



Metformin protects against gentamicin-induced hair cell death *in vitro* but not ototoxicity *in vivo*



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HIGHLIGHTS

- Our results confirm protection from gentamicin toxicity by metformin *in vitro*.
- Metformin did not protect the cochlea from gentamicin in guinea pigs *in vivo*.
- *In vivo* studies are needed to evaluate protective agents selected by *in vitro* screens.

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ABSTRACT

Metformin (N,N-dimethylbiguanidine) is a widely employed oral hypoglycemic agent for the management of type 2 diabetes mellitus. Its antioxidant properties and safe clinical use raise the possibility of preventing gentamicin-induced hearing loss in patients. Therefore, we screened the usefulness of metformin against gentamicin toxicity in murine cochlear explants and in the guinea pig *in vivo*. We confirmed in organ culture that metformin blocks the gentamicin-induced translocation of endonuclease G into the nucleus of outer hair cells and attenuates hair cell loss. *In vivo*, gentamicin treatment with 80, 100, or 130 mg/kg body weight for 14 days induced significant threshold shifts as determined by auditory brain stem responses. Metformin (30, 75, or 100 mg/kg for 14 days) was well tolerated without any indication of auditory side effects. However, co-administration of metformin with gentamicin in various permutations did not prevent loss of auditory function. On the contrary, combined treatment at higher dosages aggravated the gentamicin-induced threshold shifts and caused additional adverse reactions including body weight loss and premature deaths in some animals. These results caution against the use of metformin co-treatment with aminoglycosides and confirm the need for *in vivo* studies in order to evaluate potentially protective agents selected by *in vitro* screens.

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1. Introduction

The search for effective protection against aminoglycoside ototoxicity is receiving much attention as aminoglycosides maintain their position as powerful antibiotics to treat multi-resistant bacterial infections and tuberculosis [11]. Compelling *in vivo* and *in vitro* studies have suggested that reactive oxygen species (ROS) are important in aminoglycoside ototoxicity and that antioxidants can significantly protect against hearing loss in animal models [17]. Furthermore, two drugs have attenuated ototoxicity in the clinical

setting. Aspirin reduced the incidence of gentamicin-induced hearing loss in a double-blinded randomized clinical trial by 75% [15]. N-acetylcysteine attenuated gentamicin-induced high frequency hearing loss in hemodialysis patients in a small prospective randomized controlled open label study [7].

Proof of the concept of antioxidant protection has encouraged the search for efficacious therapeutics providing safety and easy availability. To this end, screening systems have been devised to detect potentially protective agents without the tedium of *in vivo* studies. Cell lines, cochlear explants, and zebra fish larvae have shown promise [21] but also met with problems or criticism [5].

Metformin (N,N-dimethylbiguanidine) is a potential anti-ototoxic agent, based on reports that it reduced gentamicin-induced apoptosis and cisplatin toxicity in a cell line [4]. The drug also prevents experimental gentamicin-induced nephropathy in the rat [12]. Metformin is a widely used oral hypoglycemic

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agent for the management of type 2 diabetes mellitus [20]. It also prevents oxidative stress-induced cell death through mechanisms related to mitochondrial permeability transition pore opening [22] and inhibition of lipid peroxidation. In addition, metformin scavenges hydroxyl radicals by modulating NADPH oxidase [2] and inhibits apoptotic cascades by increasing the expression of the anti-apoptotic protein Bcl-2 [19].

Metformin should be a good candidate for preventing ototoxicity based on the *in vitro* studies on gentamicin and cisplatin and the absence of adverse auditory effects during anti-diabetic therapy [1,18]. Therefore, we probed the usefulness of metformin on gentamicin-induced toxicity in murine cochlear explants and in guinea pigs *in vivo*.

2. Materials and methods

2.1. Materials

Gentamicin linked to the fluorophore Texas Red (GTTR) was kindly provided by Dr. Peter S. Steyger [13]. Anti-endothelial G (endoG) antibody was purchased from Chemicon International (Temecula, CA), rhodamine phalloidin, culture media and Alexa Fluor from Invitrogen (Carlsbad, CA), gentamicin from Sigma Chemical Co. (St. Louis, MO), and metformin from Calbiochem (Merck KGaA, Darmstadt, Germany).

2.2. Animals

For breeding, sexually mature male (6 week-old) and female (8 week-old) CBA/J mice were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN). Male Hartley guinea pigs (200–250 g) were purchased from Charles River (Wilmington, MA). Animals were maintained on a 12-h light/12-h dark schedule and had free access to water and a regular diet. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals and animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine.

2.3. Organotypic cultures of post-natal organ of Corti

The culture procedures were previously described in detail [6]. Cochleae from post-natal day 2 or 3 (p2–3) mice were immersed in cold Hank's Balanced Salt Solution, and the lateral wall tissues (stria vascularis and spiral ligament) and the auditory nerve bundle were dissected away leaving the organ of Corti. The explants were placed onto a previously prepared culture dish in 1 mL of culture medium consisting of Basal Medium Eagle, 1% serum-free supplement (Gibco #51500-056; Invitrogen, Carlsbad, CA), 1% bovine serum albumin, 5 mg/mL glucose, and 10 U/mL penicillin G. After 4 h of incubation (37 °C, 5% CO₂) 1.5 mL of medium was added to submerge the explants.

2.4. Treatment of explants

Explants were incubated for 2 days to recover from dissection stress before gentamicin treatment. The medium was exchanged for new media containing drugs and incubated for 24–72 h. Final concentrations were 3.5 μ M gentamicin, 15 μ M metformin, or 3.5 μ M GTTR; metformin was added 16 h prior to gentamicin or GTTR.

2.5. Immunofluorescent assessment

Explants were fixed with 4% paraformaldehyde overnight at 4 °C and permeabilized for 30 min with 3% Triton X-100 in PBS at room temperature (RT). Specimens were washed three times

with PBS and blocked with 10% goat serum for 30 min at RT, followed by incubation at 4 °C for 72 h with the primary antibodies (endoG, 1:1000, or Myo 7a, 1:200, in PBS). After three washes with PBS, secondary Alexa Fluor 488-conjugated goat anti-mouse antibody or Alexa Fluor 546-conjugated goat anti-rabbit antibody (each 1:200 in PBS) were applied overnight in darkness at 4 °C. Hair cells were visualized by incubating with rhodamine phalloidin or Alexa Fluor 488 phalloidin (each 1:100) at RT for 1 h; nuclei with Hoechst 33342 (2 μ g/mL in PBS, 40 min at RT). After rinsing in PBS, specimens were mounted on a slide with Prolong Gold anti-fade reagent (Life Technologies, Grand Island, NY) and imaged on an Olympus Fluoview Confocal Laser Scanning Microscope-FV500 (Olympus, Melville, NY).

2.6. Auditory brainstem response (ABR)

Guinea pigs were anesthetized with intra-peritoneal injections of xylazine (2.4 mg/kg body weight), ketamine (58.8 mg/kg), and acepromazine (1.2 mg/kg). Body temperature was maintained near 37 °C with a heating pad. ABRs were measured at 12 and 32 kHz in a sound-isolated and electrically shielded booth (Acoustic Systems, Austin, TX). Sub-dermal electrodes were inserted at the vertex of the skull, under the left ear, and under the right ear (ground). Tucker Davis Technology System III hardware and SigGen/Biosig software were used to present the stimuli (15 ms duration tone bursts with 1 ms rise-fall time) through a Beyer earphone and to average up to 1024 responses for each stimulus level. Thresholds (the lowest stimulus level with a reproducible response) were determined by reducing the intensity in 10-dB steps and in 5-dB close to threshold.

2.7. Drug administration *in vivo*

Gentamicin and metformin were dissolved in saline, pH was adjusted to 7.5, and the animals received daily subcutaneous injections for 14 days. Body weight was measured before each injection and the drug dose adjusted accordingly. If an animal was losing ~5% of its weight, 5–6 mL of saline were also subcutaneously injected.

3. Results

3.1. *In vitro* studies

3.1.1. Metformin attenuates gentamicin-induced hair cell loss

The concentration of gentamicin (3.5 μ M for 72 h) was selected from a series of preliminary experiments in murine explants (p2–3). The 72-h culture was chosen over a previous 24-h model [6] because gentamicin concentrations are lower by about two orders of magnitude and the delayed hair cell death better reflects the slow development of ototoxicity *in vivo*. The treated explants show the typical pattern of chronic aminoglycoside ototoxicity, namely damaged outer hair cells (OHCs) in a graded fashion from the base to the apex of the cochlea with essentially complete loss at the base (Fig. 1A). Inner hair cells showed no noticeable damage. Metformin alone did not cause any OHC loss at concentrations up to 1 mM but preserved OHC structure from gentamicin damage at concentrations as low as 15 μ M.

Hair cell loss was quantified in the three sections of the explants and the percentage of missing OHC was calculated against a normative database. Hair cell loss after metformin only treatment ($n=4$) remained below 1% in all regions. Gentamicin ($n=6$) caused a minor loss of 1.8% in the apex but increasing damage from the middle section ($37\% \pm 20$; means \pm s.d.) to the base ($77\% \pm 14$). Importantly, co-administration of 15 μ M metformin ($n=4$) attenuated the damage by gentamicin in the middle ($0.3\% \pm 0.3$) and basal sections ($2.2\% \pm 1.1$) almost completely and significantly ($p < 0.01$).

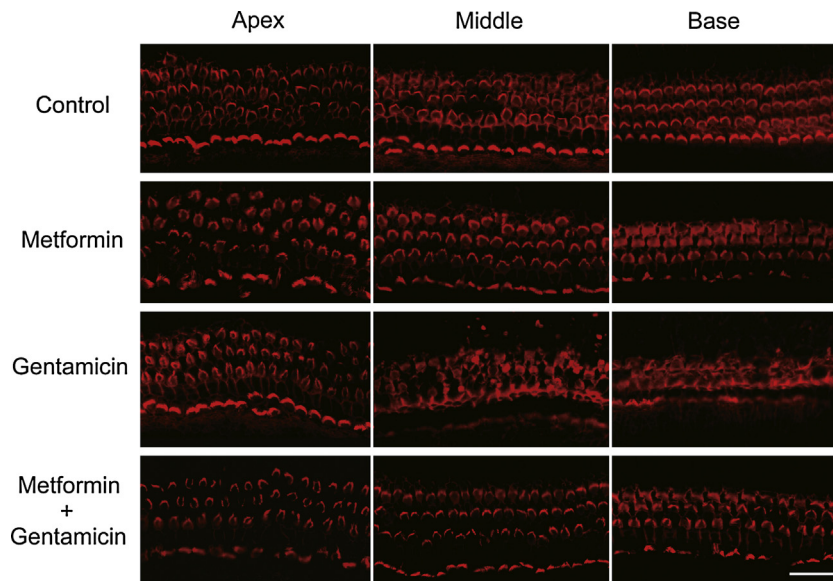


Fig. 1. Metformin protects explants from gentamicin toxicity. Gentamicin treatment ($3.5 \mu\text{M}$ for 72 h) damaged OHCs in a graded fashion with maximal damage to the base of the cochlea ($n=6$). Metformin ($15 \mu\text{M}$ for 72 h) alone did not cause any OHC loss ($n=4$) and protected hair cell integrity when co-administered with gentamicin ($n=4$). Red: rhodamine phalloidin. Scale bar = $20 \mu\text{m}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.1.2. Metformin blocks the translocation of endoG but not the entry of gentamicin

We first tested whether the protective effect of metformin might be caused by an inhibition of cellular uptake of gentamicin. Metformin did not block entry of drug into OHCs, as assessed using Texas Red-coupled gentamicin as a fluorescent probe (Fig. 2).

However, metformin attenuated apoptotic mechanisms associated with aminoglycoside ototoxicity. EndoG translocated into the nucleus of gentamicin-treated cells (Fig. 3A), consistent with previous observations *in vivo* [9]. Middle sections of 4–6 different explants per treatment, comprising >100 cells per treatment, were then quantitatively evaluated for endoG-positive nuclei (Fig. 3B). Middle sections were chosen as basal sections were unsuitable because of major loss of hair cells in gentamicin treatment. Only a few cells showed nuclear staining in the control and metformin groups. In contrast, gentamicin increased the number of endoG-positive nuclei to $26 \pm 4\%$, and this increase was significantly prevented by the additional presence of metformin in the incubations ($2 \pm 2\%$; $p < 0.01$).

3.2. In vivo studies

3.2.1. Metformin is devoid of ototoxicity

In a preliminary study, six guinea pigs received daily subcutaneous injections of 100 mg metformin/kg body weight for two weeks. The treatment was well tolerated and body weights of treated animals steadily increased from $334 \pm 41 \text{ g}$ to $401 \pm 53 \text{ g}$ ($n=6$), similar to that of control animals (from $332 \pm 18 \text{ g}$ to

$407 \pm 21 \text{ g}$; $n=4$). Blood samples were collected from three out of the six treated animals on the day after the last injections to check for renal impairment. Blood urea nitrogen (BUN) and creatinine (Cr) were in a normal range; BUN $13.0 \pm 4.0 \text{ mg/dL}$ (controls, $14.3 \pm 1.2 \text{ mg/dL}$) and Cr $0.7 \pm 0.1 \text{ mg/dL}$ (controls, $0.6 \pm 0.1 \text{ mg/dL}$).

Potential effects of metformin on auditory function were tested at 30 mg/kg, 75 mg/kg, or 100 mg drug/kg body weight per day for 14 days, and ABRs were measured three weeks after the last injections ($n=3$ each). None of the treatments caused any significant threshold shifts either at 12 or 32 kHz.

3.2.2. Metformin does not attenuate gentamicin-induced loss of auditory function

Next, we investigated whether metformin protected against gentamicin ototoxicity, starting with injections of 130 mg/kg of gentamicin for 14 days, a regimen within the range of our previous studies [10,16]. Gentamicin produced robust threshold shifts which were not attenuated by concomitant injections with 100 mg/kg metformin. On the contrary, the combined treatment aggravated the observed threshold shifts ($47 \pm 7 \text{ dB}$ at 32 kHz with gentamicin alone vs. $60 \pm 3 \text{ dB}$ for gentamicin plus metformin, $n=3$ each; $p < 0.05$). However, these results were potentially confounded by adverse reactions to the drug combination including body weight loss in 4/7 animals and 2 premature deaths, effects that were not observed with either drug alone.

We consequently reduced the dosage of gentamicin to 100 mg/kg body weight and co-injected two concentrations of metformin, 100 mg/kg or 30 mg/kg body weight. Both combinations

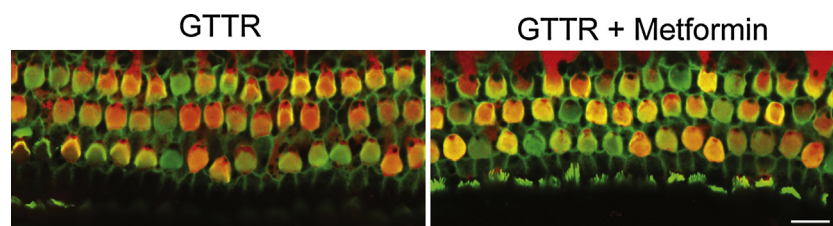


Fig. 2. Metformin does not block entry of gentamicin into OHCs in organ culture. Fluorescently tagged gentamicin ($3.5 \mu\text{M}$ for 24 h) was used to investigate the entry of gentamicin into explants. Metformin ($15 \mu\text{M}$) did not block uptake of gentamicin into OHCs ($n=3$). Green: Alexa Fluor 488 phalloidin, Red: GTTR. Scale bar = $10 \mu\text{m}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

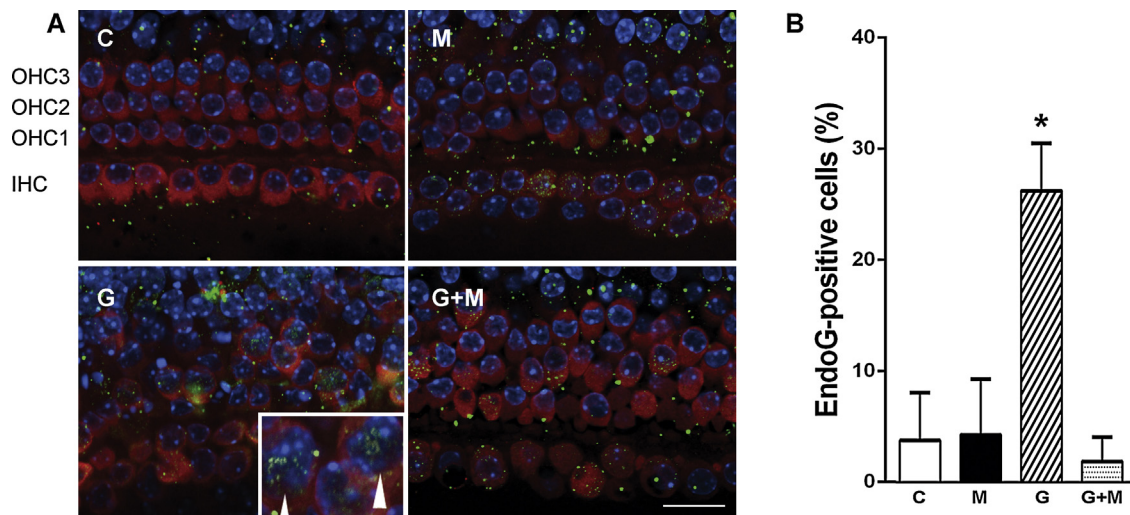


Fig. 3. Metformin blocks gentamicin-induced translocation of endoG. (A) EndoG translocated into the nuclei of gentamicin-treated cells ($3.5 \mu\text{M}$ for 72 h, arrow heads) while little or no translocation of endoG was observed in cells treated with $15 \mu\text{M}$ metformin alone or metformin in combination with gentamicin. Images are middle sections and representative of 4–6 explants per condition. IHC indicates position of inner hair cells, OHC of outer hair cells of rows 1, 2, and 3; respectively. C, control, $n=6$; M, metformin, $n=4$; G, gentamicin, $n=4$; G+M, gentamicin + metformin, $n=4$. Red: Myo 7a, Green: endoG, Blue: Hoechst 33342. Scale bar = $20 \mu\text{m}$. Insert in G shows nuclei at higher magnification with arrow heads pointing to endoG staining. (B) Middle sections of treated explants were quantified for endoG-positive cells and their percentage in each section was calculated and averaged. In each section, between 20 and 36 cells could be evaluated, for a total of >100 cells per condition. Data are means \pm s.d.; n as in (A). * Gentamicin treatment differs from all other conditions, $p < 0.05$; there are no significant differences between metformin or gentamicin plus metformin treatments (One-way ANOVA followed by Student–Newman–Keuls multiple comparisons). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

were well tolerated as judged by the weight gain of the animals. Consistent with its established pattern of ototoxicity, gentamicin at this dose again caused frequency-dependent threshold shifts averaging ~ 50 dB at 32 kHz. Co-treatment with either dose of metformin did not attenuate threshold shifts (Fig. 4A).

We continued to probe a potential window of protection by further lowering the dose of gentamicin to 80 mg/kg and concurrently reducing metformin to 75 mg/kg. Gentamicin induced a slightly smaller threshold shift but metformin remained unable to protect function (Fig. 4B).

The magnitude of threshold shifts induced by gentamicin was generally very consistent at high drug concentrations, such as 130 or 155 mg/kg body weight in our preliminary studies, but inter-animal variability increased as the dosages were lowered. For example, one animal each out of nine in each of the 80 and 100 mg

gentamicin groups did not sustain threshold shifts (≤ 0 dB). These outliers were excluded from the analysis.

4. Discussion

The salient finding of this study is the discrepancy between the protection against aminoglycoside-induced hair cell death by metformin *in vitro* and its inability to provide a functional protection *in vivo*.

Our *in vitro* experiments showing almost complete rescue by metformin of OHCs from gentamicin toxicity are consistent with findings in an auditory cell line [4] and also provide a tentative mechanistic base for a mechanism of this action. Metformin as a biguanidine exists as a cation at physiological pH and might therefore compete with cationic aminoglycosides for uptake into cells. However, metformin does not prevent entry of gentamicin into hair cells, thus suggesting an effect on intracellular pathways.

We selected the translocation of endoG as representative of apoptotic pathways based on our previous observation in mice *in vivo* following ototoxic treatment with aminoglycosides [9]. Furthermore, endoG is a particularly relevant probe because it is released in response to mitochondrial permeability change, an event that metformin is known to attenuate [8]. Consistent with the *in vivo* studies, gentamicin induces the nuclear translocation of endoG in explants, supporting an involvement of mitochondria in aminoglycoside ototoxicity [3]. Metformin indeed blocks the translocation, attesting to its mitochondrial action.

In contrast to its protective action *in vitro*, metformin failed to protect *in vivo* although our studies combined a wide range of gentamicin and metformin dosages. The combination of low doses of gentamicin (80 mg/kg) and metformin (75 mg/kg) even had a tendency to increase OHC loss as seen in cytochrome c oxidase (data not shown). There also was a trend towards aggravated threshold shifts at both 12 and 32 kHz when animals received 30 or 100 mg metformin/kg body weight in addition to 100 mg gentamicin/kg. Consistent with this indication of detrimental effects, combining 130 mg gentamicin/kg with 100 mg/kg metformin not only increased the observed threshold shifts but led to further adverse reactions, including death of the animals.

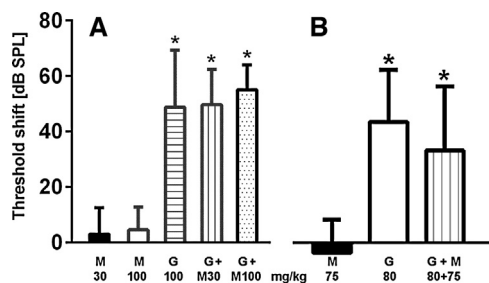


Fig. 4. Metformin does not prevent gentamicin-induced loss of auditory function. (A) Gentamicin ($100 \text{ mg/kg} \times 14$ days) induced a significant threshold shift at 32 kHz which was not attenuated by co-administered metformin. M30, 30 mg/kg metformin ($n=3$); M100, 100 mg/kg metformin ($n=3$); G100, 100 mg/kg gentamicin ($n=9$); G+M30, 100 mg/kg gentamicin + 30 mg/kg metformin ($n=3$); G+M100, 100 mg/kg gentamicin + 100 mg/kg metformin ($n=3$). (B) Threshold shift induced by a lower dose of gentamicin ($80 \text{ mg/kg} \times 14$ days) was also not attenuated by co-administered metformin. M75, 75 mg/kg metformin ($n=3$); G80, 80 mg/kg gentamicin ($n=9$); G+M, 80 mg/kg gentamicin + 75 mg/kg metformin ($n=3$). (A and B) Animals were treated and ABR recorded as described in Methods. Data are means \pm s.d. * Threshold shifts of gentamicin-treated animals differ from metformin only treatment, $p < 0.05$, but there is no significant difference between the various gentamicin treatments in the absence or presence of metformin (One-way ANOVA followed by Student–Newman–Keuls multiple comparisons).

The discrepancy between biochemical and protective actions of metformin is not an isolated observation. Cisplatin-induced functional and histological nephropathy was not prevented by metformin in a rat model *in vivo*, although metformin significantly attenuated drug-induced lipid peroxidation and reactive oxidant species and preserved enzymatic and non-enzymatic antioxidants [14]. In contrast, metformin prevented experimental gentamicin-induced nephropathy in the rat [12]. The study, however, employed a shorter course of gentamicin (3 or 6 days) than used in our *in vivo* experiments combined with a longer metformin exposure (100 mg/kg body weight \times 13 days *via* drinking water). Notwithstanding differences in drug responses between cochlea and kidney (e.g., the latter's ability to regenerate cells) and different species used in the two studies, these divergent results caution that variations of treatment parameters may introduce negative consequences of this particular drug combination. However, any therapy considered for translation to the clinic must yield robust efficacy with good safety margins. Potential protection by metformin does not conform to such a postulate.

5. Conclusion

Our results confirm protection from gentamicin toxicity by metformin in cochlear explants *in vitro*. In contrast, metformin did not protect the cochlea from gentamicin-induced ototoxicity in guinea pigs *in vivo*. These results caution against the use of metformin co-treatment with aminoglycosides and confirm the need for *in vivo* studies in order to evaluate potentially protective agents selected by *in vitro* screens.

Conflicts of interest

The authors declare no conflicts of interest.

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