Article

Roles of sirtuins in the protection against cisplatin ototoxicity

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Abstract: Cisplatin ototoxicity (CO) in chemotherapy affects an estimated 100-300 thousand cancer patients in USA annually, causing symptoms including severe hearing loss, tinnitus, and dizziness. CO is related to the accumulation of reactive oxygen species (ROS) in the cytoplasm and mitochondria of the cochlear cells, inducing damage associated mainly with outer hair cell loss. We have shown recently that honokiol (HNK), a pleiotropic antitumor agent, can protect against CO and increase animal survival in cisplatin treatment. This hearing protective effect is associated with the upregulation of sirtuin 3 (SIRT3), a member of an NAD+-dependent deacetylase family. The sirtuin family consists of 7 highly conserved members that differ in subcellular locations (cytoplasm: SIRT1&2; mitochondria: SIRT3-5; nuclei: SIRT1, 2, 6, 7), which are all key regulators of the intrinsic ROS detoxification systems. In this study, we further investigated the protective effects of HNK against CO on ribbon synapses in the inner hair cells and the potential roles of sirtuins in the cytoplasm and mitochondria. The results show that HNK also prevents the loss of ribbons induced by CO. This protective effect of HNK against CO is associated with the upregulation of SIRT1, 2, 3, and 5, suggesting the involvement of the sirtuin family.

Keywords: Drug-induced hearing loss; cochlea; outer hair cells; hearing protection; SIRT3

1. Introduction

Cisplatin and other platinum-based compounds are potent antitumor drugs used in ~40% of cancer chemotherapy regimens [1-3]. Unfortunately, platinum drugs have significant adverse effects, including nephrotoxicity [4-6], neurotoxicity [4,5,7], and ototoxicity [4,5,8,9], which limit their usage and dosage. In the cochlea, cisplatin generates reactive oxygen species (ROS) [10,11], depletes the cell's antioxidants (e.g., glutathione), and causes inflammation, high lipid peroxidation, oxidative damage of proteins, nucleic acid injury, and S-nitrosylation [12,13]. These changes activate inflammatory and apoptotic pathways [14], damaging the organ of Corti, stria vascularis (SV), the spiral ganglion neurons (SGNs) [15,16], and the inner and outer hair cells (IHCs and OHCs, for reviews, see [12,13,17-20]). The subsequent loss of hair cells leads to hearing loss. Tinnitus and dizziness are also common sequelae. The prevalence of hearing loss during chemotherapy with cisplatin is 60-80% [9,21]. In the United States, that is about hundred thousand to three hundred thousand new cases annually. Those patients cannot be helped because no FDA-approved treatment is available for hearing protection during chemotherapy.

Treatment options explored in the past include exogenous antioxidants that work as free radical scavengers (e.g., thiosulfate, amifostine [22-24]) and anti-inflammatory drugs (e.g., dexamethasone [25-28]). They were the first selected drugs tested for hearing protection against cisplatin ototoxicity (CO) in chemotherapy. Antioxidants were selected to support the cells' effort in ROS detoxification, preventing CO (for reviews, see [29,30]). The biggest concern of this approach was the possible interference with the tumor therapy in the clinic by deactivating cisplatin [30-32] or by protecting tumor cells [33]. More specific candidates, such as factors/cytokines in metabolic [34,35], inflammatory [36,37], or apoptosis [38-40] signaling pathways, were tested. Despite having promising results for these candidates, poor bioavailability and tissue toxicity prevented their use in the clinic. More importantly, ROS generated at multiple steps

along the inflammatory and apoptotic pathways damages cellular structures in both the cytoplasm and mitochondria. It is questionable whether targeting a single site in ROS production pathway is sufficient for complete protection against CO.

Our recent study on tumor-bearing mice undergoing cisplatin treatment showed that honokiol (HNK), a multi-functional molecule with antitumor effects, can protect against CO. This hearing protective effect is associated with the activation of sirtuin 3 (SIRT3) in the mitochondria, which is essential for ROS detoxification. Sirtuins belong to an NAD^+-dependent deacetylase family, which are key enzymes for stress resistance pathways and the key regulators of the intrinsic anti-ROS systems [41-44]. The sirtuin family contains seven members (SIRT1-7) in mammals, which differ in catalytic activities and subcellular locations (in the cytoplasm: SIRT1&2; mitochondria: SIRT3-5; and in the nucleus: SIRT1, 2, 6, 7). Through the regulation of ROS, sirtuins govern cellular processes including homeostasis [45], responses to stress [46], DNA damage repairing [46], inflammation [47,48], and apoptosis [47]. Sirtuins play a critical role in metabolism [49], longevity [41,50], carcinogenesis [50], etc. Since all members of the sirtuin family work through regulating ROS they may be important for hearing protection and might all be modulated by HNK. To better understand the interactions between HNK and the sirtuins, this study explores the expression of sirtuin family members in the cytoplasm and mitochondria of cochlear cells during the treatment with HNK, cisplatin, and the combination thereof, aiming to identify their roles in hearing protection against CO.

2. Results

2.1. HNK potentiates the antitumor effect of cisplatin and protects hearing in chemotherapy

A transgenic mouse model from Jackson Laboratory expressing mouse mammary tumor virus (MMTV) polyoma-virus middle T agent (PyMT) oncogene (Stock #: 002374) [114] was used in this study. The tumor-bearing female MMTV-PyMT mice were divided into four different groups which were treated with a chemotherapy regimen consisting of 3 cycles (Fig. 1A). In each cycle, 4 doses of HNK (10 mg/kg/day) and/or cisplatin (4 mg/kg/day) were administered across 4 days, followed by a 10-day recovery interval (also refer to the Materials and Methods). Treatments started on the day when the tumor size reached 500 mm^3. The tumor size of the animals in the control group reached ≥ 1500 mm^3, the limit for euthanasia, within seven days (7 out of 7, 7/7; Fig. 1B, the black curves, n=7). Most animals treated with HNK alone (HNK-only group, red traces, n=5) survived up to 14 days (4/5), with one exception (9 days, 1/5). The animals treated with cisplatin alone (Cis-only group, blue traces, n=7) survived till the 3rd cycle (6/7), but only one of the six survived till the end of the study (42 days). The other mice were euthanized for humane reasons such as significant weight loss (over 25%, 2/5) or excessive tumor size (≥1500 mm^3, 3/5), etc. All animals treated with a combination of HNK and cisplatin (HNK + Cis group, purple traces, n=7) survived till the 3rd cycle (7/7), and four out of seven mice reached the end of the study. The results showed a synergistic effect of HNK with cisplatin in tumor suppression. [51,52]

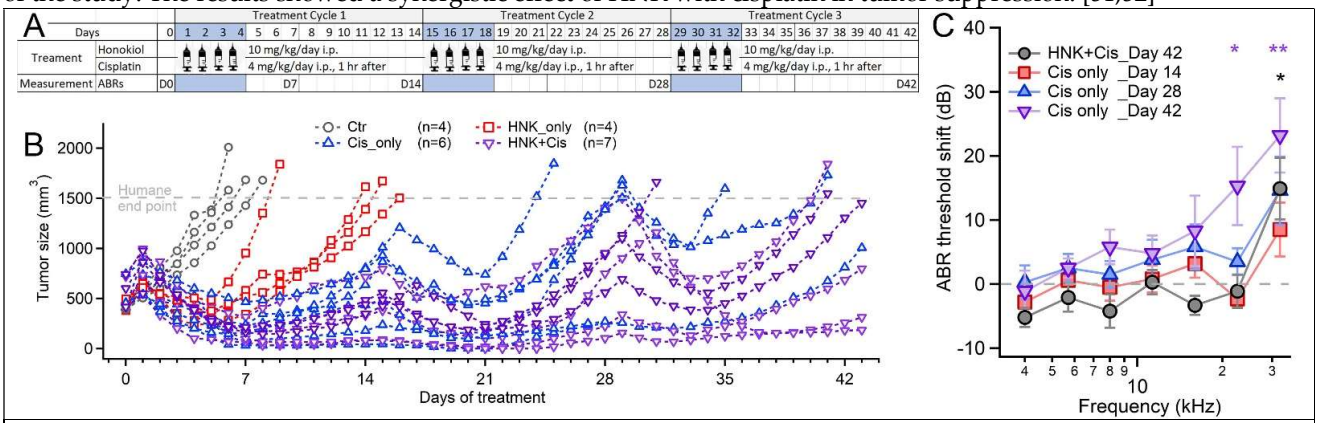

Figure 1. (A) Chemotherapy regimen for Tumor-bearing mice. (B) Tumor growth in different treatment groups. Each curve represents one animal. Treatment started when the tumor size reached 500 mm^3 and terminated when the tumor size reached 1500 mm^3. (C) ABR Threshold shift of the animals during and after the 3-cycle chemotherapy regimen, normalized by hearing baseline measured before the treatment (Day 0). Data present are mean ± standard error. **: p<0.01; *: p<0.05, compared to Day 0.

A progressive ABR threshold elevation was observed at frequencies over 16 kHz after each cycle of cisplatin treatment, as shown in Fig. 1B. In Cis-only group, a slight elevation compared to the baseline (8.5±4.2 dB, values are the mean and the corresponding standard error (SE)) was observed at 32 kHz after the 1st cisplatin treatment cycle (4mg/kg/day for 4 days), but is statistically insignificant (p=0.25, red trace). A small and still insignificant ABR threshold elevation occurred after the 2nd cisplatin treatment cycle (blue trace) at frequencies above 16 kHz. The 3rd cycle of cisplatin treatment resulted in a significant ABR threshold elevation at frequencies of 22.6 kHz (15.3 ±6.1 dB, p=0.04)) and 32

kHz (23.2±5.8 dB, p=0.01). A slight but insignificant elevation was also observed at 16 kHz (8.3±5.5 dB, p=0.16) (purple trace). In HNK + Cis group, combined administration with HNK significantly decreased this ABR threshold shift, as a smaller elevation was observed at 32 kHz only (14.9±4.8 dB, p=0.05, black trace). Note that day 42 includes ABR recordings taken on the last day during the 3rd cycle of treatment, before the endpoint of the study.

2.2. HNK prevents the loss of synaptic components induced by cisplatin treatment

In our recent publication, we histologically verified the loss of OHCs after cisplatin treatment [53]. In contrast to OHCs, the inner hair cells (IHCs) remain largely present. To verify the synaptic connections between the IHCs and the spiral ganglion neurons, we stained the pre- and post-synaptic components with antibodies for the C-terminal binding protein 2 (CtBP2, pre-synaptic component) and glutamate receptor 2 (GluR2, post-synaptic component). Fig. 2 shows typical changes for the different treatment groups. The samples came from the same frequency range along the cochleae, which was ~15-19 kHz. Fluorescence-labeled pre- (red) and post- (green) synaptic components were identifiable at the bottom of the IHCs. The yellow stains at the same location originate from the colocalization of the pre- and post-synaptic components (Fig. 2A). Cisplatin treatment decreased the number and intensity of the stained synaptic components in the cochlear samples from cisplatin group (Fig. 2B). For a quantitative study, the images were processed in Imaris software (Oxford Instruments, Abingdon, Oxfordshire, England). After digitally separating hair cells and their synapses, we determined the IHCs versus synapse number ratio. The numbers of pre-, post-, and colocalized synaptic components were counted separately (Fig. 2A' and 2B', the insets). In samples from the control group, the counts are 16.3±0.4, 16.2±0.5, and 16.0±0.4, respectively (mean ± SE, n=90 neurons from 3 samples). In samples from the cisplatin group, these numbers decreased to 12.1±0.7, 10.7±0.4, and 9.6±0.5, respectively (n=88 from 3 samples). In samples from the HNK group, the counts of the synaptic components were not affected compared to the control group (15.9±0.2, 15.7±0.4, and 15.5±0.4, respectively. Fig. 2C). In samples from the HNK + cisplatin group, combined HNK and cisplatin treatment reduced the cisplatin-induced changes (Fig. 2D). The resulting counts were 15.8±2.3, 16.2±1.8, and 15.7±2.3, respectively (n=91 from 3 samples). The ANOVA followed by a Tukey test showed that differences between the cisplatin group and all the other three groups are significant ($p < 0.01$, Fig. 3).

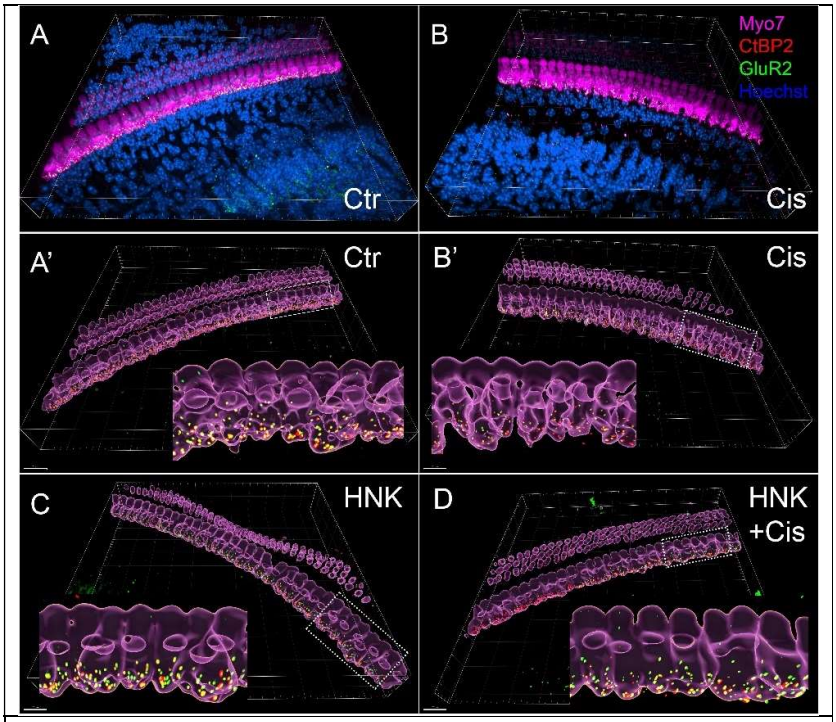

Figure 2. Confocal images showing immunostaining of the pre- (CtBP2, red) and post- (GluR2, green) synaptic components of the ribbons in the IHCs from different treatment groups: (A) and (A') control; (B) and (B') Cis-only; (C) HNK-only; and (D) HNK+Cis. The 3-D image stacks were processed to show the hair cells and synapses only. The insets are enlarged from the square areas (~19 kHz).

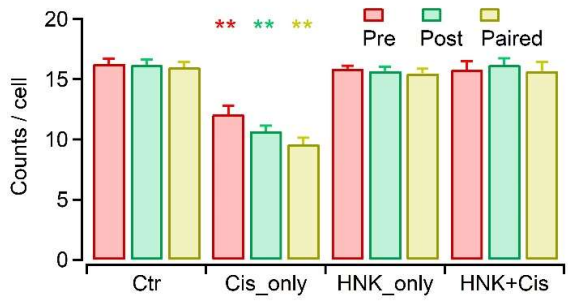

Figure 3. Quantitative study of pre-, post-, and paired synaptic components in different treatment groups at the frequency range of ~15-19 kHz. **: p<0.01, compared to all other groups.

2.3. HNK upregulates mitochondrial sirtuin expression in the cochlea

To study sirtuin expression in the cochlea, five animals from each group were euthanized one day after the administration of the last dose of the drugs in the 1st cycle (day 5). The cochleae were then harvested and immunostaining with sirtuin antibodies was performed. Fig. 4 shows the expression of mitochondrial sirtuins (SIRT3 and SIRT5) in the same location of the cochlea (frequency range: ~9-36 kHz) from different treatment groups. A baseline SIRT3 (green) and SIRT5 (red) expression was observed in the samples from the control group (Fig. 4A). In samples from the HNK group, an obvious upregulation of green and red staining was observed and colocalized (yellow), suggesting that the expression of both SIRT3 and SIRT5 was increased (Fig. 4B). The most prominent increase of SIRT3 and SIRT5 staining was in the OHCs, as shown in the orthogonal view (Fig. 4B, the arrow in the lower panel). Increased fluorescence was also observed in the IHCs (Fig. 4B, the pentagon). In the supporting cells, a possible weak increase of green (SIRT3) fluorescence was shown in some samples treated with HNK, but not decisively (Fig. 4B, the arrowheads). In the samples from the cisplatin group, no apparent increase of green fluorescence (SIRT3) was seen, but a possible slight increase of red fluorescence (SIRT5) was shown in both OHCs, IHCs, and supporting cells (Fig. 4C). In the samples from HNK + cisplatin group, upregulation of both SIRT3 and SIRT5 was apparent in the OHCs, IHCs, and supporting cells (Fig. 4D). Note that SIRT3 and SIRT5 expression was supposed to be in the mitochondria. However, it cannot be distinguished from the cytoplasm, despite the characteristic arrangement of mitochondria in OHCs. Other studies involving immunostaining mitochondria in OHCs showed similar results (e.g., [55,56]).

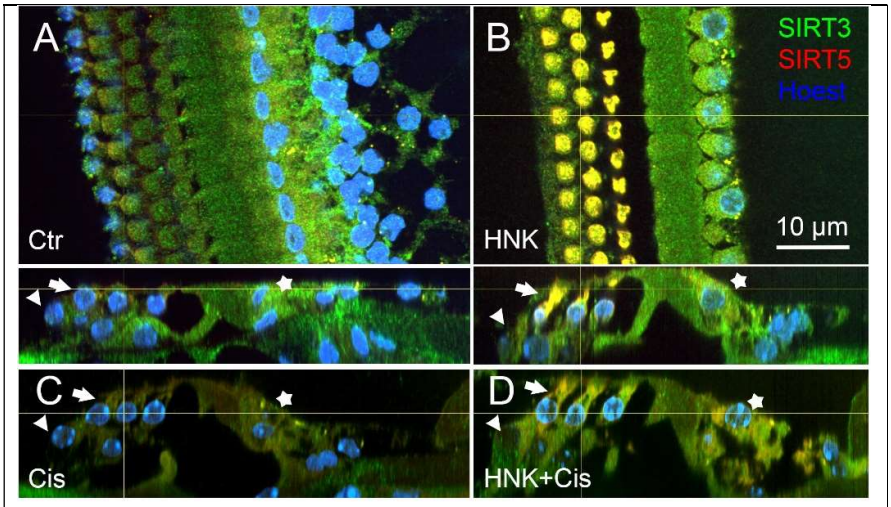

Figure 4. Surface and orthogonal views of the cochlea showing SIRT3 and SIRT5 expression in the cochleae from different treatment groups: (A) Control; (B) HNK; (C) Cis-only; and (D) HNK+Cis. SIRT3 and SIRT5 are both upregulated and colocalized (showing yellow) in the OHCs (white arrows) and IHCs (white star) in (B) and (D). Note the Control sample in (A) is flattened during mounting.

2.4. HNK upregulates cytoplasmic sirtuin expression in the cochlea

Immunostaining of cytoplasmic sirtuins (SIRT1 and SIRT2) was also perform in the cochlear samples treated with one cycle of HNK, cisplatin, and the combination of cisplatin and HNK. SIRT1 also showed a baseline expression level in the samples from the control group (Fig. 5A). HNK treatment increased the expression of SIRT1 in the OHCs and IHCs (Fig. 5B). No obvious upregulation of SIRT1 occurred in the samples from cisplatin group (Fig. 5C). SIRT1 was also upregulated in the samples from HNK + cisplatin group (Fig. 5D). A similar expression and upregulation pattern for SIRT2 was also revealed, as shown in Fig. 6.

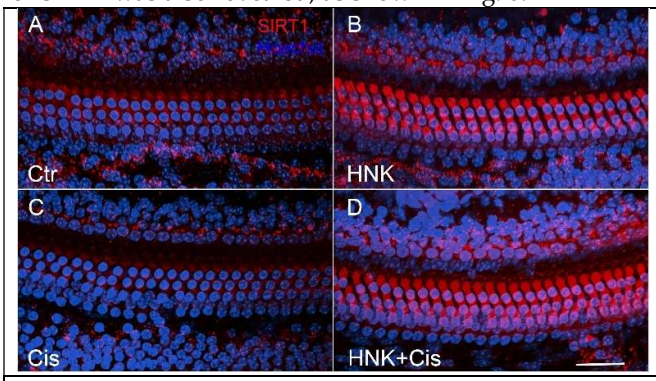

Figure 5. Projections of 3D image stacks showing SIRT1 expression in the cochleae (frequency range: ~10-16 kHz) from different treatment groups. Scale bar: 20 μm.

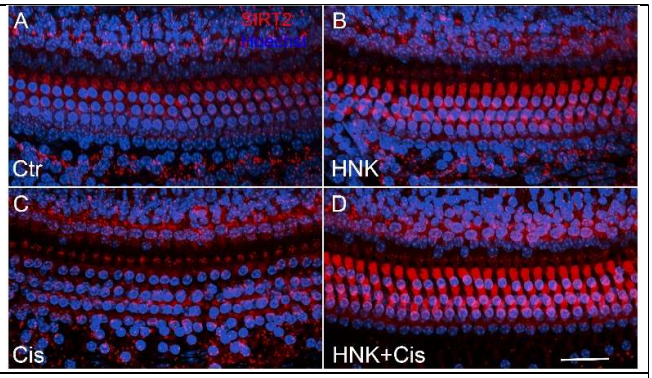

Figure 6. Projections of 3D image stacks showing SIRT1 expression in the cochleae (frequency range: ~20-30 kHz) from different treatment groups. Scale bar: 20 μm.

Since SIRT1 and SIRT2 are also expressed in the nuclei as shown in other tissues, a close examination of their expression was performed focusing on the nuclei of the hair cells and supporting cells (Fig. 7). However, no unmistakable fluorescence signal was detected in the nuclei of either hair cells (Fig. 7A) or supporting cells (Fig. 7B) in the samples from the control group (left panels). Treatment with HNK did not stimulate their expression in the nuclei, either (right panels).

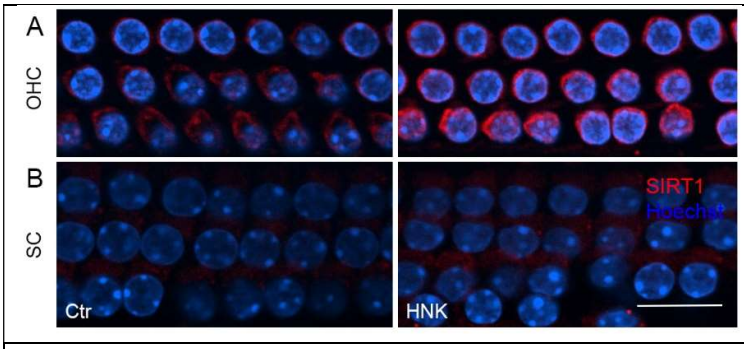

Figure 7. Enlarged images from Fig.5 A &B, focusing on the nuclei of OHC and supporting cells (SC), respectively. Scale bar: 10 μm.

3. Discussion

This study expands the findings of the protective effects of HNK against CO on the synaptic level. The potential roles of the sirtuin family members in the cytoplasm (SIRT1 and SIRT2) and mitochondria (SIRT3 and SIRT5) were also explored. The results show that cisplatin causes damage to ribbon synapses in the IHCs, which is preventable by HNK, while also protecting OHCs and improving the animals' general health during cisplatin treatment. Furthermore, HNK upregulates the expression of sirtuins in both the cytoplasm and mitochondria, suggesting the involvement of multiple members of the sirtuin family in the hearing protective effects of HNK.

3.1. HNK Protects IHC ribbon synapse in chemotherapy

OHCs are the most vulnerable cells in the cochlea against CO. Consequently, preventing OHC loss is an important goal for hearing protection against CO [57]. The quantitative study of OHC loss after cisplatin treatment in our previous publication verified the protection of OHCs by HNK [53]. On the other hand, fewer studies address the effects of CO on IHC, possibly because IHC loss is minimal during chemotherapy, as shown in our study [53] and previous publications [57,58]. Nevertheless, impairment of OHC function alone may not be fully accountable for the decrease of cochlear function after cisplatin treatment. As shown in an early quantitative study, significant hearing threshold elevation can be induced by cisplatin treatment without substantial change in OHC function [59]. In this regard, we sought to determine whether the function of IHCs was also affected by CO in this study. As shown in Fig. 2, a ~40% synaptic loss in the middle region of the cochlea occurred after three cycles of cisplatin treatment, which was prevented by HNK administration. The cisplatin-induced synaptic change agrees with other studies in which only the pre-synaptic component was studied and found to be lost during cisplatin treatment [14,60]. Our study further proves that cisplatin also induces a loss of post-synaptic components (~34%), which might be more severe than the loss of pre-synaptic components (~26%) (Fig. 3). The loss of both pre- and post-synaptic components is prevented by HNK treatment. These results suggest that acute hearing loss induced by cisplatin is also associated with the damage of SGNs, or at least the nerve endings of SGNs connecting the IHCs. Furthermore, the protective effect of HNK of SGNs may also have clinical significance since it creates a time window for (partial) hearing restoration or recovery after chemotherapy. The finding that HNK protects the central nervous system against oxidative stress [61-63], related to SIRT3 activation [64,65], supports our view of the cochlea.

3.2. Roles of sirtuin family in the hearing protective effect of HNK against CO

Sirtuins are composed of an NAD^+-dependent deacetylase family (except SIRT4, which harbors an ADP-ribosyltransferase activity), which shares a highly conserved catalytic core [42,66,67]. Expressed in diverse subcellular locations and having different substrates, sirtuins function as key regulators of the intrinsic anti-ROS systems, working through activating key enzymes for stress resistance pathways [41-44]. Through the regulation of ROS, sirtuins govern cellular processes including homeostasis [45], responses to stress [46], DNA damage repairing [46], inflammation [47,48], and apoptosis [47]. Therefore, they play critical roles in physiological functions including metabolism [49], longevity [41,50], carcinogenesis [50], etc. Among the seven members of the sirtuin family, SIRT1 and SIRT2 are the only two expressed in the cytoplasm (as well as the nucleus) [42], regulating multiple cellular processes involving metabolism and the response to oxidative stress [49,68,69]. Studies show that they play critical roles in governing general health and preventing age-associated damage [68,70,71], possibly including age-related hearing loss (ARHL) [72-74]. SIRT3-5 are the ones exclusively expressed in mitochondria. SIRT3 [75,76] and SIRT5 [77-80] fine-tune the activity of various enzymes critical for ROS detoxification through lysine deacetylation, and thereby protect cells from stresses [75,76,78], promote mitochondrial functions and integrity [65], and regulate various cellular functions including metabolism [80,81], cell survival

[82], and longevity [83]. According to the literature, activation of sirtuins by HNK is mainly associated with SIRT3, which is observed in multiple tissues and organs, including the heart [84,85], brain [64,65], and kidney [86]. Nevertheless, considering the highly conserved core structure and similarity in their catalytic activity, upregulation of other sirtuins by HNK is also expected.

Considering the central role of ROS in triggering the inflammatory and apoptotic pathways in CO [14] as well as in other types of hearing impairment (e.g., noise-induced hearing loss (NIHL) [87,88]; drug-induced hearing loss (DIHL) [9,89,90]; ARHL [91-93]; for a recent review, see [94]), sirtuins, therefore, may also play critical roles for hearing protection. However, to date, studies on the function of sirtuins in hearing protection are rare and controversial. Only one study reports the expression of all the sirtuins in the cochlea in the context of age-related changes [95]. However, the results require further verification because of the limited quality of the immunostaining and unclear pattern of sirtuin expression in different cellular compartments (e.g., cytoplasm versus nuclei). SIRT1-3 are the only sirtuins studied for their roles in hearing protection. SIRT1 has been shown to protect cochlear hair cells [74] and slows ARHL [72,73], associated with P53 [74], through regulating cellular processes such as autophagy [73]. In contrast, hemizygous SIRT1 knockout mice with a deficiency of SIRT1 prevents ARHL normally occurred in wild-type animals [96]. Noise exposure upregulates SIRT2 expression, and the treatment with a SIRT2 inhibitor reduced NIHL [97], indicating a negative role of SIRT2 in the protection of cochlea function. Nevertheless, SIRT2 upregulation is a protective mechanism in other tissues [69,98]. SIRT3 is involved in multiple intra-mitochondrial metabolic processes, including energy production, ROS generation, and detoxification [99]. The involvement of SIRT3 in hearing protection against different insults, including noise [54,100], drugs [101], and age [91,92] has also been studied. Various compounds and treatments have been tested for hearing protection through SIRT3 activation in these studies. However, no follow-up study is present, and none of the tested candidates are suitable for clinical application against CO, as discussed in our previous publication [53].

With the present study, the role of the sirtuin family in hearing protection was solidified. HNK upregulates SIRT3 in the cochlea, protecting against CO [53,54]. We also showed a severe progressive early-onset hearing loss in SIRT3 knockout mice, initiated on or before six weeks and associated with the abnormality of the synapse [53]. The current study is the first to show the upregulation of multiple sirtuin family members in the cytoplasm and mitochondria of cochlear tissue by HNK. This upregulation is accompanied by the hearing protection against CO, suggesting the significance of the entire sirtuin family (possibly also including the ones in nuclei) in this process. The role of SIRT3 in the protective effect of HNK against oxidative stress has been verified in multiple tissues, including the heart [84,85], brain [64,65], kidney [86], and cochlea [53]. The potential mechanism is related to the activation of manganese superoxide dismutase (MnSOD, or SOD2) [75,102] and isocitrate dehydrogenase 2 (IDH2) [103], which are both substrates of SIRT3 and essential for ROS reduction and detoxification [104]. This mechanism is particularly beneficial to cancer patients undergoing chemotherapy because SIRT3 expression is usually low and dysregulated in tumor cells [105-107], and the activation of SIRT3 inhibits tumor cell proliferation [81,107,108]. This dual function of HNK, i.e., synergistic with cisplatin in tumor suppression (e.g., squamous carcinoma [109]; lung cancer [110]; ovarian carcinoma [52]; colon cancer [111]; mammary tumor [53]) and protective against cisplatin toxicity to normal tissues (e.g., kidney [112]; testicles [113]; cochlea [53]), has been verified in various publications. Nevertheless, since ROS is also generated in the cytoplasm, improving mitochondrial ROS detoxification alone might not be sufficient for comprehensive protection. Cytoplasmic sirtuins (SIRT1 and SIRT2) may also play important roles in protecting against CO, as suggested by our results in the current study. Although more direct evidence is still missing, our studies provided new insight into the strategy of preventing CO without compromising the therapeutic effects of cisplatin in chemotherapy.

4. Materials and Methods

4.1 Ethics statement

All procedures were carried out following the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Northwestern University (IS00013632).

4.2. Animal groups, drug administration, and tumor growth monitoring

Female MMTV-PyMT carrier mice develop palpable tumors at the age of 65.3±8.9 days. Tumor growth was monitored every two days using a caliper since palpable. The size (volume) of the tumors was calculated with the following formula [53,114]: (length × width^2)/2. When the total size of the tumors reached 500 mm^3, the animals were randomly assigned into one of the four experimental groups: The control group (n=4) was given corn oil (solvent for HNK) and saline (solvent for cisplatin) only. The cisplatin group (n=6) was given corn oil and cisplatin (4 mg/kg/day). The HNK group (n=4) was given HNK (10 mg/kg/day) and saline. The combined HNK + cisplatin group (n=7) was given both

HNK (10 mg/kg/day) and Cisplatin (4 mg/kg/day). A chemotherapy regimen was then performed consisting of three cycles [38,53,115]. In each cycle, four doses of HNK (or corn oil) and cisplatin (or saline) were administered across four days, followed by a 10-day recovery interval. The entire chemotherapy regimen lasted for 42 days. Cisplatin and HNK (both from Sigma Aldrich, Port Washington, WI, or Tocris Bioscience, Minneapolis, MN) were both delivered through intraperitoneal (i.p.) injection, and HNK (or corn oil) was given one hour before cisplatin (or saline). Hydration with Ringer's lactated solution (RLS) was given to the treated animals daily for the first seven days of each cycle, with a dose of 25 μl/g. Animals were weighed daily, and their health was monitored throughout the study for stress and pain. Animals in critical conditions, e.g., a weight loss greater than 25%, a total tumor size over 1500 mm^3, a tumor size reaching the IACUC predetermined limit of 20 mm along one axis, or a tumor burden visibly affecting the host, were removed from the study and euthanized.

For showing sirtuin expression, another 20 animals were randomly assigned into the four groups (5 in each group) but were treated for only one cycle and euthanized on day 5.

4.2. Auditory brainstem response (ABR) measurement

The cochlear function was evaluated by ABRs, which were recorded before (day 0) and after the chemotherapy treatment was started (days 7, 14, 28, and 42). The detailed method for ABR measurement has been described in our previous publications [53,116]. Briefly, animals were anesthetized by a mixture of ketamine (80-100 mg/kg) and xylazine (2-5 mg/kg), diluted at 1:20 in RLS. The level of anesthesia was monitored every 15 minutes with paw withdrawal reflex and maintained with ketamine dilution (1:20 in RLS). The animals' body temperature was maintained at 37°C using a water-filled heating pad and monitored every 15 minutes along with other vital signs (O_2 saturation, respiratory, and pulse rates).

The acoustic stimuli were generated with a computer I/O board (KPCI 3110, Keithley, Cleveland, OH), attenuated in 5 dB steps (programmable attenuator), amplified (amplifier), and delivered with a pre-calibrated Beyer DT770Pro speaker placed in the ear canal of the animals with a speculum (quasi-free field). Tone pips with a 5 ms duration, 1 ms rise/fall time, and a maximal sound level of ≤ 107 dB SPL (sound pressure level re 20 μPa) were adopted. The frequency range was set at 4-32 kHz, with two steps per octave. To elicit ABR signals, three hypodermic needles were placed at the ipsilateral mastoid (high), vertex (low), and the body (common ground). The electrodes were connected to an ISO-80 differential amplifier (World Precision Instruments, Sarasota, FL) with an 80 dB amplification. The amplifier's high- and low-pass filters were set at 300 Hz and 3 kHz, respectively, with a slope of -12 dB/octave. A frequency devices filter (Hewlett Packard, Palo Alto, CA) with a slope of -48 dB/octave and a gain of 20 dB was used for further filtering and amplification. The stimulation signal generation and data acquisition were controlled by custom-written software in Testpoint (SuperLogics, Inc. Massachusetts, MA). The sampling rate was set at 250 kHz. Stored signals for all frequencies and sound levels were averaged 256 times or until the signal/noise ratio ≥4. ABR threshold for each frequency was defined as the minimum level required for an ABR response with a root mean square (RMS) 1.5 times above the noise floor.

4.3. Animal Euthanasia, cochlea harvest, and dissection

At the end of the treatment, the animals were euthanized by Euthasol© injection (0.1 ml, i.p.), followed by cardiac perfusion with 4% paraformaldehyde (PFA). The cochleae were then harvested, followed by an in-cochlea PFA injection, and left in PFA for 2 hours for post-fixation. Decalcification was then performed using 10% EDTA (Sigma Aldrich, St. Louis, MO) in 1X PBS (diluted from 10X PBS, Invitrogen) for 2-3 days. The full-length cochlear coil was then dissected out in 1X PBS and was cut at specific locations in the apex and base. Since the apical and basal parts tend to have artificial damages during the process of the samples, only the middle turn of the cochlea was used for immunostaining. The middle turn was also cut at specific locations into two sections (frequency range: ~9.5-19.1, and 19.1-36.5, respectively) for better immunostaining and mounting. The frequency range was determined previously by measuring the full length of the cochlea, which was correlated with the published frequency map in CBA/J mice [53,117].

4.4. Immunostaining and confocal imaging

Standard immunostaining protocols as described in our previous publications were applied[53,54]. Briefly, cochlear segments were first soaked in 30% sucrose, frozen on dry ice, and thawed at room temperature. After washing three times with 1X PBS and rinsed for 20 minutes between a shaker, the samples were blocked with 5% serum + 1% Triton. Primary antibody mixes were then added, and the samples were incubated at 37 °C overnight. Depending on the purpose, the primary antibody mix contained different primary antibodies. For synapses, it had rabbit anti-Myosin VIIa (for hair cells, Proteus Biosciences, 1:200), mouse (IgG1) anti-CtBP2 (for pre-synaptic component, BD Transduction Labs,

1:200), and mouse (IgG2a) anti-GluR2 (for post-synaptic component, Millipore, 1:500). For sirtuin family members, the antibodies for CtBP2 and GluR2 was be replaced by 1-2 sirtuin family members, including SIRT1, 2, 3, and 5 (from Santa Cruz Biotechnology or Novus Biologicals). After being rinsed three times in PBS (10 minutes each), the secondary antibody mix consisting of duck anti-rabbit (IgGH-L) AF647 (Jackson Immuno Research, 1:200), goat anti-mouse (IgG1) AF568 (Thermo Fisher, 1:200), goat anti-mouse (IgG2a) AF488 (Thermo Fisher, 1:200), and Hoechst (Thermo Fisher, 1:1000), was added and incubated in the dark for 2 hours at 37 °C. Primary antibodies from Santa Crus Biotechnology are conjugated with fluorophores (Alexa Flour 488, 546, or 594); therefore, no secondary antibody was needed for these primary antibodies. The samples will then be washed three more times with 1X PBS and mounted on slides.

Confocal imaging was performed on a Nikon W1 Dual CAM Spinning Disk confocal microscopy at the Center for Advanced Microscopy/Nikon Imaging Center of Northwestern University. Parameters for excitation and emission settings were fixed for all the panels in the same images presented in the paper. Confocal images were processed using FIJI (NIH Image) and Imaris (Oxford Instruments, Abingdon, Oxfordshire, England) afterward.

4.5. Data analysis

ABR recordings were uniformly formatted and processed using MatLab (The Mathworks, Inc., Natik, MA), and averages and standard errors of the mean (SEM) of the thresholds were calculated and presented. ABR thresholds during and after treatment were all normalized by the baseline ABR threshold measured before the treatment (day 0). For the convenience of statistical analysis, the frequencies of which the response was lost entirely were assigned a value, which was 10 dB above the maximal output of the speaker. Synapse counting was performed in Imaris using the function of surface plot. Only the dots surrounding the bottom area of the IHCs were included with a size range of 0.25±0.3 μm, and the colocalization was recognized by the software automatically. The number of synapses was normalized by the count of IHCs in the area. One-way ANOVA (two tails) was performed in Matlab for all the ABR threshold and synapse counting data. If the ANOVA indicated differences among the means, the Tukey's Honestly-Significant Difference post-hoc test was followed. The finalized data were plotted using IGOR Pro (Wavemetrics, Lake Oswego, OR). The tests were part of a statistical package provided by IGOR Pro. Statistical decisions were made for a probability of 0.05.

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