Ototoxicity: therapeutic opportunities

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Two major classes of drugs currently in clinical use can cause permanent hearing loss. Aminoglycoside antibiotics have a major role in the treatment of life-threatening infections and platinum-based chemotherapeutic agents are highly effective in the treatment of malignant disease. Both damage the hair cells of the inner ear, resulting in functional deficits. The mechanisms underlying these troublesome side effects are thought to involve the production of reactive oxygen species in the cochlea, which can trigger cell-death pathways. One strategy to protect the inner ear from ototoxicity is the administration of antioxidant drugs to provide upstream protection and block the activation of cell-death sequences. Downstream prevention involves the interruption of the cell-death cascade that has already been activated, to prevent apoptosis. Challenges and opportunities exist for appropriate drug delivery to the inner ear and for avoiding interference with the therapeutic efficacy of both categories of ototoxic drugs.

Aminoglycosides

Aminoglycoside antibiotics were developed in 1944 to treat Gram-negative bacteria that were not responsive to conventional antibiotics, such as penicillin. These compounds can be characterized by amino sugars that have glycosidic linkages. Subsequently, a number of similar compounds have been developed and are still commonly used. However, their clinical use is limited by toxic side effects that include cochlear toxicity, vestibular toxicity and nephrotoxicity. The aminoglycoside antibiotics include streptomycin, kanamycin, tobramycin, neomycin, gentamicin, amikacin and netilmicin. All display ototoxicity but vary in their preferential damage to the cochlea or vestibule.

Reactive oxygen species

The generation of reactive oxygen species (ROS) is believed to be the initiating step of aminoglycoside ototoxicity in a cascade of events that ultimately results in cell death. The formation of ROS by aminoglycosides appears to involve iron. Aminoglycoside compounds can form complexes with iron [1] that then react with unsaturated fatty acids to form superoxide ($O_2^{\bullet}$) radicals and lipid peroxides [2]. Typically, $O_2^{\bullet}$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and detoxified into water and oxygen by catalase. However, in highly oxidative conditions, endogenous antioxidant pathways can become overwhelmed, and free oxygen radicals can become abundant. Aminoglycosides, such as gentamicin, can activate inducible nitric oxide synthase (iNOS) in inner ear tissues, triggering an increase in nitric oxide [3]. Under these conditions, $O_2^{\bullet}$ can react with available nitric oxide to form the destructive peroxynitrite radical or it can directly initiate cell death. Additionally, recent evidence suggests that genes for some antioxidant enzymes

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might be downregulated by aminoglycosides. Organ of Corti (OC) cultures exposed to gentamicin displayed a 2.16-fold downregulation of a catalase gene [4]. If H_2O_2 is not degraded by catalase, iron can catalyze the conversion of H_2O_2 to highly reactive hydroxyl radicals (•OH) by the Fenton reaction [5]. Ternary complexes of $Fe^{2+/3+}$-gentamicin-phosphatidylinositol 4,5-bisphosphate, which can be oxidatively damaged to release arachidonic acid, can form. Arachidonic acid can also form a ternary complex with iron and gentamicin, which, reacts with lipid peroxides and molecular oxygen, leading to the propagation of arachidonic acid peroxidation and further cellular damage [6]. Free radicals can rapidly react with cell constituents, including cell membranes and DNA. The resulting oxidative stress can trigger apoptotic cell death (Figure 1).

Aminoglycosides preferentially damage the outer hair cells (OHCs) of the OC and/or the type I vestibular hair cell. Intriguingly, supporting cells and inner hair cells (IHCs) of the OC are mostly unaffected. One possible reason for this discrepancy is the activation and translocation of the nuclear factor (NF) κB, which is believed to have a role in ROS-induced cell signaling. Systemic administration of kanamycin to rats resulted in increased lipid peroxidation in all cell types of the OC, as indicated by immunostaining for 4-hydroxynonenal (4-HNE). This was accompanied by increased NFκB levels in nuclei of IHCs and supporting cells. However, NFκB was absent from the nuclei of OHCs, indicating that this nuclear factor is translocated to the nuclei of cells resistant to kanamycin, but not in cells that are sensitive to kanamycin. However, co-administration of salicylate or 2,3-dihydroxybenzoate facilitated translocation of NFκB into the OHC nuclei and protected them from kanamycin-induced cell damage [7].

In addition to differences in NFκB gradients within OC cell types, OHCs have lower antioxidant capacity compared with other OC cell types [8]. The level of glutathione, an endogenous intracellular antioxidant, in OHCs is lower than of the level in other cell types in the OC, and there is a gradient of OHC glutathione levels from the base to the apex of the cochlea. Apical OHCs have much higher levels of glutathione than basal OHCs [9]. Aminoglycoside-induced OHC damage originates in the basal region of the cochlea and can progress to middle and apical turns.

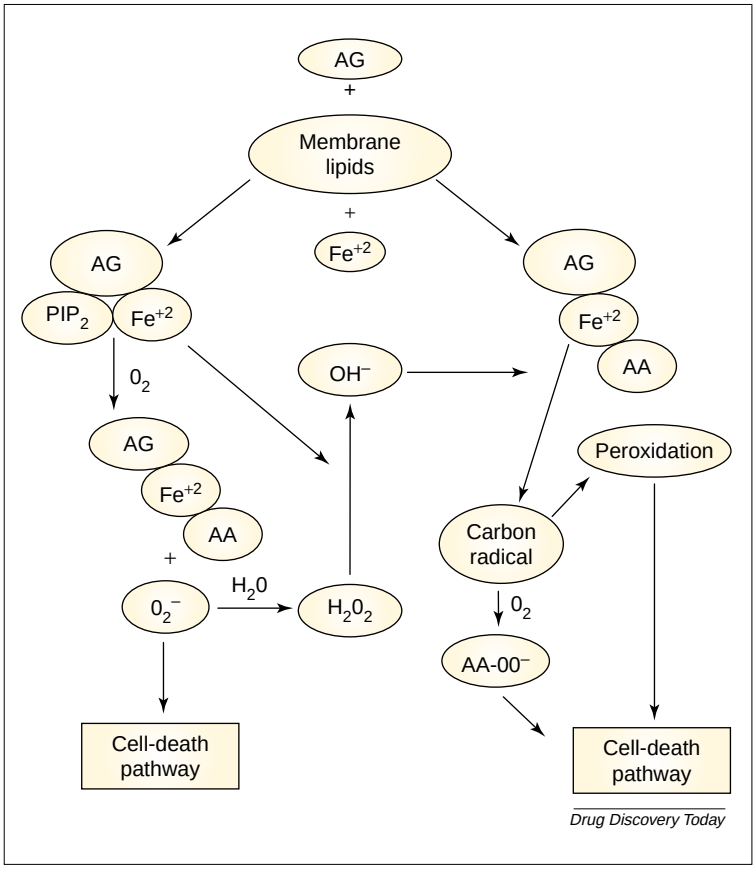

FIGURE 1

Proposed mechanisms for aminoglycoside-induced cell death in the cochlea. Aminoglycosides have been shown to form ternary complexes with lipid components of the cell membrane, including phosphatidylinositol (4,5)-bisphosphate (PIP_2) and arachidonic acid (AA) and iron (Fe^{2+}). The complex of AG, PIP_2 and Fe^{2+} can be oxidized by molecular oxygen to produce superoxide anion (O_2^-), Superoxide can then react with other cellular components or can dismutate to form hydrogen peroxide (H_2O_2). The latter can react with complexed Fe^{2+} to produce hydroxyl radicals (OH^-). AA can be released from oxidative damage to PIP_2 and can also form ternary complexes with Fe^{2+} and AG. OH^- can react with AA in the AG–Fe^{2+} to form carbon radicals, which can directly initiate peroxidation or can combine with molecular O_2 to form a peroxyl radical with AA [6]. These various ROS can then activate cell-death pathways in the OHCs in the cochlea.

Apoptotic cell death

An excess of ROS is believed to trigger cell death by apoptosis (programmed cell death). Two forms of apoptosis are currently recognized: an extrinsic death-receptor-mediated apoptosis; and an intrinsic mitochondrion-mediated cascade. Current literature, which is based predominantly on acute *in vitro* studies using cell lines or explants from neonatal rodents, supports the intrinsic apoptosis pathway as the major pathway induced by aminoglycosides in the cochlea (but it remains to be seen whether this view will prevail with additional chronic *in vivo* studies). The intrinsic apoptosis pathway is characterized by activation of G proteins, such as Ras, and GTPases, such as Rac. These events can result in activation of a family of stress-activated protein kinases, such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). The increased activity of these enzymes is accompanied by increased intracellular Ca^{2+} concentrations and release of cytochrome *c* from mitochondria. Cytochrome *c* release appears to be mediated by Bax (a protein that enhances apoptotoc cell death), which causes mitochondrial membrane damage and pore formation. However, recent evidence from hepatic tumor cells suggest that $O_2^{\bullet}$ can directly cause a profound release of cytochrome *c*, without damage to mitochondrial membranes, through a voltage-dependent anion transport channel. This phenomenon appears to modulate pore formation, but is not dependent on it [10]. Similarly, OC explants from adult guinea pigs displayed gentamicin-induced OHC death that was preceded by changes in mitochondrial membrane potentials. Co-administration

of the iron chelators, 2,2′-dipyridyl, salicylate, or cyclosporine A, a blocker of mitochondrial permeability transition, resulted in partial OHC protection. However, none of these compounds prevented gentamicin-induced condensation of chromatin in the nuclei of these cells [11]. These studies suggest that release of cytochrome *c* from mitochondria and subsequent activation of caspases-8, 9 and 3 are key steps in the cell death pathway, although other separate events also occur in the nucleus.

Aminoglycosides have been directly linked to many steps of the intrinsic apoptotic pathway in *in vivo* and *in vitro* studies. Gentamicin administration results in activation of G proteins that can be blocked by G-protein inhibitors or specific Ras inhibitors [12]. Similarly, gentamicin-induced increases in GTPase activity can be blocked using GTPase or Rac inhibitors [13]. The next stage of aminoglycoside-induced apoptosis appears to be the stress-activated protein-kinase cascade. This includes various protein kinases, such as JNK. These kinases are stored in the cytoplasm by a scaffold protein, c-Jun-interacting protein-1 (JIP1), that regulates their activity. This protein is believed to organize components of the JNK signal cascade and facilitate their phosphorylation. Activated JNK can then mediate activation of c-Jun, c-Fos, ELK-1 and activating transcription factor 2 (ATF2) in nuclei and Bcl-2 in mitochondria. Increases in JNK, c-Jun, c-Fos and Bcl-2 compounds have all been observed after aminoglycoside administration, as have cytoplasmic levels of cytochrome *c* and morphological indicators of apoptosis [14].

Approaches to protection

Approaches to otoprotection have included 'upstream' protection using antioxidants, free-radical scavengers and metal chelators. In addition, 'downstream' methods of protection have been investigated using compounds that inhibit various stages of apoptosis.

Upstream protection

Several promising agents have been investigated that prevent the initial stages of lipid peroxidation and cell damage by blocking the formation of ROS or scavenging ROS once they are formed. These include vitamin E [15], D-methionine [16] and α-lipoic acid [16]. Ebselen, an effective antioxidant and scavenger of peroxynitrite, has been shown to reduce gentamicin ototoxicity [18].

Because of the interaction of aminoglycosides with iron to form ROS, metal chelators have been investigated as protective agents. Deferoxamine, 2,2′-dipyridyl, salicylate and 2,3-dihydroxybenzoate are effective iron chelators that also function as antioxidants, and have been demonstrated to protect against aminoglycoside ototoxicity in animal studies [11,19]. Salicylate and 2,3-dihydroxybenzoate have been shown to facilitate the translocation of NFκB into the nuclei of OHCs, thus triggering anti-apoptotic pathways in these cells [7].

Flavonoids have been identified in a number of Chinese herbal extracts and have been shown to be protective against oxidative stress. These compounds possess numerous properties, including antioxidant effects, enhancement of antioxidant enzymes, free-radical scavenging and calcium stabilization. Herbal extracts that have been investigated as otoprotectants against aminoglycosides in animal studies include *Ginkgo biloba* [20], Gu Sui Bu (GSB) [21] and Tanshinone, a phenolic acid derivative of Danshen [22].

Other compounds that display antioxidant properties and have recently been investigated as protective agents against aminoglycoside ototoxicity include corticosteroids and neurotrophic growth factor. Dexamethasone, a corticosteroid, protected isolated OHCs from aminoglycoside ototoxicity, presumably by the inhibition of nitric oxide synthesis and free radical formation [23,24]. Neurotrophic growth factors have been shown to increase antioxidant enzyme activity, reduce NO formation and increase anti-apoptotic Bcl-2 proteins while inhibiting pro-apoptotic proteins. This family of compounds has shown considerable promise as protective agents. Gene therapy with transforming growth factor (TGF)-β1 and glial-cell-derived neurotrophic factor (GDNF) was shown to protect auditory function in guinea pigs [25].

Augmentation of endogenous antioxidant enzymes has been helpful in defining mechanisms of aminoglycoside ototoxicity as well as providing novel potential approaches to otoprotection. The SOD mimetic, M40403, has recently been shown to protect organotypic OC cultures from gentamicin ototoxicity [26]. Surprisingly, M40403 was not effective in protecting against the toxic effects of cisplatin in this system. Perhaps cisplatin has greater intrinsic toxicity than gentamicin, causing a greater depletion of glutathione than gentamicin, or it might cause an imbalance in complementary enzyme systems in this tissue, or it might act by pathways other than through ROS [26]. Guinea pigs whose cochleae were inoculated with adenoviral vectors for catalase, SOD1 or SOD2 were partially protected from aminoglycoside ototoxicity. Catalase and SOD2 overexpression were more effective than SOD1 [27].

Alternative otoprotective strategies have been used for aminoglycosides, including 'cell toughening' and modifying aminoglycoside kinetics with loop diuretics. The phenomenon of cell toughening in the OC, where preconditioning to low levels of stress can prepare cells to tolerate higher levels, has been studied as a means of protection from acoustic trauma. Toughening can increase levels of endogenous antioxidants, thus bolstering cellular protective mechanisms. Preconditioning with low doses of amakacin could protect against ototoxic dosages of amikacin in a guinea pig study [28].

Ototoxic synergism between aminoglycosides and loop diuretics has been well documented. However, delayed administration of ethacrynic acid has been suggested as a means of lowering perilymphatic concentrations of

TABLE 1

Effects of protective agents against aminoglycoside ototoxicity

Aminoglycoside	Protective agent	*In vivo*[a]	*In vitro*[b]	Species	Efficacy[c]	Refs
Gentamicin	2,2-DPD or cyclosporine A		X	Guinea pig	++	[11]
Gentamicin	G protein inhibitor (GDP-βs)		X	Rat	+++	[12]
Gentamicin	Ras inhibitor (FTI277)		X	Rat	+++	[12]
Gentamicin	Ras inhibitor (B581)		X	Rat	++	[12]
Gentamicin	*C. difficile* toxin		X	Rat	++	[13]
Neomycin	D-JNKI-1		X	Mouse	+++	[14]
Neomycin	D-JNKI-1	Intracochlear		Guinea pig	+++	[14]
Gentamicin	α-Tocopherol	Oral gavage		Guinea pig	+++	[15]
Gentamicin	D-methionine	i.p.		Guinea pig	++	[16]
Amikacin	Lipoic acid	i.m.		Guinea pig	+++	[17]
Gentamicin	Ebselen	i.p.		Guinea pig	+++	[18]
Gentamicin	Salicylate	s.c.		Guinea pig	+++	[19]
Gentamicin	*Ginkgo biloba* extract	Round window		Guinea pig	+++	[20]
Gentamicin	Gu Siu Bu	i.m.		Guinea pig	++	[21]
Gentamicin	Danshen	s.c.		Mouse	++	[22]
Gentamicin	Danshen		X	Mouse	+++	[22]
Kanamycin + Ethacrynic acid	Dexamethasone	Intracochlear		Guinea pig	+	[23]
Gentamicin	Dexamethasone + liver extract		X	Chinchilla	+++	[24]
Kanamycin + Ethacrynic acid	GDNF + TGF-β1	Intracochlear adenovirus		Guinea pig	++	[25]
Gentamicin	SOD analog (M40403)		X	Mouse	++	[26]
Kanamycin + Ethacrynic acid	SOD1 or SOD2	Intracochlear adenovirus		Guinea pig	++	[27]
Amikacin	Amakacin preconditioning	i.m.		Guinea pig	++	[28]
Gentamicin	Ethacrynic acid	i.v.		Guinea pig	+	[29]
Gentamicin	CEP1347	s.c		Guinea pig	+	[30]
Gentamicin	Minocycline		X	Rat	++	[31]
Gentamicin	Minocycline or p38 MAPK inhibitor (SB203580) + caspase 3 inhibitor (DEVD or ZVAD)		X	Rat	+++	[32]

[a] Abbreviations: i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.
[b] Key: X denotes that in vitro studies have been carried out.
[c] Key: +, low efficacy; ++, moderate efficacy; +++, high efficacy.

aminoglycosides, thus limiting the extent of cochlear damage. Cochlear damage was reduced when ethacrynic acid was administered 12 to 18 h after the last dose of gentamicin in guinea pigs [29].

Downstream protection

In addition to protecting the cochlea from oxidative stress and free radicals that lead to cell death, it could also be possible to slow or reverse the process of cell death. As discussed earlier, aminoglycosides have been shown to place cochlear cells into a pro-apoptotic state. As a result, trials with compounds that can block aminoglycoside-induced apoptosis have been the focus of recent research. For example, inhibition of early promoters of apoptosis, GTPases, Rho and Rac, by *Clostridium difficile* toxin B provided dose-dependent protection against aminoglycoside ototoxicity *in vitro*. This toxin also reduced the levels of c-Jun phosphorylation [13].

Phosphorylation of c-Jun via the JNK cascade appears to be a key turning point for OHC apoptosis. An inhibitor of the JNK cascade, CEP1347, has been shown to reduce aminoglycoside ototoxicity [30]. A synthetic inhibitor of JNK phosphorylation (D-JNKI-1) has shown some promise in protecting against neomycin ototoxicity. Co-administration of D-JNKI-1 resulted in nearly complete protection against neomycin-induced OHC mortality in OC explants. D-JNKI-1 also reduced neomycin-induced expression of c-Fos, a nuclear transcription factor involved in apoptosis, to near control levels. These results were also observed *in vivo* [14].

Blocking later events in the apoptotic pathway could also provide otoprotection. Minocycline, a tetracycline antibiotic, is a known inhibitor of caspases and cytochrome-*c*-release into the cytoplasm. Minocycline has recently been shown to protect against gentamicin ototoxicity *in vitro* [31]. The preservation of cell viability by minocycline is

accompanied by blocking gentamicin-induced caspase-3 activation and release of cytochrome *c* into the cytoplasm of hair cells. However, the use of a caspase-3 inhibitor alone only partially protects against gentamicin ototoxicity. Therefore, the effectiveness of minocycline might be attributed to its inhibition of upstream enzymes, such as p38 MAPK, in combination with caspase-3 inhibition. In fact, it has recently been demonstrated that caspase inhibitors combined with a p38 MAPK inhibitor, provided synergistic protection against gentamicin ototoxicity *in vitro* [32]. The most recent experimental studies of protective agents against aminoglycoside ototoxicity *in vitro* and *in vivo* are summarized in Table 1.

Platinum compounds

Cisplatin was first synthesized by Peyrone in 1845, and thus it is sometimes referred to as 'Peyrone's chloride'. In 1965, Rosenberg and Cavalieri discovered that an electrical current delivered between two platinum electrodes inhibited the proliferation of *Escherichia coli* [33]. They found that platinum complexes were formed in the presence of ammonium and chloride ions. Cisplatin was found to be the most active platinum compound in experimental tumor systems, and was introduced into clinical chemotherapy in the early 1970s. It is a highly effective agent for the treatment of a wide variety of soft-tissue neoplasms, including testicular, ovarian, cervical, bladder and lung cancer and squamous cell cancer of the head and neck. Unfortunately, nephrotoxicity, neurotoxicity and ototoxicity can occur. The clinical presentation of cisplatin includes tinnitus and high frequency sensorineural hearing loss, which can be permanent and progressive, involving the lower frequencies. With escalation of the dose of cisplatin in treatment protocols, nearly every patient can develop at least some hearing loss [34]. Cisplatin ototoxicity can involve the production of ROS [35]. Administration of ototoxic doses of cisplatin to experimental animals results in depletion of glutathione and antioxidant enzymes (SOD, catalase, glutathione peroxidase and glutathione reductase) in cochlear tissues, with a corresponding increase in malondialdehyde levels [36]. A potential source for the production of toxic, free radicals in the cochlea following cisplatin exposure is NADPH oxidase, the enzyme that catalyzes the formation of superoxide radicals. A particular isoform of NADPH oxidase, NOX3, is highly expressed in the inner ear as demonstrated by real-time PCR. *In situ* hybridization studies demonstrated that NOX3 is localized to vestibular and cochlear sensory epithelia and spiral ganglion. NOX3-transfected human embryonic kidney 293 cells pre-incubated with cisplatin showed markedly enhanced superoxide production. These exciting new findings suggest that NOX3 is an important source of ROS generation in the cochlea, which might contribute to hearing loss [37]. Because this enzyme is only expressed in the inner ear, a specific inhibitor of this enzyme could possibly be developed and then administered either systemically or locally to the round window membrane to protect against hearing loss and cochlear damage caused by cisplatin.

The superoxide radicals generated by cisplatin exposure can be transformed into hydrogen peroxide, which can be catalyzed by iron to form the highly reactive hydroxyl radical. This can react with polyunsaturated fatty acids to generate the toxic aldehyde 4-hydroxynonenal (4-HNE). After local application of cisplatin to mouse cochlea, 4-HNE and nitrotyrosine (NT) were detected immunohistochemically in auditory epithelia and in neurons damaged by cisplatin. However, in auditory hair cells, only 4-HNE- but not NT-immunoreactivity was detected. These findings suggest that the hydroxyl radical might play a crucial role in cisplatin-induced hearing loss and hair cell degeneration [38].

Cisplatin-induced ototoxicity in animal models is characterized by high-frequency hearing loss in guinea pigs, as demonstrated by changes in compound action potential thresholds after 5 daily intraperitoneal (i.p.) injections of 2mg/kg of cisplatin. This was accompanied by selective loss of outer hair cells in the basal and middle turns of the cochlea. The outer hair cells in the basal region of the cochlea demonstrated TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling)-positive staining and pathologic staining with Hoechst 33342 dye, indicating apoptotic cell death [39].

A new model for the study of cisplatin ototoxicity has been developed. This consists of a two-cycle treatment in rats, rather than an acute single high dose of cisplatin as used in other studies. Each cycle consists of four days of cisplatin injections (1 mg/kg i.p., twice daily) separated by 10 days of rest. Hearing threshold elevations and hair cell loss occurred only after the second cycle, and no mortality was reported. This model serves to eliminate potentially confounding factors that can determine the survival of a special cohort of animals [40].

Important signaling events that regulated the cell death of cisplatin-damaged cochlear cells included: the activation and redistribution of cytosolic Bax and the release of cytochrome *c* from injured mitochondria; activation of caspases-9 and 3 but not caspase 8; and the cleavage of fodrin by activated caspase-3 within the cuticular plate in damaged hair cells [41]. Because perfusing the cochlea with a caspase-8 inhibitor was not effective in preventing either cisplatin-induced hair cell death or hearing loss, it appears that the apoptosis of cochlear hair cells caused by cisplatin in guinea pigs is caspase-8 independent. Moreover, because caspase-9 and caspase-3 are activated in cisplatin-damaged hair cells, and because the intracochlear perfusion of the inhibitors of these caspases prevented apoptosis and hearing loss, it is likely that cisplatin ototoxicity is mediated by mitochondrial damage in the affected hair cells, with sequential activation of initiator and effector caspases, resulting in apoptosis, hair cell destruction and hearing loss (Figure 2).

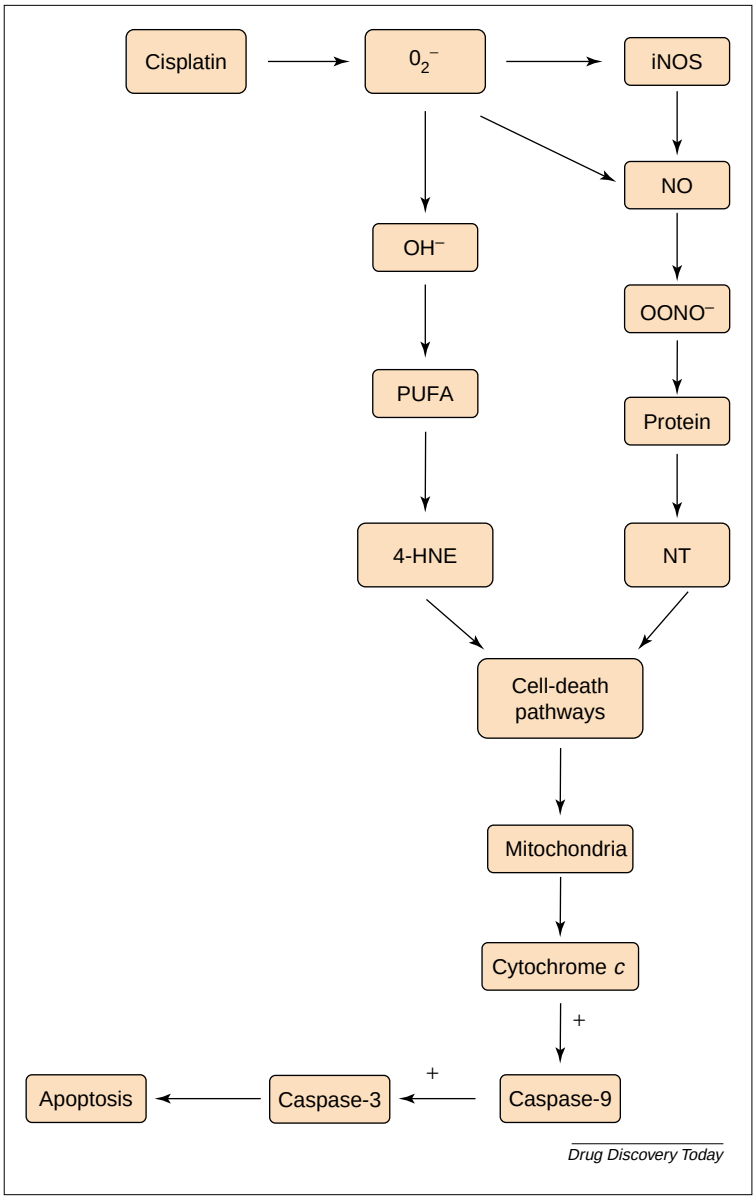

FIGURE 2

Proposed mechanisms for cell death in OHCs exposed to cisplatin. Cisplatin can generate O_2^-, perhaps through NADPH oxidase or other enzymes in the cochlea. Through the Fenton reaction, OH^- can be produced. The latter can interact with polyunsaturated fatty acids (PUFA) in the cell membrane to generate toxic aldehydes, such as 4-hydroxynonenal (4-HNE), leading to cell death. O_2^- can activate inducible nitric oxide synthase (iNOS) to generate nitric oxide (NO) which can interact with O_2^- to form peroxynitrite ($OONO^-$). The latter can react with proteins to form nitrotyrosine (NT). These toxic intermediates can then trigger cell death by causing the release of cytochrome *c* from the mitochondria, resulting in activation of downstream caspases (caspase-9 and -3), leading to apoptosis.

Cisplatin-treated animals were found to have significant increases in JNK, and perilymphatic perfusion of D-JNKI-1 prevented neither the activation and cellular redistribution of Bax nor the release of cytochrome *c* from mitochondria [41].

Upstream protection

A number of potentially protective agents containing thiol groups have been tested for efficacy against cisplatin ototoxicity in animal studies. These include: sodium thiosulfate, D- or L-methionine, diethyldithiocarbamate, methylthiobenzoic acid, lipoic acid, N-acetylcysteine, tiopronin, glutathione ester and amifostine. Perfusion of sodium thiosulfate into the cochlea of guinea pigs completely prevented cisplatin-induced hearing loss, and cochlear hair cells were well preserved in protected animals [42]. However, chronic round window application of sodium thiosulfate with an osmotic minipump provided no protection against cisplatin ototoxicity [43]. D-methionine provided excellent protection against outer hair cell loss, antioxidant enzyme depletion and auditory threshold elevations in rats pretreated with this agent prior to cisplatin administration [44]. Glutathione ester, but not glutathione, protected rats against cisplatin ototoxicity [45].

Amifostine afforded dose-dependent protection against cisplatin ototoxicity in hamsters. However, the protective agent that produced moderate to complete protection against ototoxicity also caused neurotoxicity at higher doses, manifested by prolongations in the auditory brainstem response (ABR) interpeak latency [46]. α-Tocopherol has been reported to reduce cisplatin-induced outer hair cell damage and auditory brainstem threshold elevations in rats following systemic pretreatment [47]. It blocked lipid peroxidation in the cochlea, and prevented apoptosis and outer hair cell loss accompanied by ABR threshold elevations in guinea pigs [48].

However, in another study of cisplatin ototoxicity in guinea pigs, only partial protection was seen with α-tocopherol pretreatment. When tiopronin (N-(2-mercaptopropionyl)-glycine) was combined with α-tocopherol, more effective protection was obtained. The greater effect seen with this combination was explained by the prevention of free radical formation by two different mechanisms: the thiol compound, tiopronin, by improving the antioxidant defenses of the cochlea to scavenge ROS, and α-tocopherol by inhibiting lipid peroxidation and preventing chain reactions [49]. In rats, tiopronin administered with cisplatin resulted in a significant protective effect on hair cells in the basal half and in the lower half of the middle turn of the cochlea. This cytoprotective effect was associated with a significant increase in the distortion product otoacoustic emissions (DPOAE) elicited in protected rats compared with rats treated with cisplatin alone [50]. In contrast with sodium thiosulfate, which interferes with the tumoricidal effects of cisplatin, tiopronin showed no reduction of tumor cell cytotoxicity *in vitro*. In tumor-bearing mice, tiopronin substantially reduced tumor growth in animals treated with a nontoxic dose of cisplatin, suggesting that tiopronin might enhance the antitumor properties of cisplatin [51]. Trolox® (Oxis), a water-soluble form of vitamin E, was found to be effective in reducing the ototoxicity of cisplatin when applied topically to the round window membrane of guinea pigs. Reduction in ABR threshold elevation and hair cell loss was observed in animals protected with Trolox® compared with those treated with cisplatin alone [52].

Administration of cisplatin (1mg/kg twice daily) for two cycles of four days each, separated by 10 days of rest produced significant hearing loss of 40 to 50 dB by ABR testing in rats receiving cisplatin alone. When sodium salicylate was added (100mg/kg subcutaneous, twice daily) cisplatin-induced threshold shifts were reduced and the antioxidant levels in the cochlea were restored or preserved. Salicylate might provide an antioxidant effect that antagonizes cisplatin ototoxicity [40]. Previous studies in tumor-bearing animals showed no antitumor interference in animals cotreated with cisplatin and salicylate at dosage levels where cisplatin ototoxicity was prevented by salicylate pretreatment [53].

Aminoguanidine is an inhibitor of iNOS. It is also an antioxidant that can scavenge hydroxyl radicals. Pretreatment of rats with aminoguanidine reduced the ototoxicity of cisplatin, resulting in significantly less malondialdehyde production in the cochlea and less elevation of ABR thresholds, but did not reduce the amount of nitric oxide produced. Thus, it might act as a free-radical scavenger, rather than as an iNOS inhibitor, in protecting the cochlea against cisplatin injury [54].

A significant reduction in acute ototoxicity of cisplatin in rats was observed in rats pretreated with a combined oral formulation of allopurinol and ebselen (a glutathione peroxidase mimic). Outer hair cells and auditory thresholds were preserved in rats administered the protective agents [55].

The antioxidant defense mechanisms in the cochlea might be mediated, at least in part, by adenosine receptors (ARs). The A1AR, and possibly, the A3AR might provide cytoprotection in the cochlea [56]. Local instillation of R-phenylisopropyladenosine (R-PIA) results in significant increases in cochlear glutathione peroxidase and superoxide dismutase within 90 min [57]. The application of cisplatin to the round window membrane of chinchillas results in an increase in A1AR in the cochlea at 24 and 72 h [57]. Pretreatment with the A1AR agonists, R-PIA or 2-chloro-N-cyclopentyladenosine (CCPA) provided significant protection against the loss of cochlear hair cells and ABR threshold elevations observed after cisplatin application to the round window membrane of chinchillas. These protective effects were blocked by prior application of the specific A1AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Furthermore, application of the A2AR agonist, 2-[4-(2-p-carboxy-ethyl)phenylamino]-5′-N-ethylcarboxamidoadenosine (CGS) to the round window membrane prior to cisplatin actually increased cochlear damage and threshold shift compared with cisplatin alone. These findings are consistent with the concept that the A1AR contributes significantly to cytoprotection in the cochlea, therefore protecting against hearing loss [58]. Although A1ARs in cochlear tissues are upregulated following cisplatin exposure, antagonists to this receptor do not afford protection against cisplatin ototoxicity. Rather, the agonists for the A1AR provide good protection against hearing loss and cochlear damage from cisplatin. This could occur because the upregulation of adenosine receptors represents an abortive attempt of the cochlea to protect itself from cisplatin toxicity, rather than a noxious effect of the cisplatin exposure. The interaction between adenosine A1 agonists and their receptor increase the antioxidant defenses in the cochlea and thereby afford protection of the structure and function.

Downstream protection

Intracochlear perfusion with caspase-3 inhibitor (z-DEVD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) dramatically reduced the incidence of apoptosis, hair cell loss and hearing threshold change that would otherwise occur after cisplatin administration in the guinea pig [41]. On the other hand, the intracochlear perfusion of D-JNKI-1, a cell-permeable peptide that blocks JNK-mediated activation of c-Jun, failed to prevent the mitochondrial release of cytochrome *c*. Paradoxically, the presence of D-JNKI-1 increased the sensitivity of cochlear hair cells to damage by cisplatin [41].

Addition of the p53 inhibitor, pifithrin-α to cultures of organotypic OC cells exposed to cisplatin protected the hair cells from ototoxic damage. These findings suggest that ototoxicity of cisplatin involves activation of p53 in triggering apoptotic cell death [59]. The results of the various experimental studies using *in vitro* and *in vivo* methods are summarized in Table 2.

Clinical implications and conclusions

There are certain similarities, yet other unexplained differences, in the ototoxic effects of aminoglycoside antibiotics and cisplatin. Both cause high frequency sensorineural hearing loss, which is usually permanent, and associated with loss of outer hair cells in the basal turn of the cochlea. Animal experiments suggest that both groups of drugs produce ROS in the inner ear, and these intermediates can activate cell-death pathways. Current evidence suggests that both groups of ototoxins act predominantly through the intrinsic cell death pathway, although future experiments should exclude the possible role of other pathways in addition to the intrinsic pathway. Aminoglycoside and cisplatin ototoxicity can both be reduced by the use of protective agents that block the production of or scavenge ROS. These upstream protective agents, such as antioxidants, can be administered systemically to protect against aminoglycoside or cisplatin ototoxicity, provided that interference with desired therapeutic effects have been convincingly excluded. Protective agents acting on downstream pathways can also be effective, but they might require local administration to minimize systemic side effects. It is possible to administer chemoprotective agents, anti-apoptotic drugs and other pharmaceuticals to the round window in humans to effect downstream protection against ototoxicity [60], thereby avoiding systemic toxicity from the protective agent and obviating antagonism of the therapeutic effects

TABLE 2

Effects of protective agents against cisplatin ototoxicity

Agent	*In vivo*[a]	*In vitro*[b]	Species	Degree of protection[c]	Refs
Thiosulfate	Cochlear perfusion		Guinea pig	+++	[42]
Thiosulfate	Chronic round window		Guinea pig	0	[43]
Amifostine	i.p.		Hamster	+++	[46]
Glutathione ester	i.p.		Rat	+	[45]
Diethyldithiocarbamate	i.p.		Rat	++	[36]
Methylthiobenzoic acid	i.p.		Rat	++	[36]
Ebselen	i.p.		Rat	+++	[36]
Ebselen & Allopurinol	p.o.		Rat	++	[55]
Salicylate	s.c.		Rat	++	[40]
Salicylate	s.c.		Rat	+++	[53]
α-Tocopherol	i.p.		Rat	++	[47]
α-Tocopherol	i.p.		Guinea pig	++	[48]
Trolox	Round window		Guinea pig	++	[52]
α-Tocopherol + tiopronin	i.p.		Guinea pig	++	[49]
Tiopronin	i.p.		Rat	++	[50]
Aminoguanidine	i.p.		Rat	++	[54]
R-PIA	Round		Chinchilla	++	[58]
CCPA	Window			++	[58]
Z-DEVD-fluoromethyl ketone (caspase-3 inhibitor)	Cochlear		Guinea	+++	[41]
Z-LEKD-fluoromethyl ketone (caspase-9 inhibitor)	Perfusion		Pig	+++	[41]
Pifithrin		X		++	[59]
D-JNKI 1	Cochlear perfusion		Guinea pig	0	[41]
M40403		X		0	[26]
D-methionine	i.p.		Rat	+++	[44]

[a] Abbreviations: i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; p.o., *per os*; s.c., subcutaneous.
[b] Key: X denotes that *in vitro* studies have been carried out.
[c] Key: +, low efficacy; ++, moderate efficacy; +++, high efficacy.

of aminoglycosides or cisplatin. Future studies should further elucidate the similarities and differences between the ototoxic mechanisms underlying the ototoxicity of aminoglycosides and cisplatin in order to develop more selective and specific protective strategies to minimize their ototoxicity.

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