Telmisartan Protects Auditory Hair Cells from Gentamicin-Induced Toxicity in vitro

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Keywords

Cochlea · Gentamicin toxicity · GW9662 · Hair cells · Inner ear · Organ of Corti · Peroxisome proliferator-activated receptor γ · Telmisartan

Abstract

Background: Telmisartan is an angiotensin II receptor blocker that has pleiotropic effects and protective properties in different cell types. Moreover, telmisartan has also shown partial agonism on the peroxisome proliferator-activated receptor γ (PPAR- γ). Auditory hair cells (HCs) express PPAR- γ , and the protective role of PPAR- γ agonists on HCs has been shown. **Objectives:** The objective of this study was to investigate the effects of telmisartan on gentamicin-induced ototoxicity in vitro. **Methods:** Cochlear explants were exposed to gentamicin with or without telmisartan, and/or GW9662, an irreversible PPAR- γ antagonist. **Results:** Telmisartan protected auditory HCs against gentamicin-induced ototoxicity. GW9662 completely blocked this protective effect, suggesting that it was mediated by PPAR- γ signaling. Exposure to GW9662 or telmisartan alone was not toxic to auditory HCs. **Conclusions:** We found that telmisartan, via PPAR- γ signaling, protects auditory HCs from gentamicin-induced ototoxicity. Therefore, telmisartan could potentially be used in the future to prevent or treat sensorineural hearing loss.

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Introduction

There are 466 million people worldwide who suffer from disabling hearing loss [WHO, 2018] and adult onset hearing loss is one of the 10 most common causes of disability-adjusted life years worldwide [Olusanya, 2007]. The prevalence of disabling hearing loss is increasing, and in view of the demographic development and the increase in life expectancy, this might have a much greater impact than assumed in the future [Olusanya, 2007]. It is expected that by 2050 there will be >900 million people with disabling hearing loss [WHO, 2018]. Sensorineural hearing loss is mainly caused by loss or degeneration of auditory hair cells (HCs), spiral ganglion neurons, and synaptic functions. Damage to these structures is caused by noise exposure, age, genetics, infections, and exposure to drugs like aminoglycoside antibiotics and chemotherapeutic agents. Loss of the sensory cells of the inner ear, the auditory HCs, is a main cause of sensorineural hearing loss. In addition, the loss of HCs in mammals is irreversible as no regeneration occurs. While today we are able to effectively treat conductive hearing loss, this is not the case with sensorineural hearing loss as we do not yet have any medicines specifically approved to treat hearing loss; our current therapeutic options are limited to hearing aids and cochlear implants [Wang and Puel, 2018]. To find preventive and therapeutic strategies in the future

we depend on understanding molecular mechanisms involved in hearing loss.

Telmisartan is an angiotensin II receptor blocker mainly used to treat hypertension. Angiotensin II is the major hormone in the renin-angiotensin-aldosterone system regulating blood pressure and fluid volume [Higuchi et al., 2007]. Effects of angiotensin II are mediated through the angiotensin II type 1 receptor (AT1R) and the angiotensin II type 2 receptor (AT2R). Both receptors belong to the 7-transmembrane G protein-coupled receptor family [Higuchi et al., 2007]. While most of the physiological functions of angiotensin II are imparted by the AT1R, the role of the AT2R remains less known [Higuchi et al., 2007]. Telmisartan is highly selective for the AT1R and an insurmountable antagonist at this receptor [Miura et al., 2011]. The AT1R activates several heterotrimeric G proteins, such as G_i/G_o , $G_{q/11}$, G_{12} , and G_{13} , and thus activates phospholipases A_2 , C, and D, the nuclear factor- κ B (NF- κ B) pathway, and different intracellular kinases [Higuchi et al., 2007; Hunyady and Catt, 2006]. Among the activated intracellular kinases are receptor and nonreceptor tyrosine kinases, serine/threonine kinases, mitogen-activated-signal-regulated kinases (MAPK), the Akt/protein kinase B, protein kinase C, and p70-kDa ribosomal protein S6 kinase [Hunyady and Catt, 2006; Higuchi et al., 2007]. In addition, activation of AT1R by angiotensin II leads to the formation of reactive oxygen species (ROS) and activation of small GTPases, such as Ras, Rho, and Rac [Hunyady and Catt, 2006; Higuchi et al., 2007]. The AT1R also signals via G protein-independent pathways, like β -arrestin-mediated extracellular-signal-regulated kinase activation, and the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway [Hunyady and Catt, 2006].

Besides being an AT1R antagonist, telmisartan was shown to be a partial agonist of the peroxisome proliferator-activated receptor γ (PPAR- γ) [Benson et al., 2004]. PPAR- γ belongs to the group of ligand-regulated nuclear hormone receptors (PPARs) that regulate lipid and glucose homeostasis, differentiation of inflammatory cells, but also cell proliferation [Berger and Moller, 2002]. As heterodimers with the retinoid X receptor the PPARs activate gene transcription upon ligand binding [Berger and Moller, 2002; Barish et al., 2006]. Specifically, several PPAR- γ agonists inhibit the activation of NF- κ B, MAPK, STAT, and downregulate the expression of cytotoxic and proinflammatory gene products [Berger and Moller, 2002; Collino et al., 2006; Bright et al., 2008; Hamblin et al., 2009]. Interestingly, the statin simvastatin activates

PPAR- γ [Yano et al., 2007], and in a previous study we have shown that simvastatin protected auditory HCs from gentamicin-induced toxicity [Brand et al., 2011]. In our recent study, we showed that PPAR- γ and PPAR- α proteins are expressed in the mouse organ of Corti (OC), and that they are present in auditory HCs [Sekulic-Jablanovic et al., 2017]. Moreover, we found that different PPAR- γ and PPAR- α agonists are protective against gentamicin-induced HC death [Sekulic-Jablanovic et al., 2017].

Furthermore, telmisartan has protective effects in the central nervous system [Tsukuda et al., 2009; Garrido-Gil et al., 2012; Eslami et al., 2014; Wang et al., 2014], in the cardiovascular system [Arozal et al., 2010; Maejima et al., 2011; Hu et al., 2014], and protects against gastric ulceration [Morsy et al., 2009], renal injury [Kusunoki et al., 2012; Zou et al., 2014; Ali et al., 2016], as well as hepatotoxicity [Kelleni et al., 2016]. In all these tissues, telmisartan had antioxidant properties and reduced the generation of ROS. Moreover, anti-inflammatory effects were involved in telmisartan-mediated protection.

These results prompted us to investigate a possible protective effect of telmisartan on gentamicin-damaged auditory HCs and whether the PPAR- γ antagonist GW9662 could block this effect.

Materials and Methods

OC Dissection

All animal procedures were carried out in Basel, Switzerland, according to an animal research protocol approved by the Animal Care Committee of the Canton of Basel, Switzerland. Animals were sacrificed prior to all tissue extractions.

Cochlear explants were dissected from Wistar rat pups at postnatal day 5 (Janvier Labs, Le Genest-Saint-Isle, France). After sacrifice by decapitation of the pups, the mandible was removed and the skulls were opened midsagittally, followed by removal of the brain. The temporal bones were harvested and transferred to sterile phosphate-buffered saline (pH 7.4) in sterile 60-mm Petri dishes. Removal of the extra tissue and hard cartilage allowed for exposure of the cochlear capsule, whose bony and cartilaginous layers were peeled off to reveal the membranous labyrinth. After removal of the stria vascularis, the OC was removed in its entirety from base to apex. The spiral ganglion neurons, spiral limbus, and tectorial membrane were stripped off, and the remaining cochlear tissue composed of auditory HCs, supporting cells, and part of the basilar membrane functioned as one sample.

Tissue Culture

Isolated cochlear explants were initially placed in cell culture media containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM HEPES buffer, and 30 U/mL penicillin (all chemicals from Sigma Aldrich Chemie GmbH, Steinheim, Germany, unless indicated otherwise). Follow-

Fig. 1. Experimental timeline representing addition and duration of different treatments. DMSO, dimethyl sulfoxide.

Table 1. The different treatment groups

Substance	Group					
	I	II	III	IV	V	VI
Control (dimethyl sulfoxide)	+	–	+	+	+	+
Gentamicin (LD ₅₀)	–	+	–	–	+	+
Telmisartan	–	–	+	–	+	+
GW9662	–	–	–	+	–	+

ing dissection, the culture medium was replaced with fresh cell culture medium and incubated at 37 °C in 5% CO₂ for a 24-h recovery period. For experiments with telmisartan (T8949) or with the PPAR- γ antagonist GW9662 (M6191), a concentration of 10 μ M telmisartan and/or 10 μ M GW9662, as well as an accompanying dimethyl sulfoxide (DMSO) control, were added to the recovery medium as a pretreatment (Fig. 1). Telmisartan and GW9662 media (10 μ M) had a final concentration of 0.1% DMSO; this concentration was also used in the control media and is known to cause no damage to HCs [Qi et al., 2008]. We selected the corresponding concentrations based on previous *in vitro* studies that found that 10 μ M of GW9662 blocked the effects of different concentrations of telmisartan (10, 20 μ M) [Maejima et al., 2011; Kusunoki et al., 2012; Hu et al., 2014; Su et al., 2015].

Experiments with cochlear explants were done to (1) establish a median lethal dose (LD₅₀) of gentamicin (G1397), (2) test potential toxicity of telmisartan or GW9662 on HCs, (3) understand whether telmisartan affected gentamicin-induced damage to HCs, and (4) test whether GW9662 influenced telmisartan-mediated effects on gentamicin-induced damage to HCs. Following 24-h recovery or pretreatment, explants were exposed to drugs for 48 h. Media were changed every 24 h to maintain concentration because of possible degradation after that period (Fig. 1). In order to find an LD₅₀ of gentamicin, cochlear explants were exposed to gentamicin at concentrations of 30, 60, 90, and 150 μ M. The cochlear explants were exposed to gentamicin after the 24-h recovery period, and again at hour 48, to mimic the way we would treat the explants with telmisartan and/or GW9662 in future experiments. Explants were fixed at hour 72 (Fig. 1). In the following experiments, explants were divided into the following groups (Table 1): (i) control (DMSO), (ii) gentamicin (LD₅₀), (iii) telmisartan, (iv) GW9662, (v) gentamicin (LD₅₀) and telmisartan, and (vi) gentamicin (LD₅₀), telmisartan, and GW9662.

Table 2. Numerical results of inner (IHC) and outer hair cell (OHC) numbers

	IHC	OHC
Apex		
Control	19.1±1.7	58.8±3.2
30 μ M gentamicin	18.4±2.6	43.9±13.7
60 μ M gentamicin	14.3±5.6	42.8±14.2
90 μ M gentamicin	13.1±7.1	36.5±13.2
150 μ M gentamicin	9.3±5.6	36.6±19.7
Middle		
Control	19.3±1.4	58.8±2.3
30 μ M gentamicin	18.1±3.2	51.9±9.3
60 μ M gentamicin	13.8±6.1	33.0±14.3
90 μ M gentamicin	13.3±4.9	31.0±17.7
150 μ M gentamicin	7.0±5.9	19.0±14.0
Base		
Control	19.2±1.3	58.9±1.8
30 μ M gentamicin	17.5±4.7	51.5±7.1
60 μ M gentamicin	13.4±5.8	34.7±15.9
90 μ M gentamicin	11.7±6.2	34.0±15.6
150 μ M gentamicin	8.4±5.8	16.4±12.9

Values are shown as means $\pm$ SDs. Cochlear explants were exposed to the indicated concentrations of gentamicin for 48 h. $n = 6$ –8 explants (3–4 animals) per condition. Data are expressed as the number of surviving hair cells per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant.

HC Visualization and Counts

After treatment, the explants were fixed in 4% paraformaldehyde, permeabilized with Triton solution and stained with a 1:100 dilution of Alexa Fluor 568 phalloidin (Invitrogen AG, Basel, Switzerland). All images were taken with the Nikon A1R laser confocal microscope (Nikon AG Instruments, Egg, Switzerland). The surviving HCs were counted in sections corresponding to 20 inner hair cells (IHCs) at different sites of the basal, middle, and apical turns of each cochlear explant in randomly selected fields. Both IHCs and outer hair cells (OHCs) were counted to quantify HC survival. It was considered that the HCs had undergone apoptosis if there was a gap in the normal ordered array of HCs. The results are presented as the number of surviving HCs per cochlear turn.

Fig. 2. Gentamicin ototoxicity in vitro. **a** Quantification of inner hair cells (IHCs) and outer hair cells (OHCs) exposed to the indicated concentrations of gentamicin (GM) for 48 h. **b** Representative images of phalloidin-stained hair cells (HCs). Gentamicin induced HC damage concentration dependently. $n = 6-8$ explants per condition. Scale bar for all figures, 50 μM. Data are expressed

as the number of surviving HCs per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant. Values are shown as means + SDs. ** $p < 0.01$ and **** $p < 0.0001$ versus OHC control; § $p < 0.05$, §§ $p < 0.01$, and §§§ $p < 0.001$ versus IHC control.

Statistical Analysis

All data are presented as means $\pm$ SDs. The statistical analysis was performed using Prism 7 (GraphPad software, La Jolla, CA, USA). We used two-way analysis of variance and Tukey's multiple comparisons test to analyze HC damage. The results were considered statistically significant when the p value was <0.05 .

Results

Gentamicin-Induced HC Loss in the OC

At a concentration of 30 μ M, OHC counts were only significantly reduced in the apical turn (43.9 ± 13.7 , mean $\pm$ SD, $p < 0.01$) compared to untreated OHCs (58.8 ± 3.2 ; Fig. 2a; Table 2). At a concentration of 60 μ M, the number of surviving OHCs was significantly lower in all cochlear turns (apex 42.8 ± 14.2 , middle 33.0 ± 14.3 , base 34.7 ± 15.9 , $p < 0.0001$) than the number of surviving OHCs in untreated explants (apex 58.8 ± 3.2 , middle 58.8 ± 2.3 , base 58.9 ± 1.8) and reached about 50% in the basal and middle turn (Fig. 2a; Table 2). Similar results were obtained when the explants were exposed to a slightly higher gentamicin concentration of 90 μ M. At this concentration, there were significantly fewer surviving OHCs in all cochlear turns (apex 36.5 ± 13.2 , middle 31.0 ± 17.7 , base 34.0 ± 15.6 , $p < 0.0001$) when compared to the number of surviving OHCs in untreated explants (Fig. 2a; Table 2). At a gentamicin concentration of 150 μ M, there were significantly fewer surviving OHCs in all cochlear turns (apex 36.6 ± 19.7 , middle 19.0 ± 14.0 , base 16.4 ± 12.9 , $p < 0.0001$) versus those in untreated cochlear explants (Fig. 2a; Table 2). At this concentration, much more OHCs underwent apoptosis and left a gap in the ordered rows of HCs (Fig. 2b). IHC counts were only significantly reduced at a gentamicin concentration of 150 μ M in all cochlear turns (apex 9.3 ± 5.6 , $p < 0.05$; middle 7.0 ± 5.9 , $p < 0.001$; base 8.4 ± 5.8 , $p < 0.01$) when compared to the number of surviving IHCs in untreated explants (apex 19.1 ± 1.7 , middle 19.3 ± 1.4 , base 19.2 ± 1.3 ; Fig. 2a; Table 2). The gentamicin concentration of 60 μ M was used in later experiments.

Telmisartan and GW9662 Are Not Toxic to HCs *in vitro*

IHC and OHC counts between a 0.1% DMSO vehicle control (OHCs, apex 59.9 ± 0.3 , middle 59.6 ± 1.2 , base 59.6 ± 1.3) and 10 μ M telmisartan (OHCs, apex 58.9 ± 2.2 , middle 59.8 ± 0.6 , base 59.3 ± 1.5) or 10 μ M GW9662 (OHCs, apex 59.1 ± 2.6 , middle 58.7 ± 1.8 , base 58.7 ± 1.9) did not differ significantly in all cochlear turns (Fig. 3a; Table 3). Thus, there is no HC damage induced by telmis-

Table 3. Numerical results of inner (IHC) and outer hair cell (OHC) numbers

	IHC	OHC
Apex		
0.1% DMSO control	18.5 $\pm$ 2.3	59.9 $\pm$ 0.3
10 μ M telmisartan	17.9 $\pm$ 3.5	58.9 $\pm$ 2.2
10 μ M GW9662	18.9 $\pm$ 1.5	59.1 $\pm$ 2.6
Middle		
0.1% DMSO control	19.2 $\pm$ 1.9	59.6 $\pm$ 1.2
10 μ M telmisartan	19.1 $\pm$ 2.0	59.8 $\pm$ 0.6
10 μ M GW9662	19.1 $\pm$ 1.5	58.7 $\pm$ 1.8
Base		
0.1% DMSO control	19.1 $\pm$ 1.9	59.6 $\pm$ 1.3
10 μ M telmisartan	19.5 $\pm$ 1.2	59.3 $\pm$ 1.5
10 μ M GW9662	18.3 $\pm$ 2.8	58.7 $\pm$ 1.9

Values are shown as means $\pm$ SDs. DMSO, dimethyl sulfoxide. Cochlear explants were exposed to the indicated concentrations of telmisartan and GW9662 for 72 h. $n = 6$ explants (3 animals) per condition. Data are expressed as the number of surviving hair cells per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant.

artan or GW9662 alone. The undamaged HCs are seen as 3 intact rows of OHCs and one intact row of IHCs in cochlear explants exposed to telmisartan or GW9662 alone similar to the control group (Fig. 3b).

Telmisartan Protects OHCs from Gentamicin-Induced Toxicity *in vitro*, and GW9662 Inhibits this Protective Effect

As expected, exposure to gentamicin resulted in significant OHC loss in all cochlear turns (Fig. 4a). Thus, after exposure for 48 h to 60 μ M gentamicin, there were fewer surviving OHCs in a basal to apical gradient (apex 31.7 ± 15.6 , middle 29.7 ± 14.4 , base 26.1 ± 10.9 , $p < 0.0001$) compared to untreated OHCs (apex 58.3 ± 2.5 , middle 59.4 ± 1.2 , base 59.5 ± 1.1 ; Fig. 4a; Table 4). Interestingly, in explants exposed to gentamicin and telmisartan, there were significantly more surviving OHCs in all cochlear turns (apex 47.7 ± 12.0 , middle 43.6 ± 14.5 , base 46.1 ± 15.9 , $p < 0.0001$) than in explants exposed to gentamicin alone (Fig. 4a; Table 4). In cochlear explants exposed to gentamicin and telmisartan most of the OHCs survived since the explants showed only a few missing HCs (Fig. 4b). Despite protection against gentamicin damage, OHC counts in explants exposed to gentamicin and telmisartan were still significantly lower when compared to those in untreated cochlear explants (apex $p < 0.001$, middle and base $p < 0.0001$; Fig. 4a).

Fig. 3. Telmisartan and GW9662 are not toxic to hair cells (HCs) in vitro. **a** Quantification of inner hair cells (IHCs) and outer hair cells (OHCs) in cochlear explants exposed to the indicated concentrations of telmisartan and GW9662. **b** Representative images of phalloidin-stained HCs. Exposure to 10 μ M telmisartan or GW9662 for 72 h did not cause any HC loss when compared to a

0.1% dimethyl sulfoxide control. $n = 6$ explants per condition. Scale bar for all figures, 50 μ m. Data are expressed as the number of surviving HCs per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant. Values are shown as means $\pm$ SDs.

In explants exposed to gentamicin, telmisartan, and GW9662, there were significantly fewer surviving OHCs in all cochlear turns (apex 22.7 ± 17.5 , middle 21.3 ± 15.3 , base 24.3 ± 20.0 ; $p < 0.0001$) versus those exposed to gentamicin and telmisartan (apex 47.7 ± 12.0 , middle 43.6 ± 14.5 , base 46.1 ± 15.9 ; Fig. 4a; Table 4). In the basal turn, OHC counts in explants exposed to gentamicin, telmisartan, and GW9662 were similar to those in cochlear explants exposed to gentamicin alone. In contrast, in the apical and medial cochlear turn OHC counts were also

significantly lower when compared to OHC counts in explants exposed to gentamicin alone ($p < 0.01$). Overall, explants exposed to all 3 drugs showed significantly reduced OHC counts compared to untreated explants in all cochlear turns ($p < 0.0001$). In those explants exposed to gentamicin, telmisartan, and GW9662, many OHCs were missing and left a gap in the ordered rows of HCs (Fig. 4b). In summary, GW9662 blocked the telmisartan-mediated protection on OHCs.

Fig. 4. Telmisartan protects outer hair cells (OHCs) from gentamicin-induced toxicity in vitro and GW9662 inhibits this protective effect. **a** Quantification of inner hair cells (IHCs) and OHCs in cochlear explants exposed to the indicated concentrations of gentamicin, telmisartan, and/or GW9662. **b** Representative images of phalloidin-stained hair cells (HCs). Exposure to 10 μ M telmisartan (T) in addition to 60 μ M gentamicin (GM) decreased OHC loss compared with exposure to 60 μ M gentamicin alone. Addition of 10 μ M GW9662 (GW) to gentamicin and telmisartan inhibited this

protective effect as explants showed increased OHC loss, with more gaps in the normal ordered array of HCs. $n = 10$ explants per condition. Scale bar for all figures, 50 μ M. Data are expressed as the number of surviving HCs per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant. Values are shown as means $\pm$ SDs. **** $p < 0.0001$ between 2 treated groups; # $p < 0.001$ and ## $p < 0.0001$ versus OHC control (0.1% dimethyl sulfoxide); § $p < 0.05$ and §§ $p < 0.01$ versus IHC control (0.1% dimethyl sulfoxide).

Table 4. Numerical results of inner (IHC) and outer hair cell (OHC) numbers

	IHC	OHC
Apex		
0.1% DMSO control	18.2±3.0	58.3±2.5
60 µM gentamicin	11.4±6.1	31.7±15.6
10 µM telmisartan + 60 µM gentamicin	14.7±5.7	47.7±12.0
10 µM GW9662 + 10 µM telmisartan + 60 µM gentamicin	12.5±6.6	22.7±17.5
Middle		
0.1% DMSO control	17.4±3.5	59.4±1.2
60 µM gentamicin	11.9±5.4	29.7±14.4
10 µM telmisartan + 60 µM gentamicin	15.2±4.2	43.6±14.5
10 µM GW9662 + 10 µM telmisartan + 60 µM gentamicin	10.1±5.7	21.3±15.3
Base		
0.1% DMSO control	18.2±2.2	59.5±1.1
60 µM gentamicin	14.7±5.4	26.1±10.9
10 µM telmisartan + 60 µM gentamicin	15.9±4.8	46.1±15.9
10 µM GW9662 + 10 µM telmisartan + 60 µM gentamicin	10.3±6.3	24.3±20.0

Values are shown as means ± SDs. DMSO, dimethyl sulfoxide. Cochlear explants were exposed to the indicated concentrations of gentamicin, telmisartan, and/or GW9662. *n* = 10 explants (5 animals) per condition. Data are expressed as the number of surviving hair cells per 20 IHCs, counted at different sites for the apical, middle, and basal turn of each explant.

IHC counts were significantly reduced in gentamicin-, telmisartan-, and GW9662-treated explants in the basal and medial cochlear turns (middle 10.1 ± 5.7 , $p < 0.05$; base 10.3 ± 6.3 , $p < 0.01$), and in cochlear explants exposed to gentamicin alone in the apical turn (11.4 ± 6 , $p < 0.05$) when compared to untreated cochlear explants (apex 18.2 ± 3.0 , middle 17.4 ± 3.5 , base 18.2 ± 2.2 ; Fig. 4a; Table 4).

Discussion

In the present work, we found that telmisartan protected HCs against gentamicin-induced toxicity in vitro. This protective effect was mediated by PPAR- γ . Telmisartan has pleiotropic effects and protects against damage in different cell types. Molecular events involved in telmisartan's protection include pathways that are involved in aminoglycoside ototoxicity. In a previous study we showed that PPAR- γ agonists protect HCs from oxidative stress and death [Sekulic-Jablanovic et al., 2017] and the PPAR- γ agonist pioglitazone was shown to protect against noise-induced hearing loss [Paciello et al., 2018].

Gentamicin-Induced HC Loss in the OC

The aminoglycoside gentamicin is a known ototoxic agent, and various studies on HCs that used gentamicin

as damaging agent have already shown different molecular mechanisms involved in HC survival and death [Battaglia et al., 2003; Chung et al., 2006; Brand et al., 2015].

Telmisartan and GW9662 Are Not Toxic to HCs in vitro

GW9662 is toxic to primary human peripheral blood mononuclear cell cultures [Jaudszus et al., 2014], oral squamous cell carcinoma cell lines [Masuda et al., 2005], and human colorectal adenocarcinoma cell lines [Schaefer et al., 2007]. It also potentiates neurotoxic effects in primary cultures of mouse cortical neurons, and this was discussed to be associated with non-PPAR- γ -specific effects of GW9662 [Wojtowicz et al., 2014]. Given these results and the protective role of PPAR- γ agonists on HCs demonstrated in our earlier study [Sekulic-Jablanovic et al., 2017], we wanted to rule out that GW9662 is toxic to HCs.

As demonstrated in our study, GW9662 and telmisartan are not toxic to HCs at our concentration used. Our results are consistent with previous studies, where GW9662 and/or telmisartan showed no toxic effects [Zhao et al., 2006; Garrido-Gil et al., 2012; Eslami et al., 2014; Pang et al., 2014]. These results suggest that PPAR- γ signaling is not essential for HC survival under normal conditions.

Telmisartan Protects OHCs from Gentamicin-Induced Toxicity in vitro, and GW9662 Inhibits this Protective Effect

Telmisartan significantly diminished OHC loss in all cochlear turns in explants exposed to gentamicin plus telmisartan versus explants exposed to gentamicin only. There are no other studies in the literature that investigated telmisartan-mediated effects on auditory HCs. However, our findings are consistent with other studies that demonstrated neuroprotective effects of telmisartan in various in vitro [Eslami et al., 2014; Pang et al., 2014; Wang et al., 2014] and in vivo [Jung et al., 2007; Mogi et al., 2008; Tsukuda et al., 2009; Thoenes-Reineke et al., 2011; Garrido-Gil et al., 2012; Justin et al., 2014; Wincewicz et al., 2016] experimental models. Moreover, telmisartan also has protective effects on other organ systems such as the cardiovascular system [Arozal et al., 2010; Maejima et al., 2011; Hu et al., 2014], the stomach [Morsy et al., 2009], the kidneys [Kusunoki et al., 2012; Zou et al., 2014; Ali et al., 2016], and the liver [Kelleni et al., 2016].

GW9662, a potent, irreversible, and selective PPAR- γ antagonist [Leesnitzer et al., 2002], completely inhibited telmisartan-mediated protection on HCs. These results suggest that telmisartan's protective effect on HCs is due to its agonistic activity on PPAR- γ . In previous studies, GW9662 also partially [Mogi et al., 2008; Eslami et al., 2014; Wang et al., 2014] or completely inhibited [Tsukuda et al., 2009; Garrido-Gil et al., 2012; Pang et al., 2014] telmisartan-mediated protective properties in other cellular systems than the inner ear, which is in line with our results. Expression of the AT1R or AT2R has not been investigated in the inner ear. However, of the class of angiotensin II receptor blockers, telmisartan has the strongest affinity for PPAR- γ [Marshall et al., 2006], and in our recent study we demonstrated PPAR- γ expression in cochlear explants from 5-day-old mice. We also localized PPAR- γ expression in both IHCs and OHCs, in the stria vascularis, spiral ganglion, and cochlear nerve but also in supporting cells and cells of the basal lamina of adult mice [Sekulic-Jablanovic et al., 2017]. Since we used rats in the present study, we assume that PPAR- γ is expressed similarly in the rat cochlea. Taken together, PPAR- γ expression in auditory HCs and inhibition of telmisartan's protective effect by GW9662 suggest PPAR- γ signaling as protective mechanism in HCs. These results are consistent with our previous study [Sekulic-Jablanovic et al., 2017] and with studies of other groups [Paciello et al., 2018; Park et al., 2017] that demonstrated protection from gentamicin-induced HC death and noise-induced

hearing loss mediated by PPAR agonists. Ten micromolars of both the PPAR- γ -selective agonist pioglitazone or the PPAR- γ/α dual agonist tesaglitazar offered full protection against gentamicin-induced HC loss in vitro [Sekulic-Jablanovic et al., 2017], whereas 10 μ M telmisartan only offered partial protection in our study. This difference could be explained by partial PPAR- γ agonism of telmisartan, whereas pioglitazone and tesaglitazar are full agonists.

Interestingly, in the apical and medial cochlear turns, OHC numbers in explants exposed to gentamicin, telmisartan, and GW9662 were also significantly lower when compared to OHC numbers in explants exposed to gentamicin alone. Moreover, IHC numbers were significantly reduced in gentamicin-, telmisartan-, and GW9662-treated explants when compared to untreated cochlear explants in the middle and basal turns. As GW9662 was not toxic to HCs, this raises the questions whether blocking PPAR- γ in the presence of gentamicin potentiates gentamicin-induced HC toxicity. Interestingly, Garrido-Gil et al. [2012] found similar results in that administration of GW9662 significantly increased neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neuron death in AT1R deficient mice, and it potentiated tetrabromobisphenol A neurotoxicity in primary neocortical cell cultures [Wojtowicz et al., 2014]. In contrast, this phenomenon has not been observed in other studies where GW9662 blocked telmisartan-mediated protective effects [Pang et al., 2014; Wang et al., 2014]. Therefore, possible effects of PPAR- γ inhibition on toxic events in HCs need to be further investigated in the future.

In the present study we demonstrate that telmisartan protects auditory HCs from gentamicin-induced ototoxicity. From this the question arises, by which mechanisms telmisartan can exert this PPAR- γ dependent protective effect. Since we did not investigate this in our study, we can only hypothesize about the underlying mechanisms. Telmisartan exerts protective effects in many cell types through PPAR- γ signaling [Morsy et al., 2009; Maejima et al., 2011; Kusunoki et al., 2012; Eslami et al., 2014; Zou et al., 2014]. The nuclear receptor PPAR- γ forms a heterodimer with the retinoid X receptor, and upon ligand binding it also binds to peroxisome proliferator response elements and induces gene transcription of many target genes [Berger and Moller, 2002; Fang et al., 2016]. After activation, PPAR- γ signaling regulates among others NF- κ B, tumor necrosis factor- α , and nuclear factor erythroid 2-related factor 2 signaling [Berger and Moller, 2002; Cai et al., 2018], all of which are also involved in hair cell

death and survival [Nagy et al., 2005; Dinh et al., 2008; Hoshino et al., 2011].

Main molecular mechanisms underlying telmisartan-mediated protection are anti-inflammatory [Jung et al., 2007; Tsukuda et al., 2009] and antioxidative effects [Arozal et al., 2010; Eslami et al., 2014; Jung et al., 2007; Morsy et al., 2009]. Telmisartan attenuates the expression of proinflammatory cytokines such as tumor necrosis factor- α [Thoene-Reineke et al., 2011; Tsukuda et al., 2009], interleukin-1 β , and interleukin-6 [Justin et al., 2014], decreases cyclooxygenase-2 expression [Jung et al., 2007], and increases anti-inflammatory cytokine interleukin-10 levels [Justin et al., 2014]. Moreover, telmisartan diminishes intracellular ROS, malondialdehyde as well as NADPH oxidase levels, elevates glutathione levels as well as superoxide dismutase and catalase activity [Morsy et al., 2009; Arozal et al., 2010; Eslami et al., 2014; Kelleni et al., 2016], and ameliorates lipid peroxide levels [Justin et al., 2014]. In addition, telmisartan activates the phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase-3 β (GSK-3 β) pathway [Pang et al., 2014; Wang et al., 2014]. Aminoglycoside antibiotics are mainly toxic through generation of ROS [Wang and Puel, 2018]. They have also been shown to trigger inflammatory responses [Abi-Hachem et al., 2010] and to induce the production of proinflammatory cytokines in cochlear explants in vitro [Bas et al., 2012]. The PI3K/Akt pathway is a common mechanism promoting HC survival in the inner ear [Brand et al., 2015], and it has been shown to oppose gentamicin toxicity in HCs in vitro [Chung et al., 2006]. In addition, selective GSK-3 β inhibitors diminish cisplatin-induced auditory HC death in vitro and protect against cisplatin-induced hearing loss in vivo [Park et al., 2009].

Interestingly, the PPAR- γ agonist pioglitazone protects against noise-induced hearing loss through both anti-inflammatory effects and by reducing oxidative stress [Paciello et al., 2018]. Indeed, in our recent in vitro study using a gentamicin ototoxicity model we saw that pioglitazone was able to counteract the gentamicin-induced increase in ROS and lipid peroxidation levels, and to inhibit apoptosis by enhancing the cell's intrinsic antioxidant defense system [Sekulic-Jablanovic et al., 2017]. Besides PPAR- γ , other PPAR subtypes also possess protective properties in auditory HCs [Park et al., 2017; Sekulic-Jablanovic et al., 2017; Kim et al., 2018]. Fenofibrate, a PPAR- α agonist, protects auditory HCs against gentamicin- and cisplatin-induced ototoxicity reducing oxidative stress [Park et al., 2017; Kim et al., 2018]. Interestingly, Kim et al. [2018] also showed that fenofibrate pro-

tections against mitochondrial and peroxisomal dysfunction by increasing PPAR- γ , PPAR- α , and PPAR coactivator 1 α expression and by increasing fatty acid-binding protein expression.

Taken together, these results suggest that anti-inflammatory and antioxidative effects as well as activation of the PI3K/Akt/GSK-3 β pathway may also be involved in telmisartan-mediated protection in HCs. Moreover, other mechanisms such as protection against mitochondrial and peroxisomal dysfunction may also be involved. In addition, it is possible that the involvement of these mechanisms in HC protection may vary depending on the nature of the damage and the condition of the cells. Finally, the AT1R was shown to activate ROS production and other pathways involved in HC death, such as the NF- κ B, c-Jun N-terminal kinase, and p38MAPK pathway, and to activate small GTPases, such as Ras, Rho, and Rac [Hunyady and Catt, 2006; Higuchi et al., 2007]. Although we showed that telmisartan's protection on HCs was mediated by PPAR- γ signaling, antagonistic effects at the AT1R might also be involved. Future studies will need to identify the specific molecular mechanisms involved in telmisartan's mediated protection on HCs.

Conclusion

In our study we demonstrated for the first time that telmisartan protects auditory HCs against gentamicin-induced damage in vitro. This protective effect is presumably mediated through PPAR- γ signaling, which is expressed in auditory HCs [Sekulic-Jablanovic et al., 2017].

Telmisartan is highly lipophilic and has a high volume of distribution and therefore possesses high tissue-penetrating properties [Wienen et al., 2000]. Telmisartan has been shown to penetrate the blood-brain barrier [Gohlke et al., 2001] and revealed neuroprotective properties in models of Parkinson's disease [Garrido-Gil et al., 2012], stroke [Thoene-Reineke et al., 2011], glutamate-induced neurotoxicity [Wang et al., 2014], stress-induced cognitive decline [Winiewicz et al., 2016], and Alzheimer's disease [Mogi et al., 2008; Tsukuda et al., 2009]. Together with a long terminal elimination half-life in humans (approx. 22–24 h) [Wienen et al., 2000], it is an interesting drug candidate as it could cross the blood-labyrinth barrier.

To conclude, telmisartan is a promising agent that could be used in the future to address sensorineural hearing loss.

Acknowledgment

The authors would like to acknowledge the Microscopy Core Facility of the Department of Biomedicine at the University of Basel for their technical assistance and the Animal Facility of the Department of Biomedicine for its support in animal care.

Statement of Ethics

All animal experiments were performed according to Swiss Animal Welfare laws and following an animal research protocol approved by the Animal Care Committee of the Canton of Basel, Switzerland (Kantonales Veterinäramt Basel, permission No.: 2263).

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Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

The authors have no funding or financial relationships to disclose.

Author Contributions

D.B.: conceptualization. M.C., E.W., and N.J.: acquisition, analysis. M.C., S.L., and D.B.: interpretation. D.B.: resources. D.B. and S.L.: supervision. M.C.: writing.

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