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RESEARCH ARTICLE

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Explore the effect of LLY-283 on the ototoxicity of auditory cells caused by cisplatin: A bioinformatic analysis based on RNA-seq

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Abstract

Background: Cisplatin is a commonly used chemotherapeutic drug in clinics, and long-term application will lead to hearing impairment. LLY-283, an inhibitor of PRMT5, has not been reported in deafness. Our study aimed to explore the mechanism of LLY-283 in hearing impairment.

Materials and Methods: First, we performed RNA-seq (cisplatin in the experimental group and DMSO in the control group) to obtain the biological processes mainly involved in differentially expressed genes (DEGs). CCK-8 and LDH experiments were used to observe the effect of LLY-283 on cisplatin-induced auditory cell injury. ROS experiment was used to monitor the impact of LLY-283 on oxidative damage of auditory cells. Effect of LLY-283 on apoptosis of auditory cells detected by TUNEL experiment. PCR and Western blotting were used to detect the expression of genes and proteins related to auditory cell apoptosis in LLY-283 cells. Meanwhile, we explored the effect of LLY-283 on the expression of PRMT5 in cisplatin-induced hearing impaired cells at RNA and protein levels.

Results: Biological process analysis showed that DEGs were mainly enriched in the apoptotic process involved in morphogenesis ($-\text{Log}_{10} P = 3.71$). CCK-8 and LDH experiments confirmed that LLY-283 could save cisplatin-induced auditory cell injury. ROS experiments confirmed that LLY-283 could rescue cisplatin-induced oxidative damage to auditory cells. TUNEL experiments confirmed that LLY-283 could protect cisplatin-induced apoptosis of auditory cells. Meanwhile, LLY-283 could inhibit the expression of PRMT5 in auditory cells induced by cisplatin.

Conclusion: LLY-283 can rescue cisplatin-induced auditory cell apoptosis injury. LLY-283 can inhibit the increase in PRMT5 expression induced by cisplatin.

KEYWORDS
cisplatin, hearing impairment, LLY-283, PRMT5

1 | INTRODUCTION

At present, a variety of drugs are used to treat clinical diseases, and some medications will affect the hearing of patients, resulting in hearing impairment.[1] Cisplatin is a commonly used chemotherapeutic drug in clinical treatment. Cisplatin has a pronounced therapeutic effect on the condition of tumor patients. At the same time, cisplatin also causes some side effects to patients. Among them, hearing loss caused by cisplatin is getting more and more attention.[2] At present, there is no treatment approved by the Food and Drug Administration (FDA) to prevent cisplatin-induced hearing loss.[3] Therefore, the study of cisplatin-induced deafness still has clinical significance.

Protein arginine methyltransferase (PRMT) is an enzyme family. PRMTs regulate a variety of intracellular functions critical for survival. Abnormal expression of PRMTs family can lead to many diseases.[4] Protein arginine methyltransferase 5 (PRMT5) is a kind of protein arginine methylase, as a key enzyme involved in gene transcription regulation and signal transduction DNA repair damage and cell proliferation and other biological processes.[5] At the same time, PRMT5 clearly catalyzes the symmetric methylation of multiple histone and non-histone substrates in vivo and plays an important role in regulating multiple cell processes.[6,7] In the nucleus, PRMT5 can interact with SWI/SNF, catalyze the symmetrical dimethylation of the substrates of histone H2A arginine 3 (H2AR3me2s), histone H3 arginine 8 (H3R8me2s) and histone H4 arginine 3 (H4R3me2s), and inhibit the transcription of downstream cycle regulators and tumor suppressor genes.[8,9] At present, studies on LLY-283 as a PRMT5 inhibitor in tumors have been reported, but studies on LLY-283 as a PRMT5 inhibitor in deafness have not been registered. In our research, we explored the mechanism of LLY-283 as a PRMT5 inhibitor in deafness. Robert M Campbell's team reported in 2018 that LLY-283 could act as an inhibitor of PRMT5 in tumors.[10] Peter B Dirks' team said in 2021 that LLY-283 could act as an inhibitor of PRMT5 in gliomas.[11] At present, there is no research on LLY-283 as a small molecule inhibitor in drug-induced deafness. There was no study on PRMT5 in drug-induced deafness.

Apoptosis usually occurs during development and senescence and acts as a way of cell death to maintain the homeostasis of tissue cell populations. Similarly, apoptosis in the human body is a kind of defense mechanism.[12] The common pathogenic factors of sensorineural hearing loss (SNHL) include noise interference, ototoxic drugs, ageing, and so on. These biological processes can induce programmed death of auditory hair cells through different pathways, leading to hair cell loss.[13] Bcl-2 family members are essential for regulating apoptosis, including Bcl-2, Bax, and Bak.[14]

RNA-seq technology has been widely used in scientific research in recent years.[15] By using RNA-seq technology, the whole genome expression of the research samples can be obtained quickly, and biological information such as differential genes and enriched signaling pathways can be further obtained. These functions provide research direction for researchers. In our study, RNA-seq technology was also selected for experimental verification according to the sequencing results.

Here, we first determine based on RNA-seq that cisplatin affects auditory cell injury mainly by affecting apoptosis-related biological processes. Experiments confirmed that cisplatin played a role in down-regulating cell viability in auditory cells, and LLY-283 could rescue the apoptosis of auditory cells caused by cisplatin. ROS, TUNEL assay confirmed that LLY-283 plays a protective role in auditory cell injury. Finally, we verified our hypothesis by molecular biology experiments that LLY-283, as an inhibitor of PRMT5, plays a protective role in cisplatin-induced apoptosis of auditory cells.

2 | MATERIALS AND METHODS

2.1 | HEI-OC1 and LLY-283

HEI-OC1 cell line (Gifted by Fudan University), derived from the cochlea of immortal mice, is a widely used auditory HC cell line.[16] As an anti-tumor compound (small molecule inhibitor), LLY-283 (Selleck Chemicals, Directory Number: S8883) has been gradually applied in anti-tumor research by scientists in recent years.[17] The cells were cultured in high-glucose DMEM (37 ℃, 5% CO2) of 2.5% fetal bovine serum without antibiotics.

2.2 | RNA-seq and Bioinformatics

RNA-seq samples were prepared. We prepare 6 plates of cells, including 3 plates for the control group and 3 plates for the experimental group. The cells in the control group were treated with DMSO for 24 h (DMSO), and the cells in the experimental group were treated with cisplatin dissolved (concentration of cisplatin is 30 μmol) in DMSO for 24 h (CIS). The samples were prepared according to the requirements of Wuhan Aiji Baike Biotechnology Co., Ltd., and the treated cells were collected in Trizol and stored in a cell cryopreservation tube. The original image data files obtained through high-throughput sequencing (such as Illumina HiSeqTM2000/Miseq/BQIDEQ-500 sequencing platform) are transformed into original sequences (Sequenced Reads) through Base Calling, which we call Raw Data or Raw Reads. Files stored in FASTQ format contain sequences and corresponding sequencing quality information. The software used for data processing is STAR (2.7.3a), Cufflinks (v2.2.1), and R (4.0.3).[18] We filter differential genes according to the threshold $p < 0.05$ and $|\log FC| \geq 1$.

2.3 | GO and KEGG analysis

Metascape (https://metascape.org) is a free database designed to provide comprehensive gene list annotation and analysis resources for experimental biologists.[19] We upload the list of genes we studied into the Metascape database to see where our genes are enriched in biological processes and signaling pathways for subsequent experimental studies.

2.4 | Cell viability assays

Cell Count Kit-8 (CCK-8) is used to measure cell viability according to the manufacturer's instructions. In short, HEI-OC1 cells were inoculated in 96-well plates at the density of 5 × 103 per well and cultured overnight. The cells were treated with different doses of cisplatin for 24 h and pretreated with or without LLY-283 for 2 h, and then, 100 μl CCK-8 solution produced by Dojindo Molecular Technology Co., Ltd. was added to each well and exposed to 5% CO_2 at 37℃ for 1 h. We use a flat panel reader (Bio-Rad) to measure the optical density (OD) at 450 nm. The positive control group used the same method without inoculating cells, while the negative control group did not use drugs. The relative survival rate was calculated as: (ODexperiment-ODpositive)/(ODnegative-ODpositive) × 100. According to the manufacturer's instructions, the cytotoxic LDH detection kit-WST (Dojindo Molecular Technology) was used to determine the cytolytic activity of HEI-OC1 cells.

2.5 | Detection of reactive oxygen species

The level of intracellular ROS was detected by fluorescent dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Thermo Fisher Scientific). In the presence of an oxidant, DCFH was converted into highly fluorescent 2′, 7′-dichlorofluorescein (DCF). HEI-OC1 cells (1 × 105 cells) were inoculated on slides with six-hole plates, and cisplatin injury for 24 h and LLY-283 pretreatment for 2 h were given according to the concentration. Then, HEI-OC1 cells were washed with preheated DMEM and incubated with DCFH-DA (50 μM) in the dark at 37°C. ROS was measured by fluorescence signal intensity.

2.6 | TUNEL assay

Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). According to the manufacturer's plan, TUNEL (Roche) is used for testing. HEI-OC1 cells were cultured in 12-well plates with glass slides at the density of 3×10^5 cell/ holes. The cells were treated with 30 μM cisplatin or 100 μ MLLY-283 alone for 24 h, or pretreated with 100 μ MLLY-283 for 2 h, followed by 30 μM cisplatin for 24 h. Images were taken using a fluorescence microscope (Olympus IX71).

2.7 | RT-PCR

All the experimental groups were treated with TRIzol reagent (Invitrogen) according to the production process requirements to extract total RNA. A large capacity cDNA reverse transcription kit (TIANGEN) was used to synthesize single-strand cDNA. To detect the mRNA expression of protein arginine methyl-transferase5 (PRMT5), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 40 cycles of quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) were performed with SYBR Green Master Mix (TIANGEN): pretreatment at 95℃ for 10 min and treatment at 58°C at 95℃ for 40 s and at 72℃ for 50 s. The primer sequences used for PCR amplification are as follows: PRMT5(forward, 5′-ATG GCG GCG ATG GCA GTC G-3′ and reverse,5′-CTA GAG GCC AAT GGT ATA TAG GAG CGG CC-3′); Bcl-2(forward, 5′-ACG TGG ACC TCA TGG AGT G-3′ and reverse, 5′-TGT GTA TAG CAA TCC CAG GCA-3′); Bax(forward, 5′-CCC GAG AGG TCT TTT TCC GAG-3′ and reverse,5′-CCA GCC CAT GAT GGT TCT GAT-3′); and GAPDH(forward, 5′-GGA GCG AGA TCC CTC CAA AAT-3′ and reverse,5′-GGC TGT TGT CAT ACT TCT CAT GG-3′).

2.8 | Total protein extraction and western blot

We take HEI-OC1 cells and wash them with clean PBS for three times, and then add proper amount of precooled RIPA cleavage buffer (Beyotime Institute of Biotechnology, P0013B) and protease inhibitor cocktail (Sigma-Aldrich, P8340) to each tube for 30 min at 4℃. The lysed samples were subjected to ultrasound (Sonics & Materials Inc.) for 12 times, and each time 5 s was centrifuged at 13400 *g* for 12 min to obtain soluble proteins. The protein concentration was detected by BCA protein analysis kit (Beyotime Institute Biotechnology, P0010S). After boiling at loading buffer 100℃ for 10 min, the same amount of protein samples was separated by 10% SDS-PAGE electrophoresis and transferred to 0.45 μm polyvinylidene difluoride membrane (Immobilon-P, MilliPore, IPVH00010). 5% skim milk powder was sealed at room temperature for 1 h in Tris-Buffer saline containing 0.1% Tween-20 (TBST). Next, TBST was washed three times and incubated overnight with an antibody in TBST containing 5% skimmed milk powder at 4℃. The main antibodies were anti-PRMT5 (1:1000 dilution; CST, 79998), anti-Bax (1:1000 dilution; CST, 5023), anti-Bcl-2 (1:1000 dilution; CST, 15071), anti-Cleaved Caspase-3 (1:1000 dilution; CST, 9661), anti-Cleaved PARP (1:1000 dilution; CST, 5625), and anti-GAPDH (1:1000 dilution; CST, 5174). After washing with TBST for three times, adding peroxidase-labeled secondary antibody (Invitrogen; 1:5000 diluted) shaker at room temperature and incubating at a uniform speed for 1 h. The enhanced ECL system (advansta) was used to develop, and ImageJ was used to correct the GAPDH, and the band intensity was analyzed.

2.9 | Statistical analysis

The results were shown as mean ± SEM and established through one-way analysis of variance (ANOVA) or two-tailed, unpaired Student's *t* test.[20] Statistical analyses were conducted using GraphPad Prism 8 software, with *P*-Value <0.05 considered statistically significant.

TABLE 1 Ninety differentially expressed genes (DEGs) were identified from CIS vs DMSO, including 84 up-regulated genes and 6 down-regulated genes

Upgenes	Hoxc13	Mybl1	Depdc7	Plch2	Prr7	Zfp503	Shisal2b	Ctxn1	Gm960	Dnajc19-ps
	Efnb3	Ercc5	Dusp2	Areg	Fam110c	Fam102a	Hecw2	Ptges	Nanos1	Gm14269
	Ddit4	Slc16a14	Bcl2l11	Dtx1	Asap3	Mreg	2900052L18Rik	Junb	Myh7b	Tmem95
	Mdm2	Selp	Sdcbp2	Ccl17	Il22ra1	C3ar1	Fzd5	Krt16	Slc4a11	Zfp967
	Btg2	Atf3	Lce1g	Duox1	Ccn3	Fbxo2	Hcar2	Gm10263	C1s2	Ptprv
	4921536K21Rik	1700007K13Rik	Prkaa2	Evpl	Tob1	Enc1	Adrb2	Rps12-ps9	Gm12439	Gm37008
	Rgcc	Notch1	Sgip1	Ifit1	Csf3	Phlda3	Neurl3	Ccdc153	Ctsf	1700011C11Rik
	Psca	Prrg4	Nppb	Ly6d	Plekhg6	Ikbke	Fam180a	Tigit	Gm13071	Gm21089
	Gm7780	Gm47467	4933433H22Rik	Gm50251						
Downgenes	Brinp3	Dennd1a	Gtdc1	Nebl	Cacnb2	Galnt13				

3 | RESULTS

3.1 | RNA-seq

In this study, we performed RNA-seq. We analyze the FastQ data using STAR (2.7.3a), Cufflinks (v2.2.1), and R (4.0.3). A total of 90 differentially expressed genes (DEGs) were identified, using the *P* value <0.05 and | log2 fold change |≥1 standard, 84 genes were up-regulated and 6 genes were down-regulated (Table 1). Volcano map and heatmap of these DEGs are shown in Figure 1A,B.

3.2 | GO and KEGG analysis

Metascape (https://metascape.org) is used to explore the functional enrichment of genes. $p < 0.05$ was set to the critical value. Functional annotations from Metascape showed that differentially expressed genes were mainly enriched in apoptotic process involved in morphogenesis ($Log_{10}P = -3.71$), negative regulation of cell population proliferation ($Log_{10}P = -3.58$), positive regulation of catabolic process ($Log_{10}P = -3.56$), negative regulation of intracellular signal transduction ($Log_{10}P = -3.34$), negative regulation of cell-cell adhesion ($Log_{10}P = -3.16$), Staphylococcus aureus infection ($Log_{10}P = -3.13$), negative regulation of osteoblast differentiation ($Log_{10}P = -2.79$), muscle cell proliferation ($Log_{10}P = -2.75$), multicellular organismal homeostasis ($Log_{10}P = -2.56$), formation of the cornified envelope ($Log_{10}P = -2.56$), IL-17 signaling pathway ($Log_{10}P = -2.43$), Cargo recognition for clathrin-mediated endocytosis ($Log_{10}P = -2.36$), killing of cells of other organisms ($Log_{10}P = -2.36$), protein ubiquitination ($Log_{10}P = -2.15$), and negative regulation of mitotic cell cycle ($Log_{10}P = -2.09$; Figure 2; Table 2).

3.3 | Change in the viability of HEI-OC1 cells by LLY-283

We first exposed HEI-OC1 cells to different concentrations of cisplatin (0, 1, 10, 20, 30, 40, 50, and 60 μM) for 24 h to investigate the cytotoxicity of cisplatin to HEI-OC1. As shown in Figure 3A, compared with the blank group without any treatment, exposure to cisplatin from 30 to 60 μM decreased the cell survival rate from 60.92% ± 4.282% to 35.93% ± 4.163%. According to the cell survival data, we finally chose 30 μM cisplatin for 24 h as the best condition for HEI-OC1 cell injury to study the ototoxicity of cisplatin, because the cell survival rate was significantly lower than that of the untreated control group. At the same time, in order to determine the appropriate dose of LLY-283 and its protective effect on cisplatin ototoxicity, we pretreated the cells with different concentrations of LLY-283 (0, 40, 60, 80, 100, 120, 140 μM) for 2 h, and then co-cultured with 30 μM cisplatin at 37°C for 24 h. By analyzing the experimental data of CCK-8, we confirm that the cell survival rate is indeed much higher in the presence of LLY-283 than in the absence of LLY-283, that is, cisplatin damage alone (Figure 3B). Therefore, we chose 100 μM LLY-283 related to the maximum cell

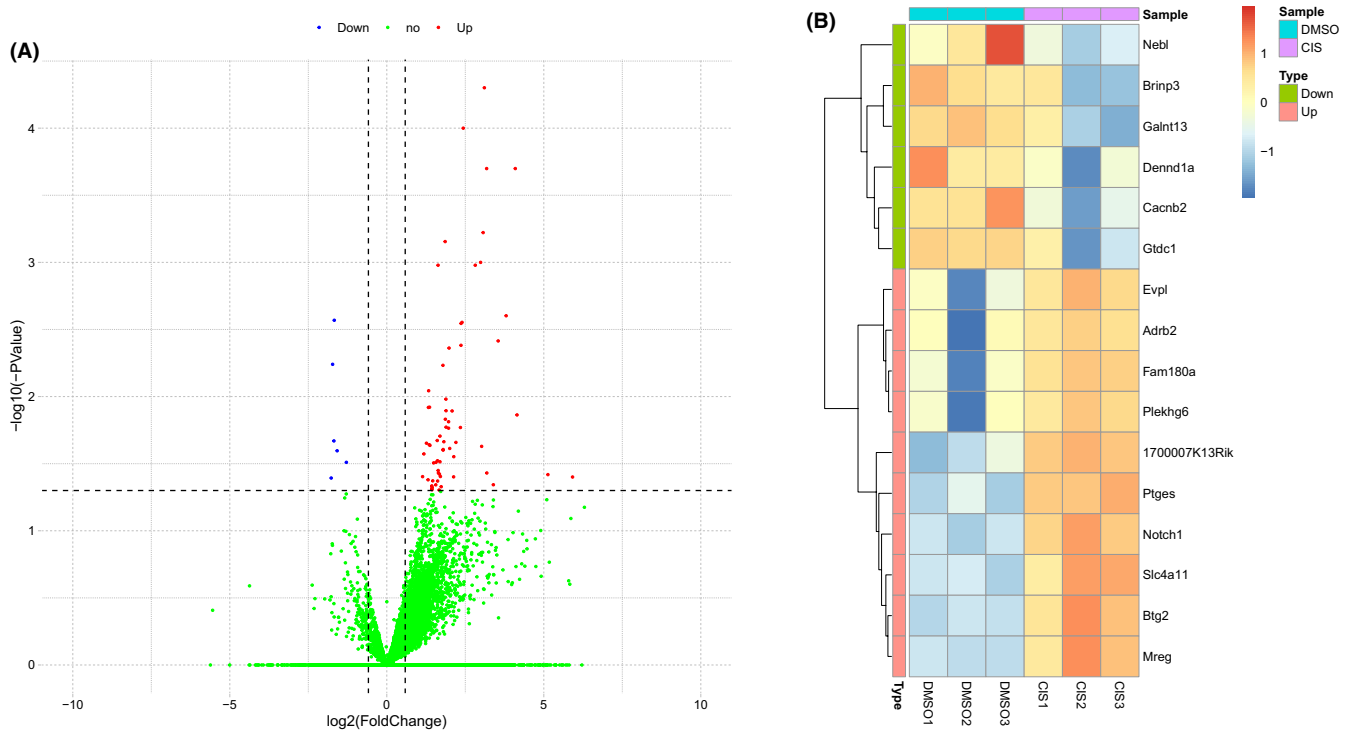

FIGURE 1 Genome changes in cisplatin-induced auditory cell injury. (A) Volcano diagram of differentially expressed genes. (B) Heatmap of differentially expressed genes

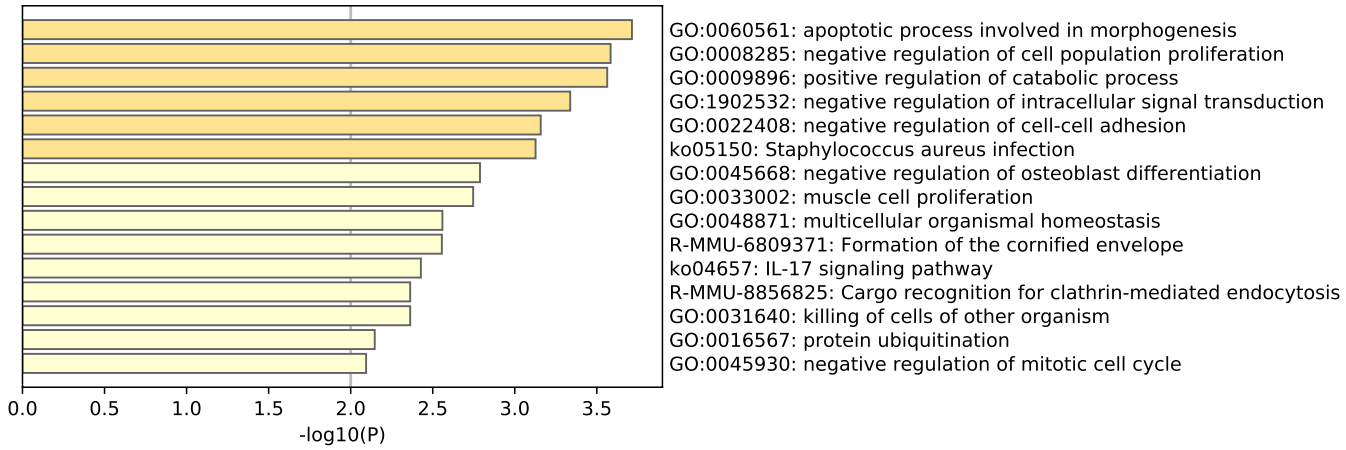

FIGURE 2 Gene ontology analysis and significant enrichment of differentially expressed genes (DEGs) in cisplatin-induced auditory cell injury. Ranking significant enriched GO terms of DEGs by Log10(P)

survival rate as the ideal concentration for follow-up experiments. Cytotoxicity LDH Assay Kit-WST is a kit for measuring cell damage by measuring the activity of lactate dehydrogenase (LDH) released by cells into the culture medium. LDH is an enzyme present in the cytoplasm. When the cell membrane is damaged, LDH is released into the culture medium. As the released LDH is stable, the detection of the amount of LDH in the culture medium can be used as an index to determine the number of dead cells and damaged cells. The results showed that the release of LDH increased with the increase of cisplatin concentration. When the concentration of cisplatin reached 30 μM, the release rate of lactate dehydrogenase reached 59.11% ± 0.5275% (Figure 3C). We also investigated the effect of LLY-283 on the release rate of lactate dehydrogenase. The results showed that with the increase of LLY-283 concentration, the release rate of lactate dehydrogenase was significantly lower than that after cisplatin injury, suggesting that LLY-283 can reduce the damage of HEI-OC1 cells induced by cisplatin (Figure 3D).

3.4 | LLY-283 can inhibit PRMT5 expression in HEI-OC1 cells

Western blot results confirmed that the protein expression of PRMT5 in the cisplatin group was significantly increased, while

TABLE 2 GO and KEGG analysis based on Metascape

GO	Category	Description	Count	%	Log10(P)	Log10(q)
GO:0060561	GO biological processes	Apoptotic process involved in morphogenesis	3	4.29	−3.71	0
GO:0008285	GO biological processes	Negative regulation of cell population proliferation	10	14.29	−3.58	0
GO:0009896	GO biological processes	Positive regulation of catabolic process	8	11.43	−3.56	0
GO:1902532	GO biological processes	Negative regulation of intracellular signal transduction	8	11.43	−3.34	0
GO:0022408	GO biological processes	Negative regulation of cell-cell adhesion	5	7.14	−3.16	0
ko05150	KEGG pathway	Staphylococcus aureus infection	3	4.29	−3.13	0
GO:0045668	GO biological processes	Negative regulation of osteoblast differentiation	3	4.29	−2.79	0
GO:0033002	GO biological processes	Muscle cell proliferation	5	7.14	−2.75	0
GO:0048871	GO biological processes	Multicellular organismal homeostasis	7	10	−2.56	0
R-MMU-6809371	Reactome gene sets	Formation of the cornified envelope	3	4.29	−2.56	0
ko04657	KEGG pathway	IL-17 signaling pathway	3	4.29	−2.43	0
R-MMU-8856825	Reactome gene sets	Cargo recognition for clathrin-mediated endocytosis	3	4.29	−2.36	0
GO:0031640	GO biological processes	Killing of cells of other organisms	3	4.29	−2.36	0
GO:0016567	GO biological processes	Protein ubiquitination	7	10	−2.15	0
GO:0045930	GO biological processes	Negative regulation of mitotic cell cycle	4	5.71	−2.09	0

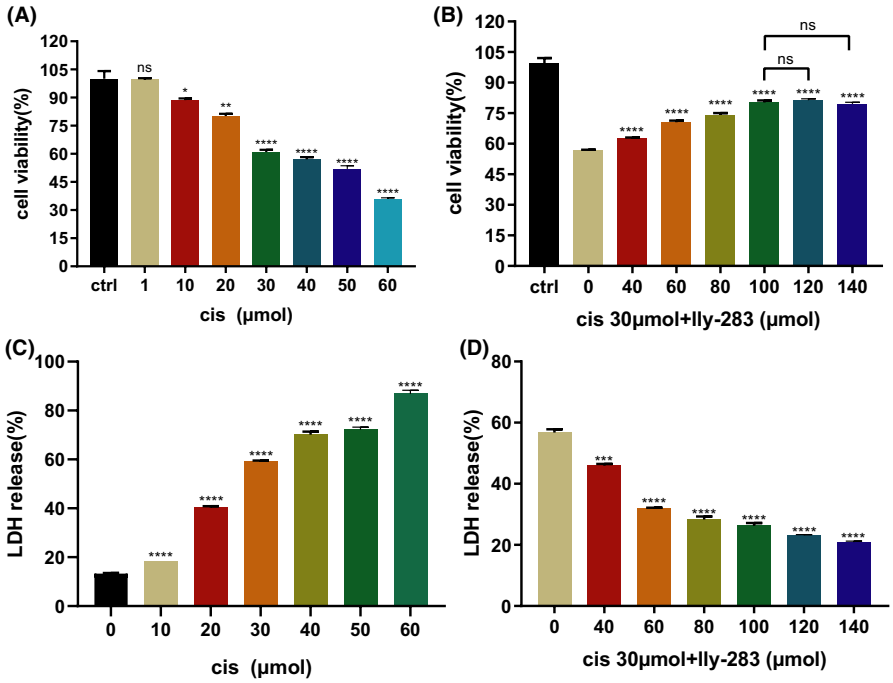

FIGURE 3 LLY-283 treatment protects auditory cells (HEI-OC1) against cisplatin-induced cell death. (A) Effects of different concentrations of cisplatin (0, 1, 10, 20, 30, 40, 50, 60 μm) on auditory cell viability. (B) Effects of different concentrations of LLY-283 (0, 40, 60, 80, 100, 120, 140 μm) on cisplatin-induced auditory cells (30 μmol) viability for 24 h. (C) Effects of different concentrations of cisplatin (0, 1, 10, 20, 30, 40, 50, 60 μm) on auditory cells were detected by lactate dehydrogenase (LDH). (D) Effects of different concentrations of LLY-283 (0, 40, 60, 80, 100, 120, 140 μm) on cisplatin-induced auditory cells (30 μmol) were detected by lactate dehydrogenase (LDH). Values were represented as the mean ± SEM from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns, not significant vs the non-treated control group

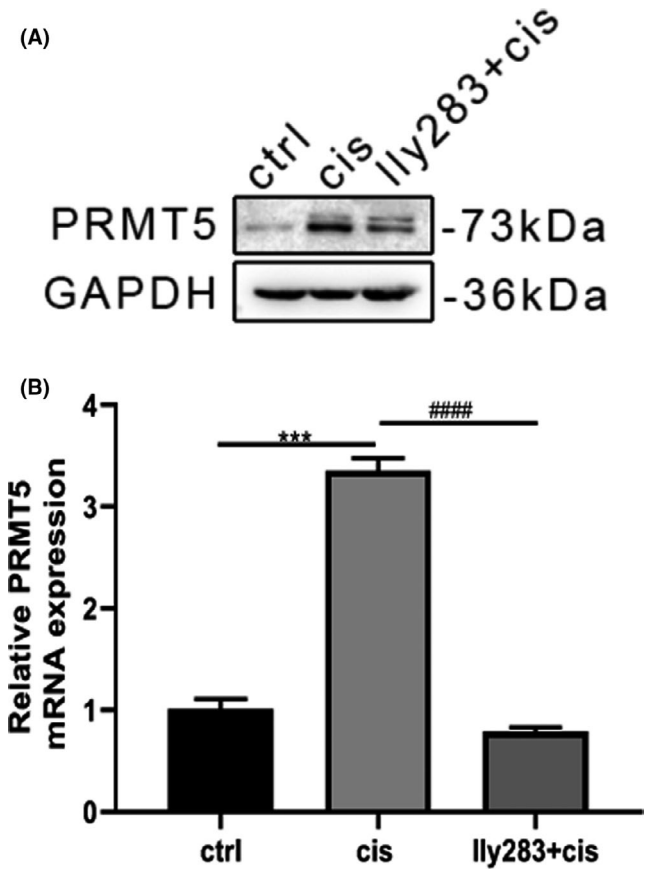

FIGURE 4 In auditory cell (HEI-OC1) cells, the expression of PRMT5 was up-regulated after cisplatin injury, but LLY-283 could inhibit the expression of PRMT5. (A) Detection of PRMT5 protein expression by Western blot, GAPDH was used as a loading control. (B) Detection of mRNA level of PRMT5 by qRT-PCR, GAPDH was used as a loading control. Data are mean ± SEM. Statistical analysis was performed using Student's t test. $^{**}p < 0.01$ vs control. $^{\#\#}p < 0.01$ vs cisplatin group

the protein expression of PRMT5 in the LLY-283 pretreatment group was significantly decreased compared with the cisplatin alone group (Figure 4A). qPCR results also showed that the expression level of PRMT5 mRNA in cisplatin-damaged group was much higher than that in the blank group and the difference was statistically significant ($p < 0.01$), while the expression level of PRMT5 mRNA in LLY-283 + cisplatin combined group was significantly lower than that in the cisplatin-damaged group ($p < 0.01$; Figure 4B).

3.5 | LLY-283 treatment protects cochlear hair cells against cisplatin-induced oxidative stress

It is reported that cisplatin exposure can increase the production of ROS in HEI-OC1 cells, so we selected DCFH-DA to detect the production of ROS in HEI-OC1 cells treated with cisplatin or cisplatin combined with LLY-283. DCFH-DA quantitative analysis showed that DCFH-DA fluorescence signal was the highest in

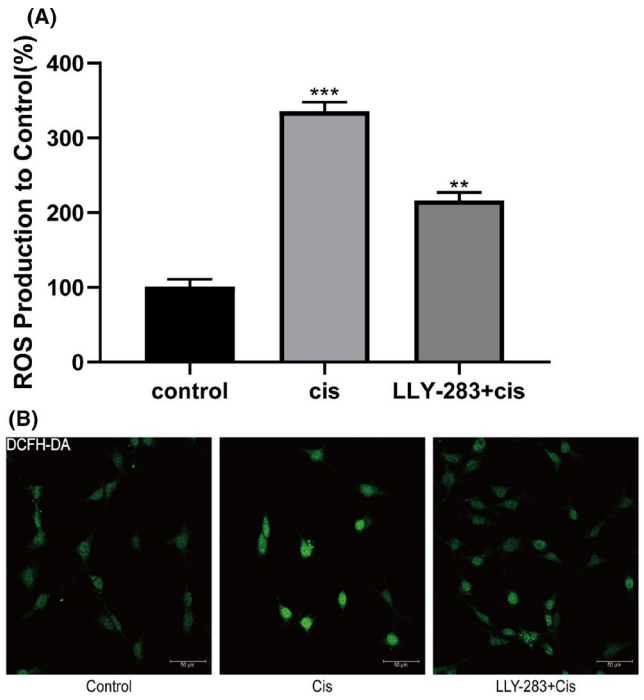

FIGURE 5 LLY-283 treatment protects cochlear hair cells against cisplatin-induced oxidative stress. (A) Effect of LLY-283 on ROS production in cisplatin-induced auditory cell injury. (B) Representative DCFH-DA (green) staining images. Data represent mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. cisplatin-induced group

the cisplatin-treated group ($p < 0.001$), while ROS production was lower in LLY-283-pretreated cells (Figure 5A,B), indicating that LLY-283 inhibited cisplatin-induced ROS production in HEI-OC1 cells.

3.6 | LLY-283 treatment decrease the apoptotic cells

In order to determine the effect of LLY-283 on cisplatin-induced apoptosis in HEI-OC1 cells, we used TUNEL method to detect the abundance of apoptotic cells. No obvious TUNEL positive cells were found in the control group and LLY-283 treatment group. There were obvious TUNEL positive cells in the cisplatin-treated group. However, the proportion of TUNEL positive cells decreased significantly in LLY-283 pretreatment group (Figure 6), suggesting that LLY-283 may protect HEI-OC1 cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced apoptosis.

3.7 | LLY-283 treatment protects HEI-OC1 hair cells against cisplatin-induced cell apoptosis through the intrinsic pathway

By detecting the expression of endogenous apoptotic factors Bax and Bcl-2 in different treatment groups, we found that

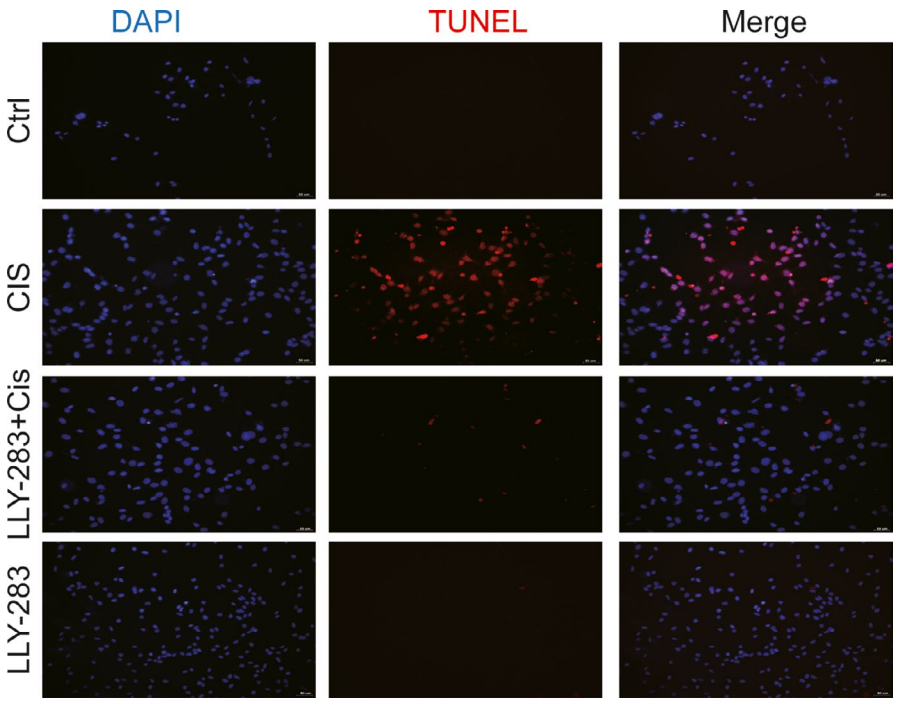

FIGURE 6 LLY-283 reduced cisplatin-induced apoptosis in auditory cells. TUNEL staining showed that apoptosis occurred in HEI-OC1 cells after different treatments. The number of TUNEL positive cells in cisplatin group was significantly higher than that in control group, while the number of TUNEL positive cells in 100 μM LLY-283 cotreatment group was significantly lower than that in cisplatin group. Scale bars = 50 μm

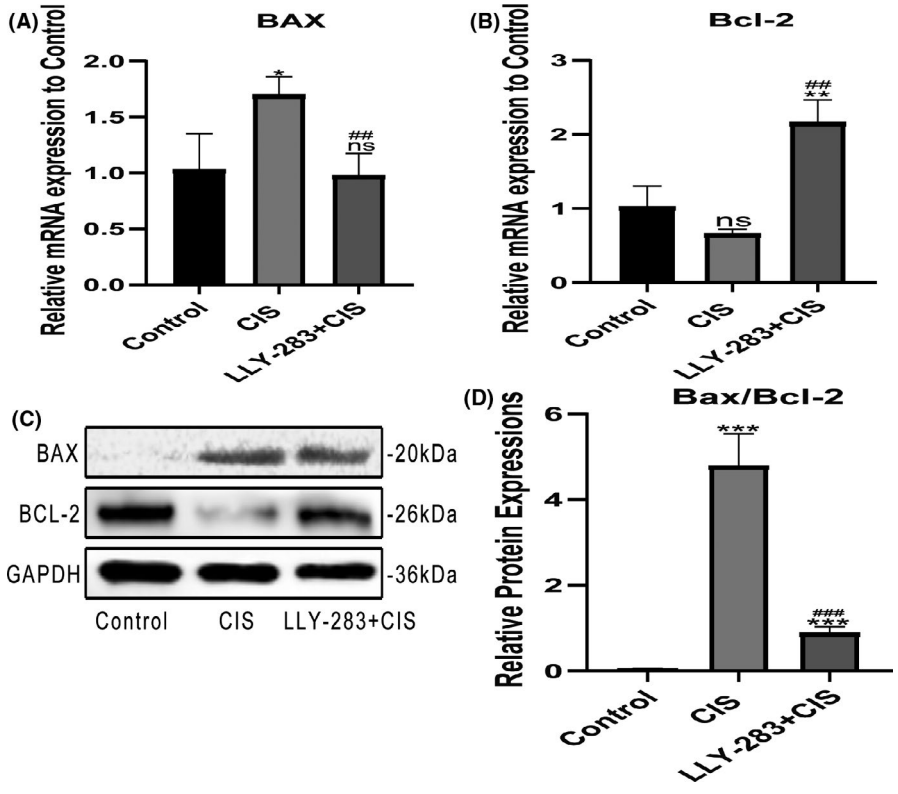

FIGURE 7 LLY-283 can protect HEI-OC1 cells from cisplatin injury by regulating the expression of endogenous apoptosis pathway molecules. (A) The expression of Bax mRNA was detected by real-time quantitative polymerase chain reaction. (B) The expression of Bcl-2 mRNA was detected by real-time quantitative polymerase chain reaction. (C) The protein expression of Bax and Bcl-2 was detected by Western blotting. (D) And ImageJ was used to analyze the relative expression of bax/bcl-2. GAPDH is used as load control. The data represent the average ± SEM from three independent experiments. $^{*}p < 0.05$, $p < 0.01$, and $^{*}p < 0.001$ compared with the control group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. 30 μM cisplatin group

cisplatin alone promoted the expression of pro-apoptotic factor Bax (Figure 7A,C) at protein level and mRNA level, while inhibited the mRNA expression of anti-apoptotic factor Bcl-2 (Figure 7C). It is worth noting that the protein and mRNA levels of Bax and Bcl-2 in LLY-283 and cisplatin co-treated group were significantly higher than those in cisplatin group ($p < 0.01$; Figure 7A–C). Western blotting showed that the ratio of Bax/Bcl-2 in cisplatin group was significantly higher than that in cisplatin group ($p < 0.001$). It is suggested that cisplatin may induce apoptosis of HEI-OC1 cells by activating endogenous apoptotic pathway, but on the contrary. The ratio of Bax/Bcl-2 in the cotreatment group was significantly lower than that in the cisplatin group

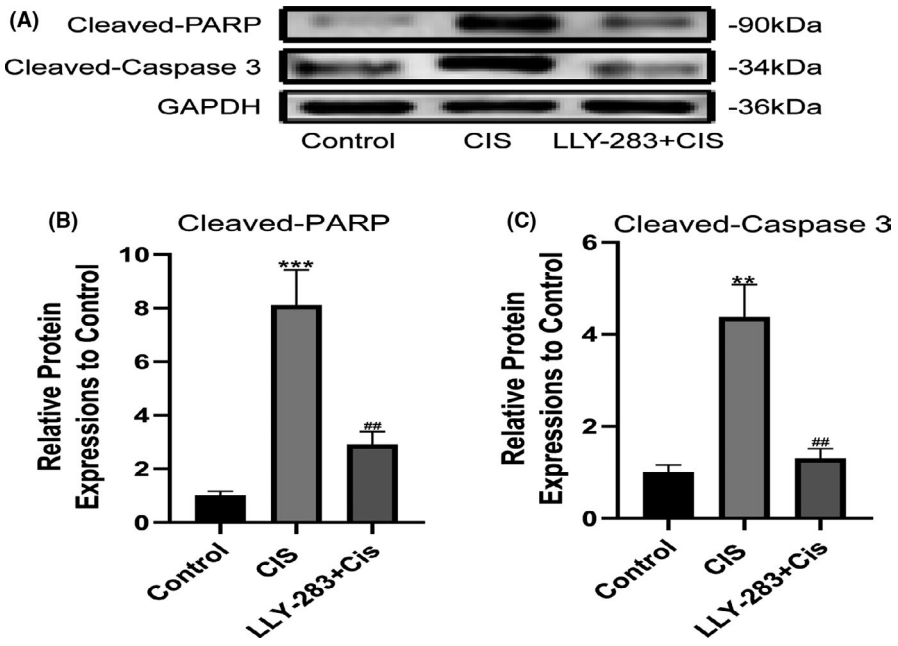

FIGURE 8 LLY-283 treatment attenuates cisplatin-induced ototoxicity via inhibiting poly (ADP-ribose) polymerase. (A) HEI-OC1 cells were incubated with 30 μM cisplatin or with or without 100 μM LLY-283 for 24 h, and Western blotting analysis was performed with anti-Cleaved Caspase-3 and anti-Cleaved PARP antibodies. (B) Quantification of the Western blot of Cleaved PARP in(A)by ImageJ. (C) Quantification of the Western blot of Cleaved-Caspase 3 in(A)by ImageJ. Data are shown as the mean ± SEM of three replicates, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control. $^{\#\#}p < 0.01$ vs. 30 μM cisplatin group

($p < 0.001$), indicating that LLY-283 has a significant protective effect on cisplatin-induced HEI-OC1 cell injury (Figure 7D).

3.8 | LLY-283 treatment attenuates cisplatin-induced ototoxicity via inhibiting poly(ADP-ribose) polymerase

The cleavage of PARP promotes the disintegration of cells and is a sign of apoptosis. Western blotting further showed that the protein expression of activated PARP was significantly increased in HEI-OC1 cells treated with cisplatin (Figure 8A,B), and cleavage caspase-3 (activated form of caspase-3) was also significantly increased (Figure 8A,C). After 100 μM LLY-283 treatment, the protein expression levels of activated PARP and activated caspase-3 were significantly lower than those in cisplatin injury group (Figure 8A–C). Taken together, these results suggest that LLY-283 can significantly reduce cisplatin-induced apoptosis in HEI-OC1 cells by inhibiting poly (ADP-ribose) polymerase.

4 | DISCUSSION

Although cisplatin is one of the most commonly used antineoplastic drugs in clinic, it can lead to progressive and irreversible hearing loss.[21] At present, the recognized mechanism of cisplatin ototoxicity is that cisplatin can produce ROS, which can not only reduce the antioxidant defense molecules glutathione and antioxidant enzymes in cochlear tissue, but also cause lipid peroxidation to increase toxic lipid peroxides, promote cell Ca2+ influx, and finally lead to apoptosis of hair cells, vascular stria and spiral ganglion neurons.[22,23] As a small molecule inhibitor, LLY-283 has not been reported in the study of sensorineural hearing loss. Our study found that LLY-283 played a protective role in cisplatin-induced sensorineural hearing loss.

In this study, we found that cisplatin-induced auditory cell injury may be related to apoptosis through RNA-seq. Therefore, our study first focused on biological processes such as apoptosis, in which LLY-283 interference was added. It was found that LLY-283 could save cisplatin-induced auditory cell apoptosis. By comparing the expression level of PRMT5 in HEI-OC1 of cisplatin-damaged and non-cisplatin-damaged mice, it was found that the expression of PRMT5 in HEI-OC1 was up-regulated after cisplatin injury, thus establishing a potential relationship between PRMT5 and cisplatin. However, how the expression of PRMT5 in HEI-OC1 is regulated by cisplatin damage is still unknown, and more research is needed to solve this problem in the future. In order to understand the function of PRMT5, we applied a pharmacological method of using LLY-283 to inhibit the activity of PRMT5. CCK-8 experiment found that HEI-OC1 treated with LLY-283 showed recovery of cell viability after cisplatin injury, and LDH test also showed that LLY-283 could significantly reduce the damage of HEI-OC1 induced by cisplatin. This observation suggests that PRMT5 may play a harmful role in the pathogenesis of cisplatin ototoxicity, on the contrary, its inhibition has a protective effect on HEI-OC1. Through the monitoring of HEI-OC1 reactive oxygen species and apoptosis, we found that LLY-283 treatment decreased the level of these indexes in the cochlea after cisplatin injury. We also found that cisplatin can induce death receptors and mitochondrial-mediated apoptosis. For example, it increases the pro-apoptotic expression of Bax and reduces the expression of Bcl-2 family members of Bax-binding proteins, thus changing the permeability of mitochondrial membrane, releasing cytochrome C, and activating more than caspase-9 and its downstream caspase--3. The cleavage of PARP is also one of the markers of apoptosis. By detecting the

expression of PARP, we also found that cisplatin can significantly increase the expression of PARP, but LLY-283 treatment can also significantly reduce it. However, excessive DNA damage can lead to overactivation of PARP-1 and cell death.[24,25] In view of the direct causal relationship among cochlear reactive oxygen species, apoptosis and occasional ototoxicity, we believe that these effects contribute to the protective effect of LLY-283 on cisplatin injury to a great extent, although other possible mechanisms cannot be ruled out because of the diversity and complexity of PRMT5 function. In addition, in view of the important role of oxidative damage in cisplatin ototoxicity-related hearing loss, it is conceivable that LLY-283 may also effectively delay the development of the disease, which is a problem that needs to be solved in future.

We understand that LLY-283, as an inhibitor of PRMT5, has a protective effect on cisplatin-induced hearing damage. The relationship between PRMT5 and cisplatin-induced hearing damage has not been studied. Therefore, in the follow-up research, we will focus on exploring PRMT5 the role in cisplatin-induced hearing damage.

To sum up, these evidence suggest that the protective effect of PRMT5 inhibitor LLY-283 on cisplatin ototoxicity may be achieved by inhibiting cochlear cell apoptosis induced by oxidative stress, which provides a basis for the application of LLY-283 in cisplatin ototoxicity intervention.

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CONFLICT OF INTEREST

Bin Zhao, Dongdong Zhang, Yixin Sun, Min Lei, Peiji Zeng, Yue Wang, Yongjun Hong, Yanchao Jiao, and Chengfu Cai declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Bin Zhao proposed suggestions for the design of sequencing data in this paper and conducted data analysis. Dongdong Zhang carried out the experiment in this paper. Yixin Sun integrated the bioinformatic data of this paper and the experimental data, and wrote the first draft of this paper. Min Lei, Peiji Zeng, Yue Wang, Yongjun Hong, and Yanchao Jiao participated in the design of the direction in this paper and recommendations. Chengfu Cai conducted a critical revision of the manuscript, and financial support for this article.

DATA AVAILABILITY STATEMENT

All data in this study can be obtained from the authors of the communication, and RNA-seq raw data will be uploaded to the GEO database for free publication.

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REFERENCES

1. Tanaka M, Hasegawa S, Nakao S, et al. Analysis of drug-induced hearing loss by using a spontaneous reporting system database. *PLoS One*. 2019;14:e0217951.
2. Moke D, Luo Q, Millstein J, et al. Prevalence and risk factors for cisplatin-induced hearing loss in children, adolescents, and young adults: a multi-institutional North American cohort study. *Lancet Child Adolesc Health*. 2021;5(4):274-283.
3. Fernandez K, Spielbauer KK, Rusheen A, et al. Lovastatin protects against cisplatin-induced hearing loss in mice. *Hear Res*. 2020;389:107905.
4. vanLieshout TL, Ljubicic V. The emergence of protein arginine methyltransferases in skeletal muscle and metabolic disease. *Am J Physiol Endocrinol Metab*. 2019;317:E1070-E1080.
5. Liang Z, Liu L, Wen C, et al. Clinicopathological and prognostic significance of PRMT5 in cancers: a system review and meta-analysis. *Cancer Control*. 2021;28:10732748211050583.
6. Tan DQ, Li Y, Yang C, et al. PRMT5 modulates splicing for genome integrity and preserves proteostasis of hematopoietic stem cells. *Cell Rep*. 2019;26(9):2316-2328.e6.
7. Hamard PJ, Santiago GE, Liu F, et al. PRMT5 regulates DNA repair by controlling the alternative splicing of histone-modifying enzymes. *Cell Rep*. 2018;24:2643-2657.
8. Chen H, Lorton B, Gupta V, Shechter D. A TGFbeta-PRMT5-MEP50 axis regulates cancer cell invasion through histone H3 and H4 arginine methylation coupled transcriptional activation and repression. *Oncogene*. 2017;36:373-386.
9. Mei M, Zhang R, Zhou ZW, et al. PRMT5-mediated H4R3sme2 confers cell differentiation in pediatric B-cell precursor acute lymphoblastic leukemia. *Clin Cancer Res*. 2019;25:2633-2643.
10. Bonday ZQ, Cortez GS, Grogan MJ, et al. LLY-283, a potent and selective inhibitor of arginine methyltransferase 5, PRMT5, with antitumor activity. *ACS Med Chem Lett*. 2018;9:612-617.
11. Sachamitr P, Ho JC, Ciamponi FE, et al. PRMT5 inhibition disrupts splicing and stemness in glioblastoma. *Nat Commun*. 2021;12:979.
12. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;35:495-516.
13. Wu J, Ye J, Kong W, Zhang S, Zheng Y. Programmed cell death pathways in hearing loss: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif*. 2020;53:e12915.
14. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*. 2002;2:647-656.
15. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. 2008;5:621-628.
16. Kalinec GM, Webster P, Lim DJ, Kalinec F. A cochlear cell line as an in vitro system for drug ototoxicity screening. *Audiol Neurootol*. 2003;8:177-189.
17. Nagatomo M, Zhang K, Fujino H, Inoue M. Et3 B/Et2 AlCl/O2-mediated radical coupling reaction between alpha-alkoxyacyl tellurides and 2-hydroxybenzaldehyde derivatives. *Chem Asian J*. 2020;15:3820-3824.
18. Chan BKC. Data analysis using R programming. *Adv Exp Med Biol*. 2018;1082:47-122.
19. Zhou Y, Zhou B, Pache L, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. 2019;10:1523.
20. Mishra P, Singh U, Pandey CM, Mishra P, Pandey G. Application of student's t-test, analysis of variance, and covariance. *Ann Card Anaesth*. 2019;22:407-411.
21. Ruggiero A, Trombatore G, Triarico S, et al. Platinum compounds in children with cancer: toxicity and clinical management. *Anticancer Drugs*. 2013;24:1007-1019.

22. Dehne N, Lautermann J, Petrat F, Rauen U, de Groot H. Cisplatin ototoxicity: involvement of iron and enhanced formation of superoxide anion radicals. *Toxicol Appl Pharmacol*. 2001;174:27-34.
23. Goncalves MS, Silveira AF, Teixeira AR, Hyppolito MA. Mechanisms of cisplatin ototoxicity: theoretical review. *J Laryngol Otol*. 2013;127:536-541.
24. Smith ME, Groves AK, Coffin AB. Editorial: sensory hair cell death and regeneration. *Front Cell Neurosci*. 2016;10:208.
25. Zhang Y, Yang Y, Xie Z, et al. Decreased Poly(ADP-Ribose) polymerase 1 expression attenuates glucose oxidase-induced damage in rat cochlear marginal strial cells. *Mol Neurobiol*. 2016;53:5971-5984.

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