechst 33258 (Sigma, St Louis, MO, USA) for 20 min at room temperature in the dark. After washing twice with PBS, the samples were immediately observed at excitation 387 nm. Fluorescent intensity was then measured at excitation of 485 nm and emission of 538 nm using a long-term real-time live cell image system (LAMBDA DG-4, Sutter Int., Novato, CA, USA). H_2O_2 20 mM was used as a positive control.

Measurement of caspase-3 activity

The enzymatic activity of caspase-3 was assayed with a caspase colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Auditory cell line lysate was prepared in a lysis buffer on ice for 10 min and centrifuged for 5 min at 14,000 rpm. The protein concentration in each lysate was measured. The catalytic activity of caspase-3 in the cell lysate was measured by proteolytic cleavage of 200 μM DEVD-pNA and colorimetric substrate for 2 h at 37 °C. The mixture incubated with no DEVD-pNA substrate was used as a negative control. The plates were read at 405 nm with a microplate reader (Spectra Max, Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis of PARP

Poly-ADP-ribose polymerase (PARP) primary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). For the assay, HEI-OC1 cells were cultured and treated with 20 μM cisplatin for 48 h in the presence or absence of metformin (1 mM, 24 h pretreatment). Cell lysates were used in Pro-prep lysis buffer (Intron Biotechnology, Seongnam-Si, Korea). The proteins (40 μg/sample) were immediately heated for 5 min at 100 °C and were subjected to SDS-PAGE on gels. Separated proteins were transferred to nitrocellulose membranes, and western blotting was performed using a gel loading kit and protein transfer system kit (BioRad, Hercules, CA,

USA). Membranes were blocked by treatment with 5 % skim milk in Tris-buffered solution (TBS) with Tween and subsequently incubated with primary polyclonal antibodies at a final dilution of 1:1,000. After three washes in TBS containing 0.1 % Tween, membranes were incubated with peroxidase-conjugated secondary antibodies (final dilution, 1:2,000) in TBS for 2 h and subsequently washed. Detection was performed by chemiluminescence using an Enhanced ChemiLuminescence kit (ECL; Amersham Life Science, Braunschweig, Germany) and subsequently using a Multiple Gel-DOC system (BioRad, Hercules, CA, USA). Beta actin was used as a control.

Statistical analysis

All values are represented as mean±SD. For data analysis, we used the SPSS 20.0 statistical program. For comparing means between the baseline and the response to agents during calcium imaging analysis, we used a paired *t* test. For the comparison of multiple groups in the MTT assay, flow cytometry analysis, ROS-, and caspase-3 activity, ANOVA was used. A *P* value of <0.05 was considered statistically significant. For multiple comparisons, Bonferroni correction was done.

RESULTS

Calcium imaging

There was no change in intracellular calcium concentrations until the application of ionomycin in the control group (P=0.889; Fig. 2A). Ionomycin was used to extract the maximal response. When cisplatin (6 mM) was applied at the 30th cycle (90 s), there was a prompt elevation of intracellular calcium concentrations (P<0.001; Fig. 2C). There was no change in intracellular calcium concentration, when metformin (1 mM) was applied at the 30th cycle (P= 0.262; Fig. 2B). However, when cells were pretreated with metformin (1 mM) for 24 h in advance, there was no significant change in intracellular calcium concentrations after the addition of cisplatin at the 30th cycle (6 mM) (P=0.877; Fig. 2D).

MTT assay

To determine whether metformin was able to prevent cisplatin-induced apoptosis, cell viability in HEI-OC1 cultures was determined by MTT assay. When cultured cells were exposed to 20 μM cisplatin and 1 mM metformin, cell viability was 53.2 (±5.4) and 95.5 (±3.3)%, respectively (Fig. 3). However, when the cells were exposed to 20 μM cisplatin after pretreatment with 1 mM metformin for 24 h, cell viability was 69.3 (±11.1)% (Fig. 3). This was significantly higher than the level of viability observed in the group treated with cisplatin alone (P=0.015).

Flow cytometry analysis

There were no significant differences in late apoptosis between the controls (1.4 %) and metformin added groups (0.6 %) (Fig. 4A, B; P=0.779). When 20 μM cisplatin was applied, the proportion of cells undergoing late apoptosis increased (59.2 %) compared with the level observed in the control group (1.4 %) (Fig. 4C; P=0.012). The proportion of late apoptosis decreased significantly when cells were exposed to 1 mM metformin (20.8 %) prior to cisplatin treatment (Fig. 4D; P=0.017).

Measurement of intracellular ROS production and Hoechst 33258 staining

Apoptosis was evaluated by the appearance of condensed and fragmented nuclei as revealed by Hoechst 33258 staining. Condensed nuclei were observed among cells exposed to 20 μM cisplatin for 48 h but not among those in the control or metformin groups (Fig. 5A–C). Cell density was also reduced in the cisplatin group; this change was attributed to an increase in the rate of apoptosis. However, when the cells were pretreated with metformin before cisplatin application (Fig. 5D), the nuclei were only slightly condensed.

There was an increase in ROS when cisplatin was applied (Fig. 5H), but when the cells were pretreated with metformin, the ROS increase was reduced in magnitude (Fig. 5I). ROS levels increased 1.8±0.1-fold after exposure to 20 μM cisplatin but ROS levels significantly decreased to 1.5±0.1-fold after metformin pretreatment (Fig. 6, P=0.008). H_2O_2 was applied for the positive control (1.9±0.6).

Measurement of caspase-3 activity

Caspase-3 activity is involved in cisplatin-induced toxicity and related to apoptotic changes in cisplatin ototoxicity. As shown in Figure 7, the administration of 20 μM cisplatin increased the activity of caspase-3 (2.5±0.7-fold over the normal control). The pretreatment of HEI-OC1 cells with metformin, however, significantly decreased caspase-3 activity (1.4±0.2-fold) as compared with cells treated with cisplatin alone (P=0.001).

Western blot analysis of PARP levels

The inhibitory effect of metformin on PARP was investigated using western blotting (Fig. 8). Cisplatin

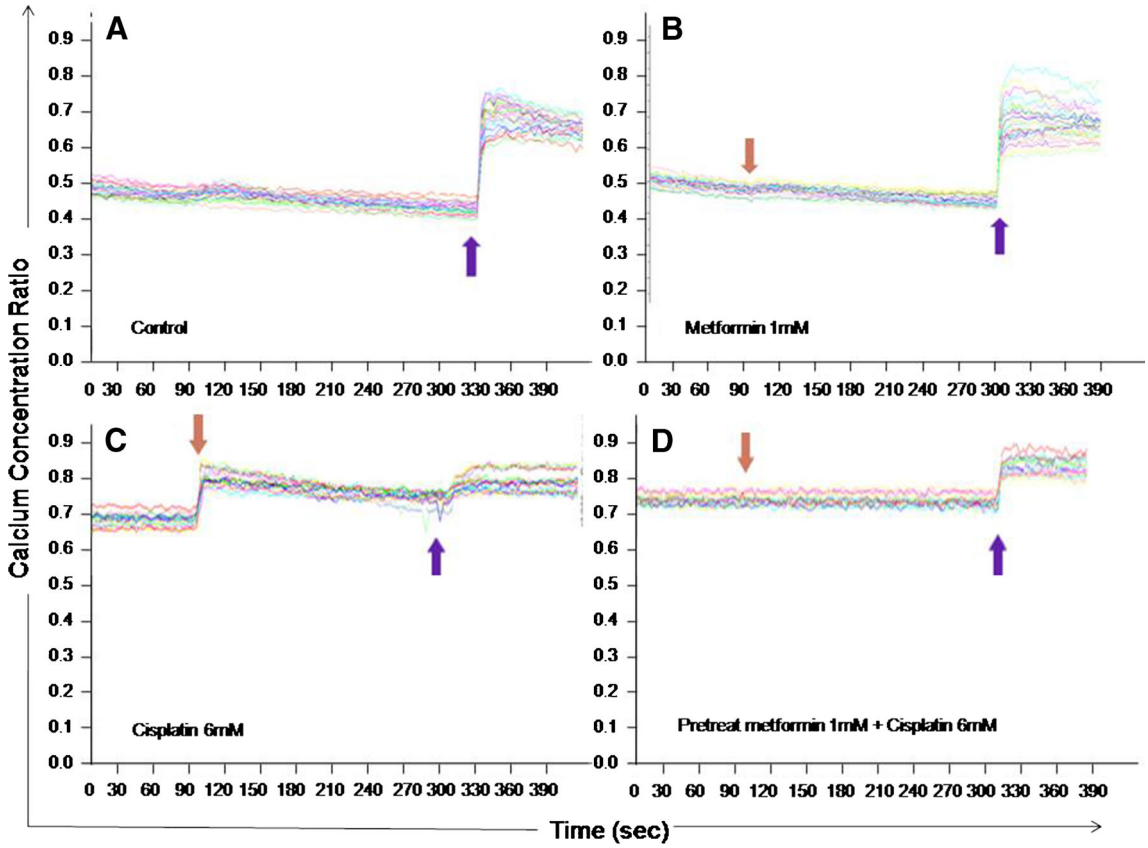

FIG. 2. Calcium imaging. In the control group, there was no change in intracellular calcium concentration until the application of ionomycin (P=0.889, **A**). There was no change in intracellular calcium concentration when metformin (1 mM) was applied (**B**) at the 30th cycle (90 s; P=0.262), but when cisplatin (6 mM) was applied, there was a prompt elevation of intracellular calcium concentrations (P<0.001, **C**). However, when cells were pretreated with metformin (1 mM) for 24 h, intracellular calcium concentrations did not change significantly after the addition of cisplatin at the 30th cycle (6 mM; P=0.877, D). *Orange arrow*, application of the agents at the 30th cycle. *Purple arrow*, application of the ionomycin around 310th cycle.

increased cleaved PARP by 3.9±1.9 times as the control. These cleavages of PARP facilitate cellular disassembly and serve as a marker of cells undergoing apoptosis. However, metformin pretreatment reduced the cleaved PARP by 2.3 ±0.4 times as the control. Thus, this result also supports an antiapoptotic effect of metformin (P=0.009).

DISCUSSION

Cisplatin is a chemotherapeutic drug used to treat a variety of malignant tumors. It is believed that DNA is the target of the antineoplastic activity, as cisplatin binds irreversibly to DNA, forming intra-strand

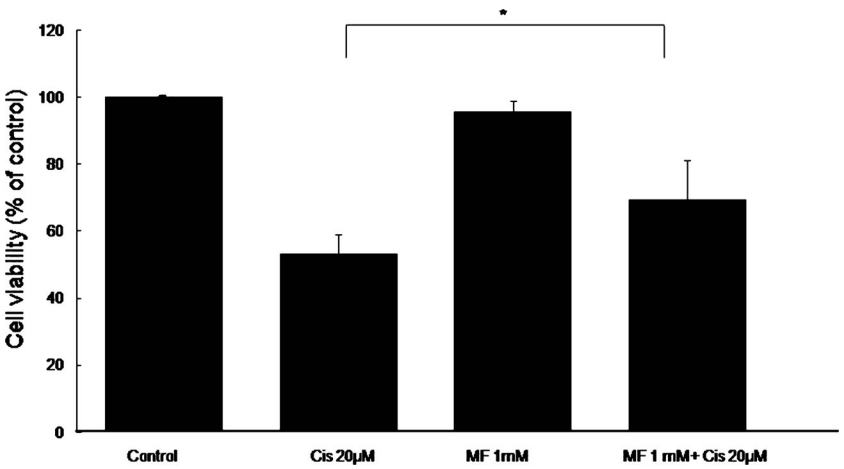

FIG. 3. MTT assay. When cultured cells were exposed to 20 μM cisplatin and 1 mM metformin, cell viability was 53.2 (±5.4) and 95.5 (±3.3)%, respectively. But when the cells were exposed to 20 μM cisplatin after pretreatment with 1 mM metformin for 24 h, cell viability was 69.3 (±11.1)% which was significantly higher than in the cells treated with cisplatin alone (P=0.015).

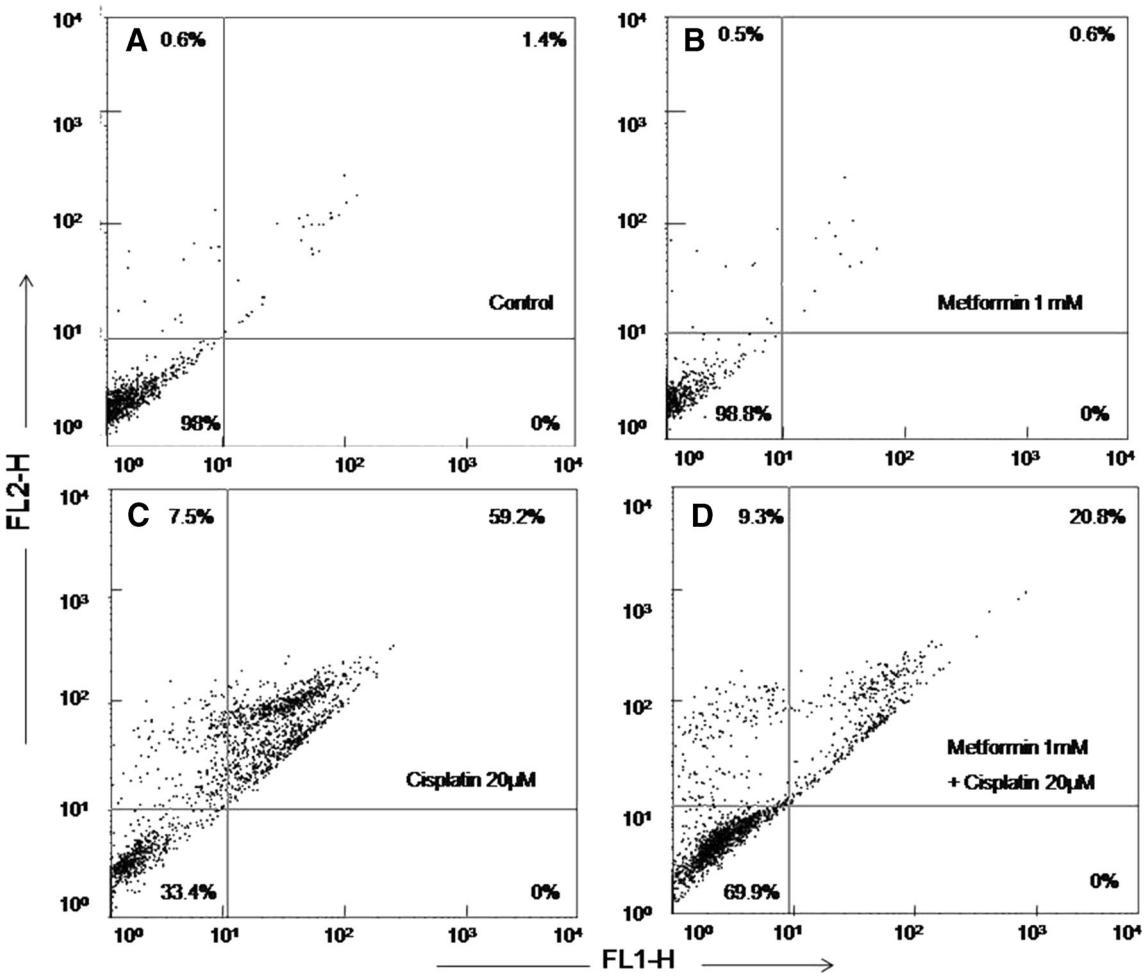

FIG. 4. Flow cytometry analysis. There were no significant differences (P=0.779) in the percentage of late apoptosis between controls (1.4 %) and metformin-added group (0.6 %). When 20 μM cisplatin was applied (**C**), the proportion of cells experiencing late apoptosis increased compared with the control (59.2 %, P=0.012). The proportion of late apoptosis decreased when 1 mM of metformin was pretreated before the application of cisplatin (20.8 %), and the differences between (**C**) and (**D**) were statistically significant (P=0.017).

crosslinks between adjacent guanine residues. However, it causes irreversible and bilateral hearing loss, and results in a disability of communication and a decreased quality of life. The loss of hearing during development, as in pediatric cancer patients, severely impedes the speech, cognition, and social development. Thus, there is a need to reduce the ototoxicity while reinforcing the efficacy of the drug's antitumor activity.

The attenuation of cisplatin ototoxicity has been observed after treatment with protective agents that are predominantly anti-oxidants. The protective effects of *N*-acetylcystein have been demonstrated in rat (Dickey et al. 2004) and guinea pig (Choe et al. 2004). An intracochlear perfusion of sodium thiosulfate was shown to inhibit the cisplatin-induced activation of cytochrome *c*, thus preventing the apoptosis of hair cells in a quinea pig model (Wang et al. 2003). No protective effect was identified in children with germ cell tumors treated with amifostine in combination with cisplatin, etoposide, and bleomycin (Marina et al. 2005; Sastry and Kellie 2005). Intra-tympanic administration of dexamethasone reduced cisplatin-induced hearing loss in guinea pig as well as mouse models (Daldal et al. 2007; Hill et al. 2008). Salicylates have been shown to be effective against cisplatin-induced outer hair cell damage (Hyppolito et al. 2006). Nonetheless, thus far, there is no ideal protective agent in clinical use.

Metformin, a drug widely used in the treatment of type II diabetes, has gained significant attention during the last decade as an anticancer drug. In diabetic patients, it has been found to inhibit gluconeogenesis in the liver by impairing oxidative phosphorylation and leading to an imbalance of the AMP/ATP ratio (Hundal et al. 2000). Consequently, metformin reduces blood glucose and insulin levels. Another study (Zhou et al. 2001) demonstrated that metformin activated adenosine monophosphate-activated protein kinase (AMPK) in both hepatocytes and

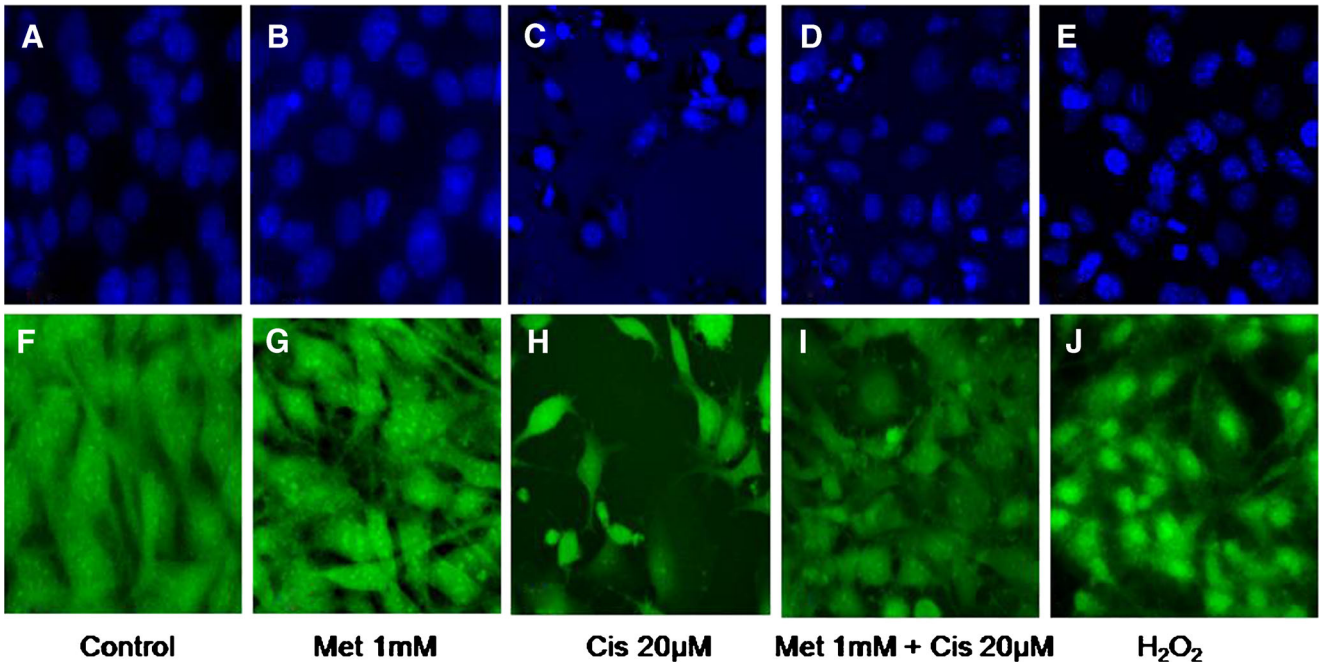

FIG. 5. Measurement of intracellular ROS production and Hoechst 33258 staining. Apoptosis was evaluated based on the appearance of condensed and fragmented nuclei in Hoechst 33258 staining (**A**–**E**). As compared with control cells and those treated with metformin (**A**, **B**), cells exposed to 20 μM cisplatin for 48 h displayed nuclei that were condensed and fragmented (**C**). Group (**C**) also exhibited reduced cell density compared with groups (**A**) and (**B**). However, when the cells were pretreated with metformin (**D**), they were less condensed and less fragmented. Intracellular ROS production was measured with DCFH-DA (**F**–**J**). Cisplatin increased ROS levels (**H**); metformin attenuated this increase in ROS levels (**I**). H_2O_2 was applied for the positive control (**E**, **J**).

skeletal muscle, which implicates the importance of AMPK in regulating both glucose production and fatty acid oxidation.

In cancer patients, AMPK activation by metformin and other activators results in inhibition of the m-TOR pathway and modulates the expression of p21, p27, and cyclin D1, which inhibit proliferation. The insulin/insulin-like growth factor-1 signaling pathway is recognized as another important pathway involved in tumor growth (Gonzalez-Angulo and Meric-Bernstam 2010; Ramandeep Rattan et al. 2011). Several studies have demonstrated that metformin can effectively inhibit the growth of colon, pancreatic, mammary adenocarcinoma, and lung carcinoma cells in AMPK-dependent and AMPK-independent mechanisms (Ben Sahra et al. 2010). Metformin treatment significantly decreased the tumor burden and mammary adenocarcinoma accumulation in the HER-2/meu mouse model for breast cancer (Anisimov et al. 2005). Moreover, recent studies have demonstrated that metformin enhances the antitumor effects of cisplatin (Rattan et al. 2011; Dong et al. 2012).

However, in our previous study of the effects of metformin on cellular ototoxicity, metformin prevented

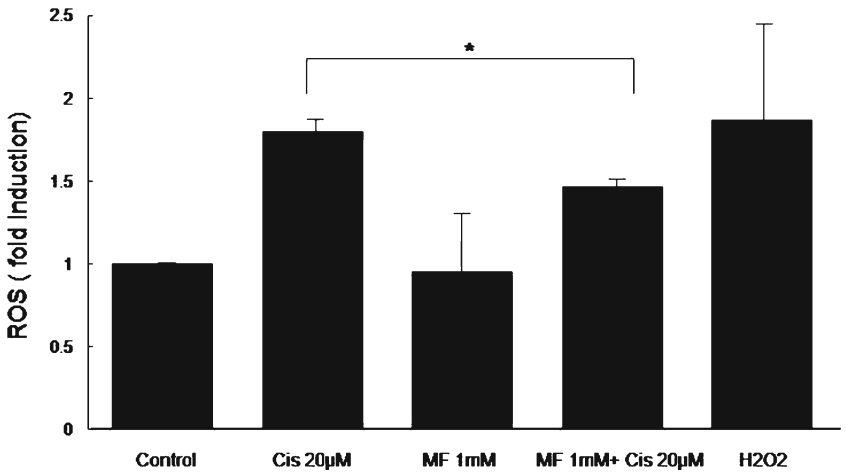

FIG. 6. Measurement of ROS production. ROS levels increased 1.8±0.1-fold after exposure to 20 μM cisplatin. ROS levels increased 1.5±0.1-fold in cells pretreated with metformin. The differences between these two values were statistically significant (P=0.008). H_2O_2 was applied as the positive control (1.9±0.6).

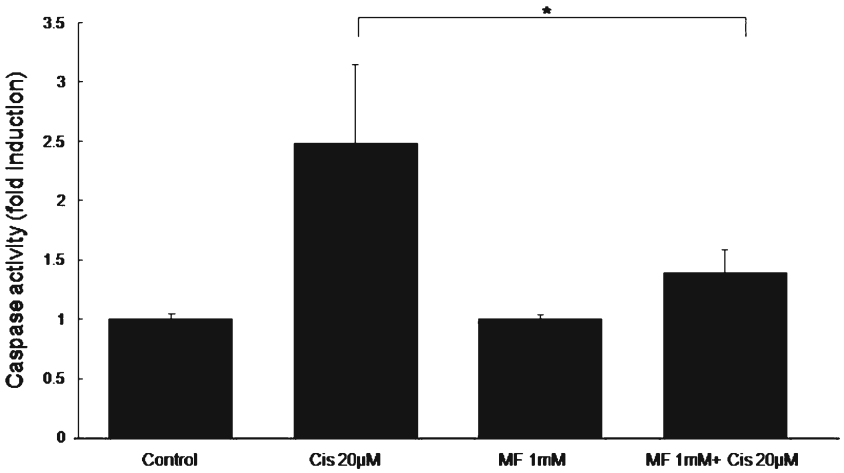

FIG. 7. Measurement of caspase-3 activity. Cisplatin increased caspase-3 activity (2.5±0.7-fold over the normal control). The pretreatment of HEI-OC1 cells with metformin, however, significantly decreased caspase-3 activity (1.4 ±0.2-fold over the normal control), as compared with cells treated with cisplatin alone (P=0.001).

gentamicin-induced apoptosis through calcium-modulating and ROS-reducing anti-apoptotic effects (Chang et al. 2011). Metformin inhibited increases in intracellular calcium concentrations and reduced the levels of caspase-3 and PARP, indicating that the drug regulates the apoptosis through the intrinsic apoptotic pathway. Other studies on cellular toxicity have proposed that metformin induces calcium retention and modulates the mitochondrial permeability transition, thereby preventing cell death because of the release of cytochrome *c* through PTP (Guigas et al. 2004; Lablanche et al. 2011). Ullah et al. also demonstrated that metformin inhibits the apoptotic cascade by increasing Bcl-2 expression, repressing the activation of caspase-9 and caspase-3 and reducing the cleavage of PARP-1 (Ullah et al. 2012).

In the internal pathway of apoptosis, mitochondria releases apoptogenic factors through PTP into the cytoplasm to activate caspases. Caspases are a family of cysteine proteases and are critical mediators of cell apoptosis, which play an important role in the apoptotic process (Grutter 2000). Caspase-3 can activate DNA fragmentation factor, which in turn activate endonucleases to cleave nuclear DNA, and ultimately leads to cell death (Lee et al. 2007). In addition, caspase-3 is responsible for the proteolytic cleavage of many key proteins, including PARP, which is important for cell viability. The cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver et al. 1998).

In the current study, metformin prevented cisplatin-induced cell death in auditory cell line by regulating intracellular calcium concentration, preventing a sequential apoptotic cascade, and reducing ROS production, as reported previously (Chang et al. 2011). Metformin significantly decreased caspase-3 activity and reduced the cleaved PARP. In Hoechst stain, the cells pretreated with metformin had less condensed and fragmented nuclei. In addition, the amount of ROS production was reduced and the change of intracellular calcium concentration decreased when metformin was used.

Although our experiment was conducted with an HEI-OC1 cell line designed to evaluate the ototoxicity of the drug, its conditions are not typical condition of cochlear cells as it is cultured under permissive conditions (33.8 °C, 10 % CO_2). Therefore, in order to validate the protective effect of metformin in

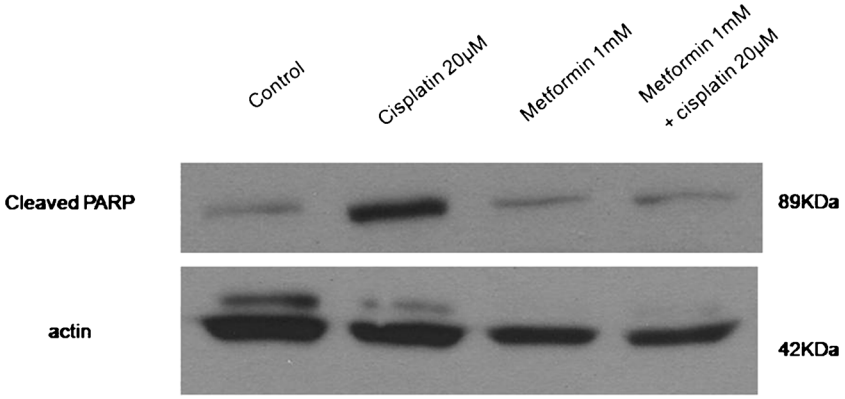

FIG. 8. Western blot analysis of PARP. Cisplatin increased PARP cleavage, which facilitates cellular disassembly and thereby identifies cells undergoing apoptosis. Cisplatin increased cleaved PARP by 3.9±1.9-fold over the normal control. However, metformin pretreatment reduced the cleaved PARP by 2.3±0.4-fold over the control. The difference between two groups were statistically significant (P=0.009).

cisplatin ototoxicty, these results obtained in vitro should be corroborated by in vivo studies. Moreover, metformin is a hydrophilic base which exists at physiological pH as the cationic species (>99.9 %). The oral absorption, hepatic uptake, and renal excretion of metformin are mediated very largely by organic cation transporters and plasma membrane monoamine transporter. It is not yet found which receptors or transporters are responsible for its cellular uptake, either in cochlea in vivo or HEI-OC1 cells in vitro. As both cisplatin and metformin are hydrophilic, it is possible that metformin competes with cisplatin for transporters or receptors, even in in vitro condition. In addition, it has to be validated by in vivo study in the future.

Although metformin is rather a safe agent when administered to the patients (Goodarzi and Bryer-Ash 2005), its clinical application of cisplatin-induced ototoxicity remains unclear, because the problem as to whether metformin enhances or suppresses the efficacy of cisplatin in the treatment of cancer cells remains unsolved. Further in vivo studies are necessary and should focus on evaluating whether metformin can simultaneously enhance the therapeutic action of cisplatin and decrease the frequency of adverse side effects.

CONCLUSIONS

This is the first study to investigate the protective effects of metformin against cisplatin induced ototoxicity in an auditory cell line. In experiments on HEI-OC1 cells, we found that metformin inhibited the increase in intracellular calcium, enhanced cell viability, and prevented ROS production.

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REFERENCES

Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA, Kovalenko IG, Poroshina TE, Semenchenko AV, Provinciali M, Re F, Franceschi C (2005) Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Exp Gerontol 40:685–693

Banfi B, Malgrang B, Knisz J, Steger K, Dubois-Dauphin M, Krause KH (2004) NOX3, a superoxide-generating NADPH oxidase of the inner ear. J Biol Chem 279:46065–46072

Ben Sahra I, Le Marchand-Brustel Y, Tanti JF, Bost F (2010) Metformin in cancer therapy; a new perspective for an old antidiabetic drug? Mol Cancer Ther 9:1092–1099

Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, Forte MA (2006) The mitochondrial permeability transition from in vitro artifact to disease target. FEBS J 273:2077–2099

Boulikas T, Vougiouka M (2003) Cisplatin and platinum drugs at the molecular level: review. Oncol Rep 10(6):1663–1682

Chang J, Jung HH, Yang JY, Choi J, Im GJ, Chae SW (2011) Protective role of antidiabetic drug metformin against gentamicin induced apoptosis in auditory cell line. Hear Res 282(1–2):92–96

Choe WT, Chinosornvatana N, Chang WK (2004) Prevention of cisplatin ototoxicity using transtympanic *N*-acetylcysteine and lactate. Otol Neurotol 25:910–915

Clerici WJ, DiMartino DL, Prasad MR (1995) Direct effects of reactive oxygen species on cochlear outer hair cell shape in vitro. Hear Res 84:30–40

Daldal A, Odabasi O, Serbetcioglu B (2007) The protective effect of intratympanic dexamethasone on cisplatin-induced ototoxicity in guinea pigs. Otolaryngol Head Neck Surg 137(5):747–752

Davis CA, Nick HS, Agarwal A (2001) Manganese superoxide dismutase attenuates cisplatin-induced renal injury; importance of superoxide. J Am Soc Nephrol 12(12):2683–2690

Dehne N, Lautemanrun J, Petrat F, Rauen U, de Groot H (2001) Cisplatin ototoxicity: involvement of iron and enhanced formation or superoxide anion radical. Toxicol Appl Pharmacol 174:27–34

Dickey TD, Muldoon LL, Kraemer DF, Neuwelt EA (2004) Protection against cisplatin-induced ototoxicity by *N*-acetyl-cysteine in rat model. Hear Res 193:25–30

Dong L, Zhou Q, Zhang Z, Zhu Y, Duan T, Feng Y (2012) Metformin sensitizes endometrial cancer cells to chemotherapy by repressing glyoxalase I expression. J Obstet Gynaecol Res 38(8):1077–1085

El-Mir MY, Detaille D, Villanueva GR et al (2008) Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. J Mol Neurosci 34:77–87

Gonzalez-Angulo AM, Meric-Bernstam F (2010) Metformin: a therapeutic opprotunitiy in breast cancer. Clin Cancer Res 16:1695–1700

Goodarzi MO, Bryer-Ash M (2005) Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. Diabetes Obes Metab 7(6):654–665

Grutter MG (2000) Caspases: key players in programmed cell death. Curr Opin Struct Biol 10(6):649–655

Guigas B, Detaille D, Chauvin C et al (2004) Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study. Biochem J 382:877–884

Hill GW, Morest DK, Parham K (2008) Cisplatin-induced ototoxicity: effect of intratympanic dexamethasone injections. Otol Neurotol 29(7):1005–1011

Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumann WC, Petersen KF, Landau BR, Shulman GI (2000) Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes 49:2063–2069

Hyppolito MA, de Oliveira JA, Rossato M (2006) Cisplatin ototoxicity and otoprotection with sodium salicylate. Eur Arch Otorhinolaryngol 263(9):798–803

Jacotot E, Costantini P, Laboureau E, Zamzami N, Susin SA, Kroemer G (1999) Mitochondrial membrane permeabilization during the apoptotic process. Ann N Y Acad Sci 887:18–30

Kalinec GM, Webster P, Lim DJ, Kalinec F (2003) A cochlear cell line as an in vitro system for drug ototoxicity screening. Audiol Neurootol 8:177–189

Lablanche S, Cottet-Rousselle C, Lamarche F, Benhamou P-Y, Halimi S, Leverve X, Fontaine E (2011) Protection of pancreatic INS-1 b-cells from glucose- and fructose-induced cell death by inhibiting mitochondrial permeability transition with cyclosporine A or metformin. Cell Death Dis 2:e134

Lee JE, Nakagawa T, Kim TS, Endo T, Shiga A, Iguchi F, Lee SH, Ito J (2004a) Role or reactive radicals in generation of the auditory

system of mice following cisplatin treatment. Acta Otolaryngol 124:1131–1135

LEE JE, NAKAGAWA T, KITA T, KIM TS, ENDO T, SHIGA A, IGUCHI F, LEE SH, ITO J (2004B) Mechanisms of apoptosis induced by cisplatin in marginal cells in mouse stria vascularis. ORL J Otorhinolaryngol Relat Spec 66:111–118

LEE MK, KANG SJ, PONCZ M, SONG KJ, PARK KS (2007) Resveratrol protects SH-SY5Y neuroblastoma cells from apoptosis induced by dopamine. Exp Mol Med 39(3):376–384

MACGLASHAN D JR (1989) Single-cell analysis of Ca++ changes in human lung mast cells: graded vs. all-or-nothing elevations after IgE-mediated stimulation. J Cell Biol 109:123–134

MARINA N, CHANG KW, MALOGOLOWKIN M, LONDON WB, FRAZIER AL, WOMER RB, RESCORLA F, BILLMIRE DF, DAVIS MM, PERLMAN EJ, GILLER R, LAUER SJ, OLSON TA, CHILDREN'S ONCOLOGY (2005) Amifostine does not protect against the ototoxicity of high-dose cisplatin combined with etoposide and bleomycin in pediatric germ-cell tumors: a Children's Oncology Group study. Cancer 104(4):841–847

MCKEAGE MJ (1995) Comparative adverse effect profiles of platinum drugs. Drug Saf 13:228–244

OLIVER FJ, DE LA RUBIA G, ROLLI V, RUIZ-RUIZ MC, DE MURCIA G, MURCIA JM (1998) Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. J Biol Chem 273(50):33533–33539

RATTAN R, GRAHAM RP, MAGUIRE JL, GIRI S, SHRIDHAR V (2011) Metformin suppresses ovarian cancer growth and metastasis with enhancement of cisplatin cytotoxicity in vivo. Neoplasia 13(5):483–491

ROLAND JT JR, COHEN NL (1998) Vestibular and auditory ototoxicity. In: Cummings CW, Frederickson JM, Harker LA, Krause CJ, Schuller DF, Richardson MA (eds) Otolaryngology & Head and Neck Surgery, 3rd edn. Mosby, St. Louis, pp 3186–3197

RYBAK LP, KELLY T (2003) Ototoxicity: bioprotective mechanisms. Curr Opin Otolaryngol Head Neck Surg 11:328–333

RYBAK LP, HUSAIN K, MORRIS C, WHITWORTH C, SOMANI S (2000) Effect of protective agents against cisplatin ototoxicity. Am J Otol 21:513–520

SASTRY J, KELLIE SJ (2005) Severe neurotoxicity, ototoxicity and nephrotoxicity following high-dose cisplatin and amifostine. Pediatr Hematol Oncol 22(5):441–445

ULLAH I, ULLAH N, NASEER MI, LEE HY, KIM MO (2012) Neuroprotection with metformin and thymoquinone against ethanol-induced apoptotic neurodegeneration in prenatal rat cortical neurons. BMC Neurosci 13:11

WANG J, FAULCONBRIDGE RVL, FETONI A, GUITTON MJ, PUJOL R, PUEL JL (2003) Local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. Neuropharmacology 45:380–393

ZHOU G, MYERSR LY, CHEN Y, SHEN X, FENYK-MELODY J, WU M, VENTRE J, DOEBBER T, JUJI N, MUSI N, HIRSHMAN MF, GOODYEAR LJ, MOOER DE (2001) Role of AMP-activated proten kinase in mechanism of metformin action. J Clin Investig 108:1167–1174