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The Targeted Antioxidant, Catalase-SKL, Protects Against Cisplatin-induced Ototoxicity in HEI-OC1 Cells

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Abstract

Hearing loss is the fastest growing and one of the most prevalent chronic health conditions in the world. Cisplatin, a drug widely used in the treatment of many cancers, has been shown to be particularly damaging to the inner ear, causing ototoxicity in 70-100% of patients treated. Previous studies demonstrated the accumulation of reactive oxygen species (ROS) in hair cells as a major contributor to hair cell death in response to ototoxic drugs, noise exposure, and age-related hearing loss. The antioxidant enzyme catalase, predominantly localized in the peroxisome of cells, plays a crucial role in regulating cellular oxidative stress by degrading ROS. In the present study, we investigated whether our novel antioxidant CAT-SKL – a stable, cell penetrating, peroxisome-targeted derivative of catalase – is able to offer protection against cisplatin-induced ototoxicity in the HEI-OC1 immortalized mouse cochlear cell line. Using immunocytochemistry and western blot techniques, we confirmed that CAT-SKL was successfully internalized when delivered to cells *in vitro*. A WST-1 cell viability assay demonstrated that CAT-SKL is not only non-toxic to cells derived from the inner ear, but significantly improved cell viability compared to untreated cells in a dose-dependent manner. As expected, 15µM cisplatin treatment induced a significant decrease in cell viability of HEI-OC1 cells. This decrease was ameliorated by pretreatment with 10µM CAT-SKL. Furthermore, even at a cisplatin concentration of 30µM, pretreatment with CAT-SKL was still able to improve cell viability. In addition, cisplatin treatment resulted in increased levels of ROS as detected by dihydroethidium staining and induced peroxisomal proliferation. Pretreatment with 10µM CAT-SKL was able to attenuate cisplatin-induced damage due to its antioxidant properties, thereby decreasing ROS

formation and peroxisomal proliferation. At present, there is no widely accepted pharmacotherapy which protects against ototoxic pharmaceuticals. The results of this project demonstrate a safe and effective prophylactic intervention for protection against ototoxic drug-induced hearing loss.

Introduction

Hearing loss is the fastest growing and one of the most prevalent chronic health conditions in the world[1]. According to the WHO, it affects approximately 360 million people, which is over 5% of the world's population[2]. The consequences of hearing loss and tinnitus, the perception of a ringing sound in the ears that often accompanies hearing loss, are extensive[3]. It has tremendous implications on an individual's ability to communicate effectively, which can seriously impact patients in their professional and personal lives, leading to isolation, withdrawal, and exacerbation of other psychological disorders[4]. There is also clear evidence linking hearing loss to an increased risk of developing dementia, Alzheimer's disease, and the acceleration of age-related cognitive decline[5]. Given the prevalence and widespread consequences of hearing loss, there is a crucial need to develop novel compounds that can be administered prophylactically to limit permanent damage to the auditory system.

Causes of Hearing Loss

Some common causes of hearing loss and tinnitus include exposure to loud noise[6]; ototoxic drugs such as the aminoglycoside antibiotics (e.g. kanamycin, gentamicin, and neomycin[7]) and platinum-based chemotherapeutic agents (e.g. cisplatin, oxaliplatin, and carboplatin[8,9]). Aminoglycoside induced hearing-loss occurs in approximately 20% of patients undergoing short-term treatments[10]. In comparison, cisplatin therapy is associated with a higher occurrence of hearing deficits[10]. According to Rybak et al., the incidence of cisplatin-mediated ototoxicity is estimated to occur in approximately 75-100% of patients treated with the medication[9].

Despite the ototoxicity of these platinum-based chemotherapeutic agents and

aminoglycosides, these drugs still remain in use. This occurs either because the risk of damage is relatively small, or because no comparative alternative treatment options are available[10]. In particular, there is a large demand for aminoglycoside antibiotics in developing nations due to their broad-spectrum efficacy against most infections and low cost of production[10].

Cisplatin is widely used as a chemotherapeutic agent, either singly or in combination therapy[11]. It is used in the treatment of head, neck, lung, bladder, cervical, and gastrointestinal cancers[11]. More recently, other platinum-based chemotherapeutic agents, such as carboplatin and oxaliplatin, have been developed. However, they are used in very limited applications: carboplatin is used in the treatment of specific types of breast cancers and oxaliplatin is used in combination therapy against colorectal cancer[10]. Although cisplatin is the most ototoxic in the family of platinum-based chemotherapeutic drugs, it is still used far more often than others because it is approximately 40 times more effective against certain cancers compared to carboplatin or oxaliplatin[10].

Mechanism of Cisplatin-Induced Ototoxicity

Cisplatin or aminoglycoside induced hearing loss is generally bilateral and begins in the higher frequency region of the inner ear[12]. With prolonged treatment, this damage will extend to the lower frequencies[12]. Furthermore, aminoglycosides can also elicit vestibulotoxicity which manifests as a loss of balance with or without vertigo[10]. Both cisplatin and aminoglycosides cause nephrotoxicity by accumulating in the kidneys[10]. Previous studies using multiple cell systems, including an immortalized cochlear cell line, have identified the mammalian copper transporter 1 (CTR1) as the major influx

transporter of platinum-based drugs, such as cisplatin, and the glycoprotein transporter megalin in the case of aminoglycosides[8,10]. Although these drugs are both nephrotoxic and ototoxic, the renal toxicity is reversible, while any damage to the inner ear is usually permanent[9]. Studies have shown that in hair cells, cisplatin acts to generate a substantial amount of reactive oxygen species (ROS) while depleting endogenous antioxidant enzymes that would normally be able to neutralize this increase[11]. Moreover, cisplatin tends to accumulate in cochlear tissues and integrates itself into the DNA, causing inefficient and dysfunctional protein and enzyme synthesis[11]. Previous studies by Ding et al., showed that high levels of extracellular cisplatin do not induce hair cell loss or stereocilia damage[8]. It is instead the intracellular accumulation of cisplatin that results in its ototoxic effects. Because of the cochlea's unique anatomical position and isolation, it resembles a closed system, and its ability to remove these accumulated toxins, such as cisplatin, is very limited. This leads to ROS overload, and when combined with an inefficient antioxidant system, apoptosis will occur[10]. Research has shown the down-regulation of cochlear antioxidant enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase, and glutathione reductase increases the vulnerability of the cochlea to damage associated with normal aging[13].

The Role of Reactive Oxygen Species in Hearing Loss

Reactive oxygen species are typically formed as a byproduct during the normal cellular metabolism of oxygen[14]. However, during times of environmental stress, ROS levels can increase and damage cells by interacting with DNA, RNA and other cellular components[14,15]. Numerous studies have shown that oxidative stress is a key factor in the pathophysiology of noise trauma. For example, in noise-exposed animals, a

significant increase in oxidative stress markers was observed in the cochlea[16]. Many studies have attempted to offset this oxidative stress by administering dietary antioxidants to noise-exposed animals, with limited effectiveness[17]. Moreover, administration of dietary antioxidants that appeared to lessen noise-induced hearing loss in preclinical models were unable to provide equally promising results when administered to humans[18].

The Role of Catalase in Hearing loss

Normally found in the peroxisomes of cells, the endogenous antioxidant, catalase, plays an integral role in protecting cochlear hair cells from oxidative stress[19]. Catalase catalyzes the degradation of hydrogen peroxide to water and oxygen ($2H_2O_2 -> 2H_2O + O_2$), thus preventing the oxidative damage in the cell caused by hydrogen peroxide[20]. The enzyme is a tetramer with a total molecular weight of 240kDa, and each tetrameric molecule contains four heme groups[21]. Studies involving both rodents and humans have confirmed that it would be highly desirable to therapeutically increase, or at least preserve, catalase following noise exposure. For example, transgenic mice that overexpress catalase were resistant to age-related hair cell death and hearing loss[15]. Furthermore, research on animal models has shown that in the days following noise exposure, catalase levels decrease[22,23]. Finally, human polymorphisms in the catalase encoding gene have been linked to an increased vulnerability to noise-induced hearing loss[19]. These studies suggest that compounds that augment or preserve the catalase system in the cochlea may be protective against noise-induced hearing loss.

The Creation of a Recombinant Derivative of Catalase

Similar to dietary antioxidants, flooding the body with supplemental catalase is not an effective option because it needs to effectively penetrate the cells undergoing oxidative stress, and then enter their peroxisomes. As a first step towards overcoming this obstacle, Dr. Paul Walton has succeeded in engineering a recombinant derivative of catalase (Patent No. US20060141598 A1)[24]. Normally, the catalase protein contains a KANL sequence at the carboxyl terminus. Although KANL functions as a peroxisome targeting sequence, its targeting efficiency is limited. It was found that other proteins that were more effectively targeted to the peroxisomes contained a more efficient motif (SKL) at its carboxyl terminus. Thus, in the recombinant derivative of catalase, the KANL sequence of catalase was replaced with the SKL sequence to form Catalase-SKL (CAT-SKL). Upon its administration, CAT-SKL can penetrate cells via a cell penetrating peptide (CPP) at its amino terminus. The CPP is an arginine-rich region that enhances the uptake of the modified catalase into cells. Previous studies conducted by Giordano et. al. demonstrated the efficacy of CAT-SKL in reducing Amyloid-beta (Aβ)-induced neurotoxicity, which is a major contributor to the pathologies associated with Alzheimer's disease[25]. CAT-SKL administration was able attenuate ROS formation, neurite formation and restore overall cell viability. In addition, CAT-SKL has also been shown to be efficacious in reducing ischemia reperfusion injury induced cell death, which occurs due to generation of oxidative stress in ventricular myocytes[26].

The HEI-OC1 Cell Line

Thus, the aim of this project was to design a high-throughput system for testing the otoprotective effects of CAT-SKL. To further investigate the cellular mechanisms

involved in CAT-SKL protection against ototoxic agents, the house ear institute-organ of corti 1 (HEI-OC1) cell line was used[27]. This cell line, derived from the auditory organ of the transgenic Immortomouse™, expresses specific markers of cochlear hair cells such as prestin and myosin 7a[27]. Studies have provided substantial evidence that typical ototoxic drugs such as cisplatin, kanamycin, and gentamicin-induced caspase-3 activation in these cells, while non-ototoxic drugs such as penicillin did not[27]. Therefore, this cell line has been used widely as an *in vitro* system to investigate the molecular mechanisms involved in ototoxic compounds or otoprotective properties of new pharmacological drugs.

Because HEI-OC1 cells are vulnerable to damage by ototoxic agents, I hypothesized that cisplatin would decrease the viability of HEI-OC1 cells. Additionally, I hypothesized that addition of CAT-SKL would protect against the reduced viability of HEI-OC1 cells caused by exposure to cisplatin.

Materials and Methods

HEI-OC1 Cell line

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line, derived from the cochlea of the Immorto-mouse™, was provided by Dr. F. Kalinec[27]. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 33°C in a humidified incubator with 5% CO2. HEI-OC1 cells were passaged using 0.25% trypsin with EDTA (Gibco).

Drug treatments

Cisplatin (Sigma-Aldrich; 15663-27-1) and Catalase-SKL (made by Dr. Paul Walton; stock concentration at 32 mg/mL dissolved in sterile PBS) were added to their respective culture mediums at assay specific concentrations, followed by incubation at 33°C for assay specific time periods. Menadione (Sigma-Aldrich; M5625), a known inducer of cellular oxidative stress, was dissolved in 100% ethanol at a stock concentration of 50mM. It was used as a positive control for oxidative stress at a concentration of 100μM dissolved in media.

Detection of cell viability

HEI-OC-1 cells were counted and seeded at density of $1x10^4$ cells in medium onto a 96-well plate and incubated overnight for attachment. Each well contained a volume of 100μL. Cells were then treated with CAT-SKL (1μM, 2.5μM, 5μM or 10μM) for a 24-hour pretreatment period followed by 48 hours of cisplatin (15μM, 20μM or 30μM) exposure. Cell proliferation reagent WST-1 (Sigma-Aldrich; 5015944001) was then added to each well at a ratio of 1:10 (10μL of WST-1 per well). After 1.5 h of incubation, cell viability was determined by measuring the absorbance at 450 nm using a BioTek Epoch

Microplate Spectrophotometer. Each assay was performed in triplicate, with six replicates run on each plate.

Immunohistochemistry for detection of Catalase-SKL internalization, reactive oxygen species, and peroxisomal proliferation

HEI-OC1 cells were plated on circular coverslips with 2 coverslips placed in each well of a 6-well plate. Each well was seeded with $8x10^4$ cells in fresh medium. After 24 h incubation, coverslips were treated with CAT-SKL (1μM, 2.5μM, 5μM or 10μM) for a 24-hour pretreatment period followed by 48 hours of cisplatin (15μM, 20μM or 30μM) exposure. Cells were then washed for 5 min twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The solution was aspirated and coverslips were placed into a humidity chamber and washed twice for 5 min with PBS. A permeabilization solution comprised of 0.5% Triton X-100 solution in PBS was added the coverslips for 30 min. The coverslips were washed again for 5 min three times with PBS. Finally, a blocking solution comprised of 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS was added to each coverslip. Coverslips were incubated with the blocking solution for 1 h at room temperature.

To track CAT-SKL internalization, a biotinylated form of CAT-SKL (bCAT-SKL) was used. Alexa Fluor™ streptavidin 488 (Thermo Scientific; S11223) at a dilution of 1:400 in the blocking buffer at room temperature for 1 h was used to detect the presence of bCAT-SKL. Coverslips were then washed three times for 5 min each with PBS. Then, coverslips were mounted with ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (Thermo Scientific; P36962) onto glass slides.

To visualize peroxisomes, after incubation with blocking solution, coverslips were incubated with the primary antibody, rabbit polyclonal anti-PMP70 (70kDa-peroxisomal membrane protein; Affinity BioReagents; PA1-650), at a dilution of 1:200 in the blocking buffer at 4^{o}C overnight. Coverslips were then washed three times for 5 min each with PBS, then incubated for 1 h at room temperature with secondary antibody, Alexa Fluor^{TM} 594 donkey anti-rabbit IgG (Thermo Scientific; A-11008). Coverslips were washed again at 5 mins each with PBS for three times. Coverslips were mounted with ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (Thermo Scientific; P36962) onto glass slides.

Cells were imaged with a Zeiss LSM 800 confocal Airyscan microscope equipped with ZenWorks software. Images were captured with a ×63 oil immersion objective at room temperature.

To detect reactive oxygen species, dihydroethidium (DHE) stain (Thermo Scientific; D1168) was used. The media was aspirated and coverslips were rinsed 3 times in PBS for 5 min. The coverslips were incubated with 10μM of DHE for 30 min, covered in the dark. Finally, the coverslips were rinsed three times with PBS for 5 min each. A small drop of PBS was placed on a glass slide and one at a time, coverslips were mounted onto the glass slide and sealed with clear nail polish. The slides were then immediately visualized and imaged with a Zeiss LSM 800 confocal Airyscan microscope equipped with ZenWorks software. Images were captured with a ×63 oil immersion objective at room temperature.

Western blot

HEI-OC1 cells were plated at a density of $8x10^5$ cells per well of a 6-well plate. After 24 h incubation, cells were treated with CAT-SKL for 24 hours. Media was then aspirated and cells were twice with cold PBS. 1x lysis buffer was made containing 2x immunoprecipitation buffer, sodium orthovanidate (100mM), sodium fluoride (100mM), cOmplete™Mini EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich; 11836170001) dissolved in ddH_2O. Lysis buffer was added to the cells and cell lysates were scraped using a rubber policeman. Solutions were transferred to 1.5mL tubes and placed on ice for 30 min, vortexing periodically. Solutions were then centrifuged at 4°C for 15 min at 12,000RPM. Supernatant was transferred to newly labeled 1.5mL tubes. Protein concentration was determined using a bicinchoninic acid (BCA) assay (Thermo Scientific; 23225) in a 96 well plate. After determining the concentration, 30µg of protein/lane was separated by electrophoresis on 12% SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting. The nitrocellulose membrane was blocked with 3% BSA solution for 1 h and then incubated overnight at 4°C with rabbit polyclonal anti-catalase (1:200; Thermo Scientific; PA5-29183) antibody and mouse monoclonal anti-GAPDH (1:7500; Milipore, Billerica, MA). Primary antibodies were detected using goat anti-rabbit IRdye 800 (1:10,000; LI-COR Biosciences) and Alexa Fluor 680 goat anti-mouse (1:10,000; Invitrogen) secondary antibodies. Membranes were scanned and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analysis

GraphPad Prism version 6 was used for all statistical analysis. All data are presented as mean ± SEM. When a One-way ANOVA for non-repeated measures was performed, the Dunnett's post hoc test was used to compared between treatments and control. When a Two-way ANOVA for non-repeated measures was performed. Tukey's post hoc test was used for multiple comparisons. Data was determined to be statistically significant when $p<0.05$.

Results

CAT-SKL is successfully internalized in HEI-OC1 cells

Internalization of the CAT-SKL was documented in HEI-OC1 cells, with entry confirmed within 24 hours of addition. To demonstrate CAT-SKL internalization in HEI-OC1 cells, CAT-SKL was tracked using a biotinylated form of the recombinant enzyme (Fig. 1a). Immunofluorescence analysis confirmed catalase staining was noticeably increased in CAT-SKL treated cells relative to untreated cells. To confirm that treatment of HEI-OC1 cells with 10μM of CAT-SKL did indeed lead to an increase in the intracellular level of the exogenous catalase protein, a western blot was performed (Fig. 1b). Endogenous expression levels of the catalase protein (60kDa) are comparable between the untreated group and the CAT-SKL treated group with both showing a band of relatively equal intensity at 60kDa. However, the cells of the CAT-SKL treated group showed an additional band at 65kDa when probed with an anti-catalase antibody. This signifies the successful internalization of exogenous CAT-SKL, which has a slightly higher molecular weight due to the additional cell penetrating peptide on the recombinant protein.

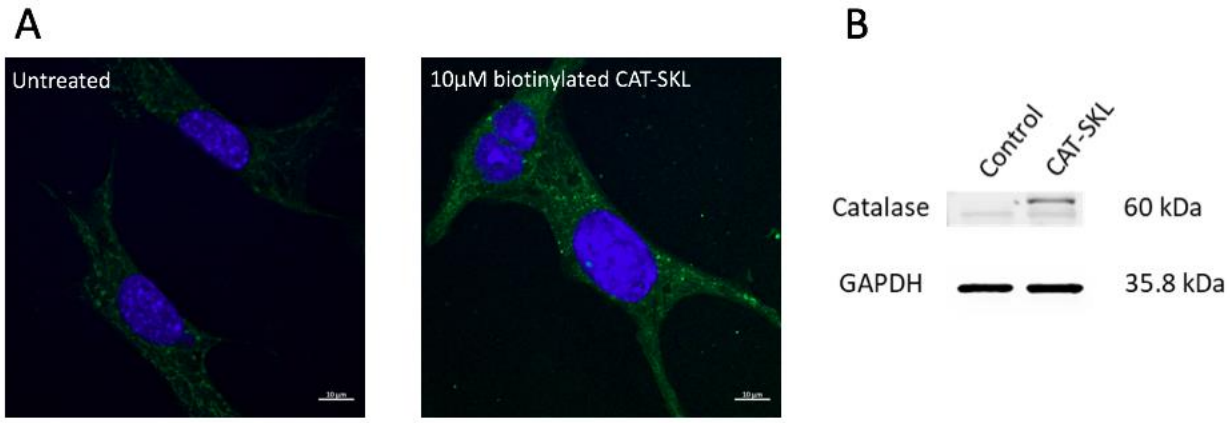

Figure 1. **CAT-SKL uptake in HEI-OC1 cells.** A) Immunocytochemistry showed HEI-OC1 cells stained for 4′,6-diamidino-2-phenylindole (DAPI) (blue) and streptavidin (green). Cells incubated with 10µM CAT-SKL for 24 hours showed a noticeable increase in streptavidin staining compared to untreated cells (Bars, 10µM). B) Western blot was also performed to confirm the internalization of CAT-SKL in HEI-OC1 cells. Cells were either incubated with CAT-SKL (10µM) for 24 hours or untreated. The membrane was probed with anti-catalase antibody (1:200). GAPDH (1:7500) was used as a loading control. Results are shown from the same probed blot.

CAT-SKL is not harmful to HEI-OC1 cells

To confirm CAT-SKL is not harmful to HEI-OC1 cells, a WST-1 cell proliferation assay was performed. HEI-OC1 cells were incubated with 1µM, 2.5µM, 5µM, or 10µM of CAT-SKL for 24 hours. The cell viability of each group was obtained by measuring the absorbance at 450nm using a spectrophotometer. The background absorbance was measured for a blank well filled with media only and this value was subtracted from all samples. To compare cell viability between CAT-SKL treated cells and control cells, a one-way non-repeated measure ANOVA was performed. When ANOVA indicated significant treatment effects ($p<0.05$), means were separated using Dunnett's multiple comparison's test, comparing all groups to the untreated control group. The ANOVA revealed a significant main effect for treatment ($F_{(5,102)}=89.73$, $p<0.0001$). The results of post-hoc testing revealed that the addition of CAT-SKL at 1µM or 2.5µM does not significantly alter cell viability compared to untreated cells ($p>0.05$). However, at higher concentrations of CAT-SKL (5µM and 10µM), there is a significant increase in cell viability compared to untreated cells ($p<0.0001$). Menadione, a known cellular oxidative stressor, was used as a positive control. As expected, the addition of menadione at 100µM for 1h caused a significant reduction in cell viability ($p<0.0001$).

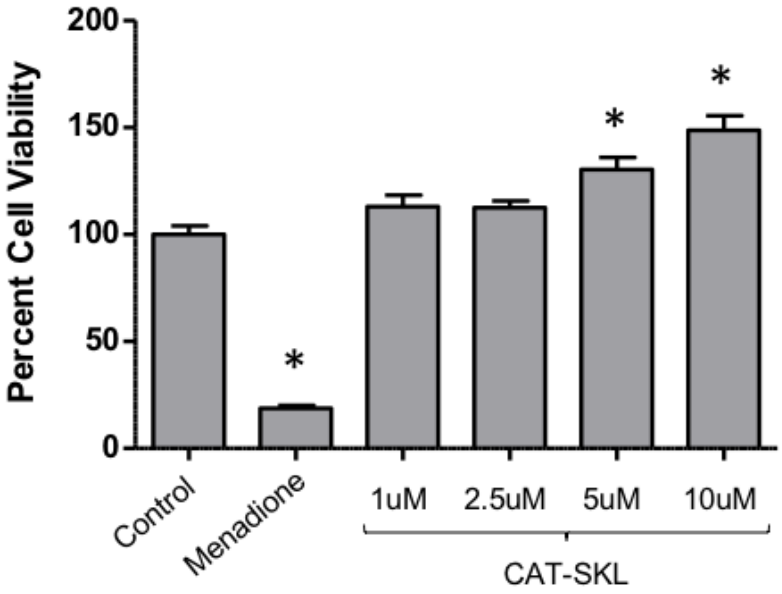

Figure 2. **CAT-SKL is not harmful to HEI-OC1 cells.** HEI-OC1 cells were incubated with CAT-SKL for 24 hours prior to assessment of cell viability using the WST-1 cell proliferation assay. Menadione, a known oxidative stress inducing reagent was used as a positive control. Addition of menadione (100µm) for 1 hour induced a significant decrease in cell viability. Incubation with CAT-SKL at 1µM and 2.5µM for 24 hours did not result in a significant deviation from the control. However, addition of CAT-SKL at 5µM and 10µM significantly increased cell viability. Statistical analysis was performed using a one-way ANOVA, non-repeated measures. When ANOVA indicated significant treatment effects ($p<0.05$), means were separated using Dunnett's multiple comparison's test, comparing all groups to the untreated control group. Bars represent mean ± SEM for triplicate samples (N=3, n=18).

CAT-SKL protects against cisplatin-induced ototoxicity

Next, we examined the protective effects of CAT-SKL against cisplatin-induced toxicity in HEI-OC1 cells. To accomplish this, WST-1 cell proliferation reagent was used. HEI-OC1 cells were pretreated with either 1μM, 2.5μM, 5μM, or 10μM of CAT-SKL for 24 hours and subsequently challenged with 15μM of cisplatin, which has been previously reported as the IC_{50} of cisplatin, for 48 hours. To compare the cell viability between CAT-SKL pretreated cells, control cells and cisplatin only cells, a one-way non-repeated measure ANOVA was performed. When ANOVA indicated significant treatment effects ($p<0.05$), means were separated using Dunnett's multiple comparison's test, comparing all groups to the cisplatin only (15μM) group. The ANOVA revealed a significant main effect for treatment ($F_{(5,103)}=10.52$, $P<0.0001$). As expected, cells treated with cisplatin alone had a significant reduction in cell viability compared to untreated cells (Fig. 2b; $p<0.01$). CAT-SKL was protective against cisplatin exposure in a dose dependent manner. Pretreatment with CAT-SKL at 1μM and 2.5μM did not demonstrate a protective effect against cisplatin exposure as there were no significant differences in percent cell viability as compared to cells treated with cisplatin only ($p>0.05$). Pretreatment with CAT-SKL at 5μM and 10μM exhibited a protective effect against cisplatin exposure as evidenced by a significant increase in cell viability compared to cells treated with cisplatin alone ($p<0.01$).

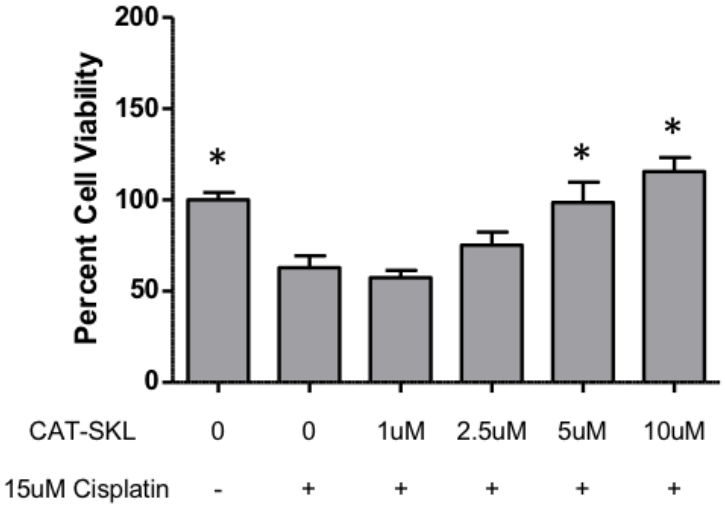

Figure 3. **CAT-SKL is protective against cisplatin-induced ototoxicity.** HEI-OC1 cells were incubated with CAT-SKL for 24 hours before assessment of cell viability using the WST-1 cell proliferation assay. Incubation with cisplatin at 15μM showed a significantly reduced percent cell viability compared to untreated cells. Pretreatment with CAT-SKL at 1μM and 2.5μM followed by cisplatin challenge did not significantly improve percent cell viability. However, pretreatment with CAT-SKL at 5μM and 10μM, followed by cisplatin challenge, did significantly increase the percent cell viability compared to the cells exposed to cisplatin only. Statistical analysis was performed using a one-way ANOVA, non-repeated measures. When ANOVA indicated significant treatment effects ($p < 0.05$), means were separated using Dunnett's multiple comparison's test and comparing all groups to the cisplatin treated group. Bars represent mean ± SEM for triplicate samples (N=3, n=18).

CAT-SKL improves cell viability even at high concentrations of cisplatin

Having established the protective effect of CAT-SKL in our cisplatin-induced HEI-OC1 ototoxicity model, we next sought to investigate whether CAT-SKL was able to offer sufficient protection against higher doses of cisplatin. To accomplish this, WST-1 cell proliferation reagent was used. Cell viability was determined in HEI-OC1 cells pretreated with 10μM of CAT-SKL for 24 hours and subsequently challenged with 15μM, 20μM, and 30μM of cisplatin for 48 hours (Fig. 4). A two-way ANOVA revealed a significant main effect for CAT-SKL treatment ($F_{(1,135)}$=153.3, $p<0.0001$), a significant main effect for cisplatin treatment ($F_{(3,135)}$=80.75, $p<0.0001$), as well as a significant interaction between CAT-SKL and cisplatin treatments ($F_{(3,135)}$=4.115, $p<0.01$). As expected, Tukey's post hoc test showed increasing the concentration of cisplatin (15μM, 20μM, and 30μM) resulted in significantly greater reductions in cell viability relative to untreated cells ($p<0.0001$). Additionally, it was observed that pretreatment with CAT-SKL (10μM) increased the cell viability after challenge by cisplatin such that cells pretreated with CAT-SKL, then treated with cisplatin at both 15μM, 20μM, no longer exhibit significant differences in cell viability compared to untreated cells ($p>0.05$). Moreover, at all three concentrations of cisplatin (15μM, 20μM, and 30μM), pretreatment with CAT-SKL then challenge by cisplatin, significantly improved the cell viability compared to cisplatin only cells ($p<0.0001$). Surprisingly, pretreatment with CAT-SKL was able to improve cell viability even at the highest dose of cisplatin that was tested (30μM).

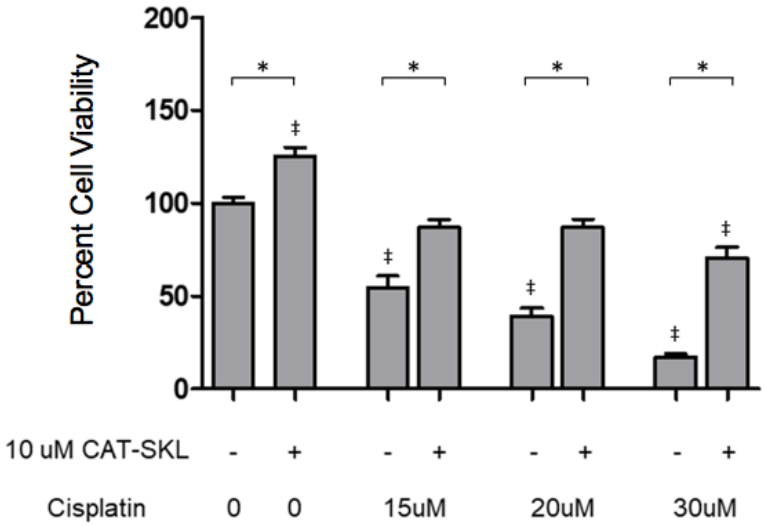

Figure 4. **CAT-SKL is able to improve cell viability even at high concentrations of cisplatin.** HEI-OC1 cells were incubated with CAT-SKL for 24 hours before assessment of cell viability using the WST-1 cell proliferation assay. Incubation with cisplatin at 15µM, 20µM, or 30µM showed a significant reduction in percent cell viability compared to untreated cells in a dose dependent manner. Pretreatment with CAT-SKL at 10µM followed by cisplatin challenge for 48 hours at all three concentrations significantly improved the percent cell viability. Most noticeably, pretreatment with CAT-SKL at 10µM was able to improve the decrease in percent cell viability caused by 30µm of cisplatin. Two-way ANOVA was performed, means were separated using Tukey's post hoc test. (*) represent significant differences within cisplatin treatment groups; (‡) represent significant differences from untreated cells. Bars represent mean ± SEM for triplicate samples (N=3, n=18).

CAT-SKL ameliorates cisplatin-induced production of reactive oxygen species

Using dihydroethidium staining, we examined the protective effects of CAT-SKL against cisplatin-induced production of reactive oxygen species in HEI-OC1 cells. HEI-OC1 cells were either left untreated, treated with 10μM of CAT-SKL alone, treated with 30μM of cisplatin alone, or pretreated with 10μM of CAT-SKL prior to exposure to 30μM of cisplatin. As predicted, treatment with 30μM of cisplatin caused a noticeable increase in red fluorescence, signifying high levels of superoxide radicals within the cells. Pretreatment with CAT-SKL decreased the cisplatin-induced increase in superoxide radicals, as evidenced by a decrease in red fluorescence. Incubation with CAT-SK alone did not cause a noticeable change in the levels of reactive oxygen species compared to the untreated cells.

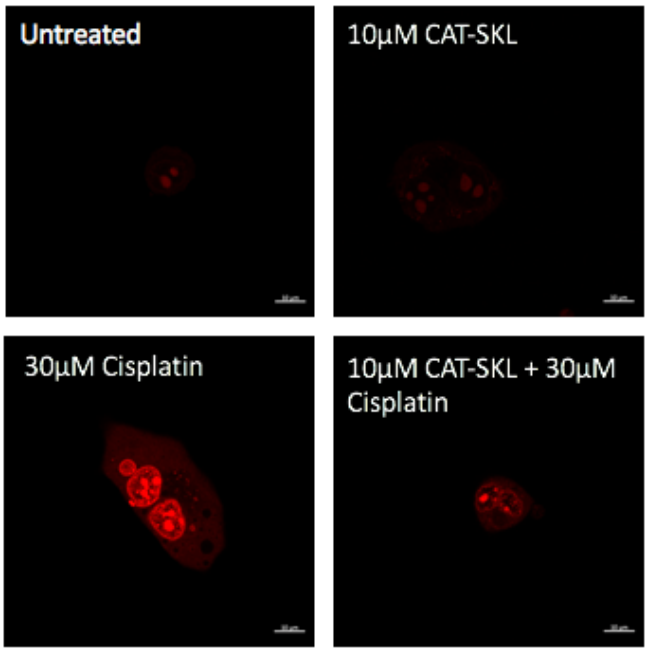

Figure 5. **CAT-SKL restores oxidative equilibrium to cisplatin challenged cells.** HEI-OC1 cells were incubated with CAT-SKL for 24 hours before assessment of cellular superoxide radical content using a dihydroethidium (DHE) staining (red). Incubation with cisplatin (30µM) showed noticeably higher levels of red fluorescence. Pretreatment with CAT-SKL (10µM), followed by cisplatin challenge was able to attenuate the ROS content. Similar levels of red fluorescence were observed between untreated and CAT-SKL (10µM) group, suggesting CAT-SKL administration does not result in increased ROS production (scale bars, 10µm).

CAT-SKL attenuates cisplatin-induced peroxisomal proliferation

Previous studies have reported proliferation of peroxisomes in cells undergoing either the aging process or diseased states[28,29]. This proliferation has been postulated to compensate for the compromised peroxisome function and further assist the mitochondria in metabolizing the overwhelming formation of ROS[24]. To detect peroxisomal proliferation, immunocytochemistry was performed on HEI-OC1 cells. Cells were stained for DAPI (DNA stain) and anti-PMP70 (70kDa-Peroxisomal membrane protein), a common peroxisome marker. HEI-OC1 cells were pretreated with CAT-SKL (10μM) for 24 hours prior to exposure to either 15μM or 30μM cisplatin for 48 hours. CAT-SKL only and untreated cells were also stained as control groups. In both control and CAT-SKL only groups, no noticeable difference in peroxisomal staining or distribution was observed. As predicted, cells treated with either 15μM or 30μM of cisplatin alone exhibited increased amounts of anti-PMP70 staining in both the cell body and cell processes, signifying peroxisomal proliferation. Pretreatment with CAT-SKL (10μM) followed by cisplatin challenge (15μM or 30μM) showed noticeable attenuation peroxisomal proliferation and resulted in peroxisomal staining more akin to that seen in untreated cells.

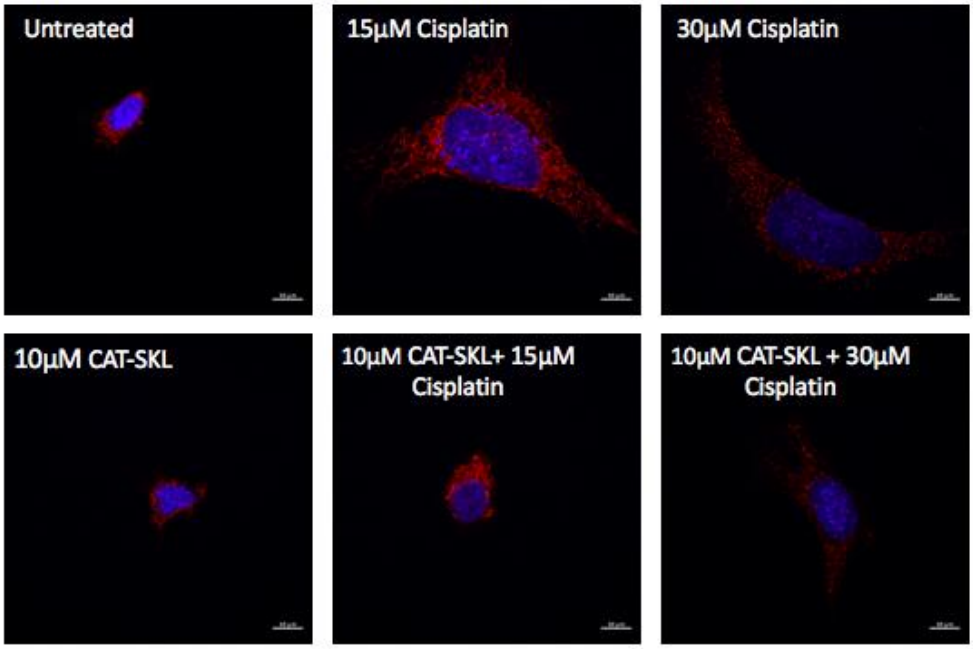

Figure 6. **CAT-SKL attenuates cisplatin-induced peroxisomal proliferation.** Immunocytochemistry was performed on HEI-OC1 cells stained for DAPI (blue) and anti-PMP70 (red). HEI-OC1 cells challenged with cisplatin (15µM and 30µM) exhibited a greater expression of the peroxisomal marker PMP70. Pretreatment with CAT-SKL diminished these changes, resulting in peroxisomal staining and distribution more comparable to that seen in untreated cells (scale bars, 10µm).

Discussion

In this study, we investigated the protective effects of the targeted antioxidant, catalase-SKL, on cisplatin-induced ototoxicity in HEI-OC1 cells. We demonstrated that administration of CAT-SKL is not only innocuous to HEI-OC1 cells, but will also significantly improve cell viability at high concentrations. In addition, treatment of HEI-OC1 cells with the ototoxic compound, cisplatin, resulted in decreased cell viability, increased levels of reactive oxygen species, and induced peroxisomal proliferation. Pretreatment with CAT-SKL (10μM) was able to attenuate cisplatin-induced damage due to its antioxidant properties. By decreasing ROS levels, the toxicity induced by cisplatin was lessened thereby increasing cell viability, decreasing ROS formation, and decreasing the proliferation of peroxisomes.

The results of this study add to a growing body of literature demonstrating the effectiveness of CAT-SKL as a targeted antioxidant to reduce cellular stress caused by ROS. Previous studies explored the efficacy of CAT-SKL in reducing cell death caused by amyloid β-derived diffusible ligands (ADDL)[25]. ADDLs have commonly been used to stimulate accumulation of Aβ-formation, which is a major precursor in Alzheimer's disease[25]. Their research demonstrated that CAT-SKL administration was able to protect against ADDL-induced cytotoxicity in rat primary cortical and hippocampal cultures[25]. Namely, CAT-SKL improves overall cell viability, stimulates neurite formation and reduces ROS production[25]. Furthermore, the antioxidant capabilities of CAT-SKL have also been demonstrated on a model of ischemia-reperfusion injury (IRI) in neonatal rat ventricular myocytes[26]. Again, this disease is primarily driven by the excessive generation of ROS. Administration of CAT-SKL showed both increased

catalase content and activity in myocytes, proving its successful internalization, as well as a reduction in cell death in response to IRI[26]. Finally, according to a previous study by Young et. al., inflammatory skin diseases, including psoriasis, are exacerbated by the increased expression of inflammatory cytokines[30]. With addition of CAT-SKL, they demonstrated a significant decrease in inflammatory cytokines including TNF-α[30]. Taken together, these studies unequivocally demonstrate the widespread efficacy of CAT-SKL as a powerful antioxidant enzyme.

Despite numerous studies on the efficacy of CAT-SKL in combating diseases mediated by the excessive generation of ROS, its efficacy against cisplatin-induced cell death, specifically in ameliorating its ototoxic effects, has not been defined. Previous studies have been conducted exploring usage of other compounds in reducing cisplatin-induced ototoxicity, but have received mixed results. Because the majority of cisplatin enters hair cells through mammalian copper transporter 1 (CTR1), it was believed that competitively inhibiting this transporter with copper sulfate would reduce the ototoxic effects[8]. Ding et. al. did confirm the protective effects of copper sulfate in attenuating cisplatin-induced hair cell loss in an organotypic culture[8]. However, their study also showed at higher levels of copper sulfate, the protective agent itself was also able to induce cytotoxicity. Research conducted by Han et. al. also explored the usage of laminarin, superoxide and hydroxyl radical scavenger widely used in traditional Chinese medicine, in reducing cisplatin-induced ototoxicity in the HEI-OC1 cell line[31]. Their study did demonstrate that administration of laminarin was able to inhibit the mitochondrial apoptotic pathway through a reduction of Bax mRNA and an elevation of Bcl-2 mRNA, which indicated protection[31]. However, despite its protective effects, high doses appear

to cause cytotoxic effects instead of the beneficial effects[31]. Thus, the development of a safe and effective protective agent against cisplatin-induced ototoxicity is of utmost importance.

Our study confirmed the successful internalization of CAT-SKL in HEI-OC1 cells using immunocytochemistry and western blotting (Fig. 1). In addition, results from our WST-1 cell viability assay showed that the administration at higher concentrations of CAT-SKL significantly improves their cell viability to 130% compared to untreated cells (Fig. 2). Also using a WST-1 assay, a similar increase in cell viability of over 100% was observed by Choung et. al. Their study focused on using Korean Red Ginseng (KRG) to prevent gentamicin-induced hearing loss in rats[32]. Like cisplatin, gentamicin-induced ototoxicity is also dependent on excessive generation of ROS[32]. KRG is a known inhibitor of ROS generation, and upon administration, reduced gentamicin-induced cell death[32]. It was demonstrated that administration of KRG, was able to induce a significant increase in cell viability compared to control groups – approximately 140% at 200pg/mL[32].

Previous studies have demonstrated successful reduction of ROS formation in the HEI-OC1 cell line through the usage of antioxidants. Dong et. al. indicated sodium hydrosulfide, NaHS, is able to exert antioxidant effects due to its role as an oxygen scavenger[33]. They used the HEI-OC1 cell line and the cells were challenged with gentamicin, an aminoglycoside antibiotic, known to induce oxidative stress and apoptosis[32,33]. DCFH-DA fluorescence was used to confirm the increase in ROS production upon gentamicin administration[33]. NaHS pretreated groups had a noticeable reduction in fluorescence[33]. Furthermore, cells pretreated with NaHS then challenged

with gentamicin also exhibited greater cell viability as detected by the MTT assay, a cell viability assay similar to the WST-1 cell viability assay[33]. Similarly, we observed an increase in ROS production, using DHE staining, in HEI-OC1 cells challenged with cisplatin (Fig. 5). This increase in red fluorescence was then attenuated upon pretreatment with CAT-SKL, our antioxidant. We also observed a significant increase in cell viability in HEI-OC1 cells pretreated with CAT-SKL then challenged with cisplatin at all 3 concentrations (15μM, 20μM, and 30μM) when compared to cisplatin only cells (Fig. 4). This data suggests we are able to successfully induce oxidative stress in the HEI-OC1 cell line and subsequently rescue the cells from ROS stress upon administration of an antioxidant.

As a marker of cell condition, we performed immunocytochemistry on HEI-OC1 cells using anti-PMP70 (70kDa Peroxisomal Membrane Protein) antibody, a common marker of peroxisomes (Fig. 6). Previous studies have reported the unique ability of peroxisomes to proliferate and multiply in response to nutritional and extracellular environmental stimuli[24,28]. This proliferation is typically characterized by the increase in the number and size of peroxisomes, and an induction of peroxisomal enzymes[28]. Furthermore, it has been observed that mitochondrial abnormalities were accompanied by the proliferation of peroxisomes[28]. Studies by Feher et. al. observed noticeable peroxisomal proliferation in patients experiencing age-related macular degeneration in the cells of the retinal epithelium[34]. It is believed this peroxisomal proliferation occurs to compensate for reduced peroxisome function and to further assist the mitochondria in metabolizing the pathological levels of ROS[25,34]. As predicted, when HEI-OC1 cells were treated with 15uM and 30uM of cisplatin, we observed a proliferation of

peroxisomes in both the cell bodies and cell processes. Furthermore, when cells were pretreated with CAT-SKL then challenged with cisplatin, this proliferation was noticeably attenuated in both groups.

Limitations of the Study and Future Directions

The HEI-OC1 cell line is a well-established cell line, widely used in the testing of ototoxic compounds[27,31,33]. However, a limitation of this study is the translational potential to a mouse model. Currently, we have established the IC_{50} of cisplatin and demonstrated successful protection with CAT-SKL pretreatment, but in the future, the physiological concentration of cisplatin must be established in order to determine the protective capabilities of CAT-SKL at those concentrations. Furthermore, the metabolism, absorption, and toxicity of CAT-SKL on other organ systems in an *in vivo* mouse model need to be evaluated in order to confirm its safety.

Moreover, we have yet to determine a dose response curve for the timing of CAT-SKL administration. Though we know CAT-SKL is internalized in HEI-OC1 cells at 24 hours, we do not know its protective capabilities if it is co-administered with cisplatin. Our study has successfully demonstrated the protective abilities of CAT-SKL on cisplatin-induced ototoxicity. Therefore, we plan to further explore its protective effects on reducing ototoxicity induced by other compounds such as gentamicin, an aminoglycoside antibiotic. Although CAT-SKL is able to protect against cisplatin-induced ototoxicity, its potential interference with the therapeutic effects of cisplatin have yet to be verified. Another future step would be to elucidate the activity and expression of key proteins in the mitochondrial apoptotic pathway to evaluate the extent of protection conferred by CAT-SKL. Furthermore, although CAT-SKL internalization in a cell line has

proven to be successful, the presence of a non-discriminatory cell penetrating peptide may become a barrier when administering CAT-SKL into a mouse. Therefore, our lab is investigating the efficacy of removing the cell penetrating peptide from the CAT-SKL enzyme and instead using exosome encapsulation of the enzyme to allow for delivery. Exosomes are not only highly resistant to degradation in the blood stream, but studies have shown viruses can be used to modify exosomes in order to target them to very specific locations in the body[35,36].

Conclusion

There currently exists a great need to develop a safe and effective protective agent against cisplatin-induced ototoxicity. The results of this project have proved to yield promising steps towards that goal. Our study has shown that CAT-SKL is not only harmless to HEI-OC1 cells, but at high concentrations, actually improves overall cell viability. Moreover, we demonstrated that cisplatin administration results in decreased cell viability, increased ROS formation, and induced peroxisomal proliferation – all of which was attenuated with CAT-SKL pretreatment. At present, there is no widely accepted pharmacotherapy which protects against ototoxic pharmaceuticals. Therefore, with the results of this project, we will be one step closer to developing a safe and effective prophylactic pharmaceutical therapy for protecting against ototoxic drug-induced hearing loss.

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