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# Protective role of edaravone against cisplatin-induced ototoxicity in an auditory cell line

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ABSTRACT

Edaravone is a neuroprotective agent with a potent free radical scavenging and antioxidant actions. In the present study we investigated the influence of edaravone on cisplatin ototoxicity in auditory cells. Cell viability was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide cell proliferation assay. Oxidative stress and apoptosis were assessed by reactive oxygen species (ROS) measurement, Hoechst 33258 staining, caspase-3 activity assay, and immunoblotting of PARP. Pre-treatment with 100 μM of edaravone prior to application of 15 μM of cisplatin increased cell viability after 48 h of incubation in HEI-OC1 cells (from 51.9% to 64. 6% viability) and also, attenuated the cisplatin-induced increase in reactive oxygen species (ROS) (from 2.3 fold to 1.9 fold). Edaravone also decreased the activation of caspase-3 and reduced levels of cleaved poly-ADP-ribose polymerase (PARP). We propose that edaravone protects against cisplatin-induced ototoxicity by preventing apoptosis, and limiting ROS production in HEI-OC1 cells.



## 1. Introduction

Cisplatin (cis-diamminedichloroplatinum II) is an important chemotherapeutic agent used in the treatment of solid tumors such as ovarian, cervical, testicular, lung, and head and neck cancers. Cisplatin acts in the tumor cells through mechanisms such as DNA damage and production of reactive oxygen species (ROS), which lead to cell death by apoptosis. Cell death can also occur via necrosis when the cell is exposed to high concentrations of cisplatin (Wang and Lippard, 2005). However, the administration of cisplatin is accompanied by dose-limiting adverse effects such as nephrotoxicity, ototoxicity, neurotoxicity, gastrointestinal tract toxicity and bone marrow toxicity (Saleh and El-Demerdash, 2005). Nephrotoxicity can be managed with saline hydration and administration of diuretics, and some other side effects can be reduced with fractionated doses of medication. However, 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). There are several mechanisms by which cisplatin damage the auditory and vestibular system and trigger ototoxicity. The first mechanism is through the covalent binding of cisplatin to guanine bases in DNA forming inter- and intra-strand chain crosslinking which induces the p53 and apoptosis. A second mechanism refers to the generation of free radicals, specifically ROS, which can increase lipid peroxidation, alter enzyme and structural proteins, and cause apoptotic cell death (Casares et al., 2012; Gutteridge and Halliwell, 2010). In organotypic cultures, when activated by cisplatin, the nicotinamide adenine dinucleotide phosphate oxidase 3 isoform (NOX 3) produces superoxide radical ($O2\cdot-$) (Bánfi et al., 2004; Rybak, 2007). The superoxide radical may interact with unsaturated fatty acids in the lipid bilayer of the cell membrane to generate aldehyde 4-hydroxynonenal, which is highly toxic and may lead to cell death. Also the increase in aldehyde 4-hydroxynonenal concentration is associated with an increased calcium influx to the outer hair cells, leading to apoptosis (Ikeda

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et al., 1993). The superoxide anion may also inactivate antioxidant enzymes (Pigeolet et al., 1990), and cause the pro-apoptotic Bax protein to release cytochrome c from the damaged mitochondria to activate caspases 9 and 3 (Rybak et al., 2009).

Edaravone (MCI-186, 3-methyl-1-phenyl-pyrazolin-5-one) which is clinically used to treat acute cerebral infarction and acute myocardial infarction (Mishina et al., 2005; Tsujita et al., 2004) is a free radical scavenger and can interact with both peroxyl and hydroxyl radicals to form oxidized compounds (Asplund et al., 2009). Edaravone is reported to reduce the amount of oxidative DNA damage and lipid peroxidation in barotraumatic inner ear (Maekawa et al., 2009). Edaravone also suppresses streptomycin-induced vestibulotoxicity, and protects the cochlea from acoustic trauma in guinea pigs (Horiike et al., 2003; Takemoto et al., 2004). Furthermore, edaravone protects hair cells of zebrafish against cisplatin and preserved ultrastructure of mitochondria (Hong et al., 2013).

While previous experimental studies have investigated the antioxidant activity of edaravone, the effect of edaravone on cisplatin induced ototoxicity has not been evaluated thoroughly. The purpose of the present study is to investigate the mechanism of edaravone on cisplatin ototoxicity in auditory cell line.

## 2. Methods

### 2.1. HEI-OC1 cell culture

The HEI-OC1 cell line is extremely sensitive to ototoxic drugs, expresses several molecular markers which are characteristic of organ of Corti sensory cells (Kalinec et al., 2003), and therefore the HEI-OC1 cell line can be a useful study model of ototoxic drugs. The cells were maintained in high-glucose Dulbecco's modified eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 50-U/mL interferon-γ (PEPROTECH, USA) without antibiotics at 33 °C and 10% $CO_2$ in air.

### 2.2. MTT assay to identify cell viability

The uptake and conversion of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazdiumbromide (MTT, Sigma, St Louis, MO, USA) to crystals of dark violet formazan depends on cell viability. Thus, HEI-OC1 cells (2 x $10^4$ cells/well of 48-well plate) were incubated with 15 μM of cisplatin (a concentration which is known to result in 50% of cell viability in our experiments) for 48 h, and the dose-dependent or time-dependent effects of cisplatin were measured using an MTT assay. After pre-test of application of various concentrations of edaravone, the concentration of 100 μM edaravone was adopted since higher concentration seemed to influence the growth of HEI-OC1 cell (Fig 1). In order to examine the effects of edaravone on cisplatin ototoxicity in the auditory cell line, the cells were pretreated with edaravone (100 μM) for 24 h, and then were exposed to cisplatin (15 μM, 48 h). These protocols were maintained throughout the experiments. For the MTT assay, 200 μL of the MTT solution (5 mg/mL) was added to cells and the plates were incubated for 3 h at 33 °C in a 10% $CO_2$ and 90% air mixture. The resulting insoluble violet formazan crystals were centrifuged and the pellets dissolved in DMSO (500 μL/well). Optical density was measured using a microplate reader at 570 nm (Spectra Max, Molecular Devices, Sunnyvale, CA, USA).

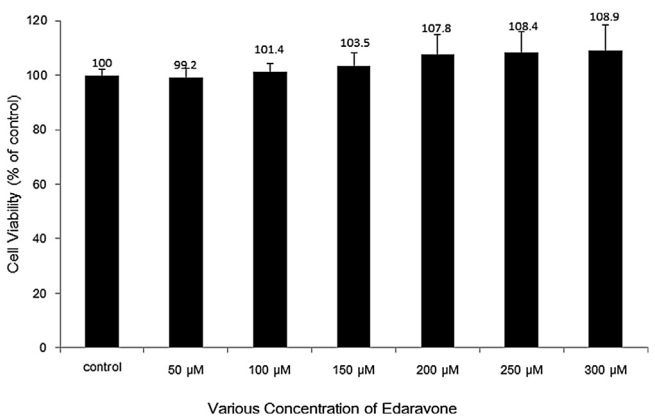


**Fig. 1. Effect of edaravone on HEI-OC1 cells** HEI-OC1 cells were cultured with various concentrations (0, 50, 100, 150, 200, 250, 300 μM) of edaravone for 72 h. The concentrations more than 150 μM had effect the growth of HEI-OC1 cell (cell viability of 103.5 ± 4.8%). In order to rule out the growth effect of edaravone, we adopted the 100 μM of edarvaone for the further study (cell viability of 101.4 ± 3.0%).

### 2.3. Intracellular ROS measurement

The intracellular ROS level was measured using a fluorescent dye, 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; Calbiochem, Darmstadt, Germany). In the presence of an oxidant, DCFH is converted into highly fluorescent 2′, 7′-dichlorofluorescein (DCF). For ROS assay, HEI-OC1 cells were treated with 15 μM cisplatin for 48 h in the presence or absence of edaravone (100 μM, 24 h pretreatment). Following this incubation, the cells were treated with 50 μM DCFH-DA and were further incubated for 30 min. The samples were measured using a FACScan flow cytometry (BD Biosciences, Heidelberg, Germany) at a 488 nm excitation wavelength and a 530 nm emission wavelength (10,000 cells/sample), and the mean fluorescence intensity was calculated by histogram statistics using BD CellQuest Pro software (BD Biosciences).

### 2.4. Fluorescence microscopy

Cells were grown on the Cell Culture Slides (SPL, Gyeonggido, Korea). Cells were washed twice with serum-free medium without phenol red and incubated with 50 μM DCFH-DA in serum-free medium without phenol red for 30 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 and mounting, the fluorescence images from multiple fields of view were obtained using an olympus ix71 microscopy with a long-term real-time live cell image system (LAMBDA DG-4, Sutter Int., Novato, CA, USA).

### 2.5. Measurement of caspase-3 activity

The enzymatic activity of caspase-3 was assayed with a caspase3/CPP32 fluorometric assay kit (Biovision, Milpitas, California, 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 50 μM DEVD-pNA and fluorometric 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 by microplate reader (Spectra Max, Molecular Devices, Sunnyvale, CA, USA) at a 400 nm excitation filter and a 505 nm emission filter.

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