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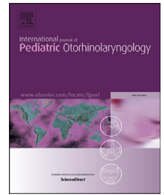

Protective effects of 1,2,3-triazole derivative KPR-A020 against cisplatin-induced ototoxicity in murine cochlear cultures

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ABSTRACT

Cisplatin (*cis*-diaminedichloridoplatinum(II), *cis*-[$PtCl_2(NH_3)_2$]) is an effective chemotherapeutic agent in the treatment of several types of malignant solid tumors but its clinical use is associated with ototoxicity. Several studies have investigated the effect of antioxidants on cisplatin-induced ototoxicity in mice. The triazole KPR-A020 has been shown to play a protective role against mitochondrial dysfunction by reducing the production of mitochondrial reactive oxygen species (ROS). The effect of KPR-A020 on cisplatin-induced ototoxicity was examined using cultures of cochlear explants. Healthy mice were randomly divided into 4 groups: control, treated with cisplatin alone (CP), treated with cisplatin and KPR-A020 (CP + KPR-A020), and treated with KPR-A020 alone (KPR-A020). The cochlear explants were harvested for histological and immunohistochemical examinations. Biochemical analyses of the explants revealed that pre-treatment with KPR-A020 prevented an increase in mitochondrial ROS levels. Moreover, the CP + KPR-A020 group showed better hair cell survival than the CP group. Immunohistochemical examinations of cochlear explants stained with anti-caspase-3 revealed greater immunopositivity in the CP group. The CP + KPR-A020 group showed significantly less immunopositivity than the CP group ($P < 0.05$). Thus, it appears that KPR-A020 protects hair cells in the organ of Corti from cisplatin-induced toxicity by decreasing the production of mitochondrial ROS. The results of this study suggest that KPR-A020 can be used as an antioxidant and antiapoptotic agent to prevent hearing loss caused by cisplatin induced-oxidative stress.

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1. Introduction

Cisplatin (*cis*-diaminedichloridoplatinum(II), *cis*-[$PtCl_2(NH_3)_2$]) is a highly effective chemotherapeutic agent that is widely used to treat a variety of soft tissue neoplasms, including those associated with ovarian, testicular, cervical, lung, bladder, and head and neck cancer. Once in the cytoplasm of a cell, it is well established that cisplatin undergoes a conversion to its aquated derivative, *cis*-[$PtCl(NH_3)_2(OH_2)]^+$, driven by the approximately 13-fold lower concentration of chloride in the cytoplasm relative to the extracellular fluid [1–3]. This aquated derivative is recognized to be responsible for the anti-cancer activity of the drug, because of its ability to react with DNA, eventually forming a bifunctional adduct, which inhibits DNA replication and initiates apoptosis [4].

However, serious side effects include nephrotoxicity, neurotoxicity, and ototoxicity. In particular, the ototoxicity limits the dose that can be administered. Some audiometric studies have reported

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elevated hearing thresholds in 75–100% of patients treated with cisplatin [5]. This is particularly problematic in children receiving cisplatin. Risk factors that increase the risk for ototoxicity from cisplatin in children include younger age, larger cumulative doses, pre-existing hearing loss, renal disease [6,7], and irradiation of the brain or skull base [8]. Both *in vitro* and laboratory animal studies show that cisplatin interacts with cochlear tissues such as the outer hair cells of the organ of Corti, the stria vascularis, the spiral ligament, and the spiral ganglionic cells, generating a robust reactive oxygen species (ROS) response [9–13].

ROS that are physiologically produced in mitochondria are involved in signaling pathways that mediate adaptive responses to stress. It also helps to regulate cellular growth and differentiation [14]. However, the overproduction of ROS can be accumulated in a cell which results in altering enzymatic and ion channel activities, and even inducing apoptosis [15].

It has been reported that certain triazole-based compounds, including KPR-A020, inhibit RANKL-induced osteoclast differentiation and bone resorption [16]. Triazoles are heterocyclic compounds comprising three nitrogen and two carbon atoms. Triazoles can bind to various biological molecules, including enzymes and receptors. Consequently, triazole-based compounds have been shown to have diverse biological activities, including anti-inflammatory, anti-tumor, anti-tubercular, and anti-fungal activities [17,18]. In addition, many triazole derivatives have low toxicity, high bioavailability, and few side effects [19,20]. Therefore, researchers have attempted to use triazole derivatives as therapeutic agents against infections, cardiac diseases, and seizures [21]. However, there have been few reports describing the direct antioxidant effect of triazole derivatives on oxidant-induced ototoxicity. Different compounds containing 1,2,3-triazoles have been reported to have interesting antiproliferative activities [22–26]. This recently led us to investigate the synthesis of different terpenes coupled to triazole rings using click-chemistry [27–29] and to assess their antiproliferative activities. The antifungal activity of triazoles is well known; fluconazole, itraconazole, voriconazole, and posaconazole are the most used antifungal agents in the clinic [20].

In this study, we investigated the use of KPR-A020, a triazole derivative, as a protective agent against cisplatin-induced ototoxicity by evaluating its effect on mitochondrial ROS production and apoptosis in murine cochlear cultures.

2. Materials and methods

2.1. Culture of murine cochlear explants

Primary cochlear explants were prepared from 'postnatal day 3' Institute for Cancer Research (ICR) mice, purchased from Hyochang Science (Daegu, Republic of Korea). Culturing of mouse cochlear explants was performed as described previously [30]. Dissected organs of Corti were attached to four-well culture dishes and subsequently incubated in the culture medium under a humidified atmosphere of 5% CO_2 at 37 °C, which contained high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and ampicillin (10 μg/mL; Life Technologies, Carlsbad, CA, USA). The cultured organs of Corti were divided into four groups for KPR-A020 treatment: control (CT, $n = 4$), cisplatin alone (CP, $n = 4$), treatment with KPR-A020 prior to cisplatin (CP + KPR-A020, $n = 4$), and KPR-A020 alone (KPR-A020, $n = 4$). After a 16 h incubation, organs of Corti were treated with KPR-A020 (1 mM in DMSO; Sigma, St. Louis, MO, USA) and diluted in culture medium for 1 h under a humidified atmosphere of 5% CO_2 at 37 °C. After the 1 h incubation, cisplatin (20 μM; Ildong Pharmaceutical Co., Daegu, Republic of Korea) was added to the CP group and CP + KPR-A020 group.

All animal experiments were conducted in accordance with the guidelines of the 'Institutional Animal Care and Use Committee' of Kyungpook National University. These experiments were approved by the 'Committee on the Ethics of Animal Experiments' of Kyungpook National University.

2.2. Histological evaluation

To investigate the protective effects of KPR-A020 against cisplatin-induced ototoxicity in the organ of Corti, we examined the morphology of inner hair cells (IHCs) and outer hair cells (OHCs) within the organs of Corti. At the end of a 30 h or 48 h incubation period, all cochlear explants were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA, pH 7.4) in PBS for 15 min, and permeabilized with 0.1% Triton X-100 in PBS (PBS-Tx) for 30 min at room temperature (RT) [9]. Permeabilized samples were blocked with 5% normal goat serum diluted in PBS-Tx for 1 h at RT and then stained with either Alexa Fluor 488- or 555-conjugated phalloidin (1:1000; Invitrogen-Molecular Probes, Eugene, OR, USA) in PBS-Tx for 3 h at RT. The specimens were rinsed three times with PBS and mounted on glass slides using Fluoromount (Sigma, St. Louis, MO, USA). Images were captured using Zeiss Axio Imager A2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.3. Determination of mitochondrial ROS levels

MitoSOX-red (Invitrogen, Carlsbad, CA, USA) is a novel fluorogenic indicator of superoxide molecules generated specifically from mitochondria. At the end of the 30 h incubation period, all cochlear explants were washed with PBS and stained for 10 min with MitoSOX-red (5 μM) under a humidified atmosphere of 5% CO_2 at 37 °C. After washing with PBS, the specimens were visualized using a Zeiss Axio Imager A2 fluorescence microscope.

2.4. Immunohistochemical analysis and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

To assess apoptotic cell death in the organs of Corti, we conducted immunohistochemistry using antibodies directed against active caspase-3. We also evaluated DNA fragmentation using the TUNEL assay. At the end of the 48 h incubation period, all cochlear explants were fixed, permeabilized, and blocked as previously described. Following blocking, the specimens were stained with antibodies directed against active caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA) and diluted in the blocking solution at 4 °C overnight. Samples were then washed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:1000; Invitrogen, La Jolla, CA, USA) and diluted in the blocking solution for 1 h at RT. Following this, F-actin was labeled for 3 h at RT with Alexa Fluor 555-conjugated phalloidin in PBS-Tx and mounted on glass slides using Fluoromount.

DNA fragmentation was evaluated as a marker of apoptotic cell death, using the TUNEL assay according to the manufacturer's protocol (Promega, Madison, WI, USA). The cochlear explants were fixed with 4% PFA in PBS for 15 min at RT and then washed with PBS. They were then permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in distilled water for 10 min at 4 °C, followed by staining with TUNEL working solution in the dark for 30 min at 37 °C. F-actin was labeled with Alexa Fluor 555-conjugated phalloidin stain in PBS-Tx for 3 h at RT. The specimens were mounted on glass slides using Fluoromount and visualized using a Zeiss Axio Imager A2 fluorescence microscope.

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