Design, Synthesis, and Biological Evaluation of a New Series of Carvedilol Derivatives That Protect Sensory Hair Cells from Aminoglycoside-Induced Damage by Blocking the Mechanoelectrical Transducer Channel

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ABSTRACT: Aminoglycosides (AGs) are broad-spectrum antibiotics used for the treatment of serious bacterial infections but have use-limiting side effects including irreversible hearing loss. Here, we assessed the otoprotective profile of carvedilol in mouse cochlear cultures and in vivo zebrafish assays and investigated its mechanism of protection, which we found, may be mediated by a block of the hair cell’s mechanoelectrical transducer (MET) channel, the major entry route for the AGs. To understand the full otoprotective potential of carvedilol, a series of 18 analogues were prepared and evaluated for their effect against AG-induced damage as well as their affinity for the MET channel. One derivative was found to confer greater protection than carvedilol itself in cochlear cultures and also to bind more tightly to the MET channel. At higher concentrations, both carvedilol and this derivative were toxic in cochlear cultures but not in zebrafish, suggesting a good therapeutic window under in vivo conditions.

INTRODUCTION

Aminoglycosides (AGs) are broad-spectrum antibiotics widely prescribed to treat severe bacterial infections.1−3 Despite being highly efficacious, they cause unfortunate side effects such as reversible nephrotoxicity and irreversible hearing loss, with the latter occurring in up to 25% of treated patients.4 The AGs can enter the sensory hair cells of the inner ear through both endocytic processes5 and specialized cation channels, the mechanoelectrical transducer (MET) channels that are located at the tips of the stereocilia and are responsible for the detection of sounds and body movements.6−9 The mechanism of ototoxicity is not fully understood and it differs amongst the various AGs, with neomycin and gentamicin, for instance, being shown to activate different cell-death pathways once inside zebrafish lateral line hair cells.10−12 Once inside the cell, they are thought to interact with various targets such as ribosomes, the endoplasmic reticulum, and mitochondria,13,14 leading to the production of cytotoxic levels of reactive oxygen species (ROS) which, in turn, cause apoptosis.15 It is this AG-induced hair cell death that underlies the hearing loss associated with clinical drug treatments. Aside from the redesign of novel AGs15,16,17 or hair cell regeneration approaches,18 methods aimed at preventing this use-limiting side effect have primarily focused on either preventing the entry of AGs into hair cells by identifying blockers of the MET channel14 or by reducing the cellular accumulation of ROS, often by application of antioxidants, in an attempt to prevent the induction of apoptosis.14,19 Recent efforts in this field have led to the identification of otoprotective agents able to reduce or prevent the AG-induced hearing loss both in vitro20−23 and in vivo models of AG ototoxicity.24

Here, we investigated the otoprotective potential of carvedilol, an FDA-approved nonselective α1- and β-adrenergic blocker (Figure 1), used clinically for the treatment of hypertension, angina, and symptomatic chronic heart failure. Carvedilol has previously been reported to protect against

Figure 1. Structure of carvedilol, a nonselective α1- and β-adrenergic blocker.

Received: August 22, 2018
Published: May 14, 2019
neomycin damage in lateral line hair cells of zebrafish when tested at 10 μM against 200 μM neomycin.22 In addition, carvedilol did not abrogate the antimicrobial properties of neomycin, making any interference with the bactericidal activity of AGs unlikely22 and making it an ideal chemical starting point for further investigation.

In this study, we report its protective properties against gentamicin-induced hair cell damage in mouse cochlear cultures. We propose the mechanism by which it offers otoprotection with data supporting the block of the MET channel as being responsible for its otoprotective effect. We synthesized and evaluated a series of novel carvedilol derivatives aimed at improving the protective efficacy and affinity for the MET channel, whilst concurrently reducing toxicity.

■ RESULTS

**Carvedilol Protects Mammalian Sensory Hair Cells from Gentamicin Damage.** Mouse cochlear cultures were used to assess whether carvedilol can protect mammalian hair cells from the death induced by exposure to 5 μM gentamicin for 48 h. A concentration of 5 μM gentamicin is optimal, as it is close to the estimated concentration of 1 μM gentamicin reached in the endolymph in vivo at the onset of ototoxic symptoms,25 and it kills >90% of the basal cells while sparing the apical cells, consistent with the predominantly high-frequency hearing loss observed in patients treated with AGs.3 On an average, incubation with 5 μM gentamicin caused a loss of 86% of outer hair cells (OHCs) from the mid-basal region of the cochlea, with 110 ± 2.2 (n = 26) OHCs present in a 300 μm long segment of the control and only 15 ± 2.2 (n = 26) in the gentamicin-treated culture (p < 0.001) (Figure 2A,B).

![Figure 2](image)

Figure 2. Protective effect of carvedilol against gentamicin-induced damage in a mouse cochlear culture assay. Control cultures exposed to either 0.5% DMSO (A) or 5 μM gentamicin + 0.5% DMSO (B) for 48 h. When coincubated with 5 μM gentamicin, carvedilol was found to be partially protective at 5 μM (C), fully protective at 10 and 20 μM (D,E respectively), and generally cytotoxic at ≥30 μM (F). The asterisk in (E) indicates an example of IHC hair bundle disruption. Scale bar is 50 μm.

Subsequently, mouse cochlear cultures were coincubated with 5 μM gentamicin together with escalating concentrations of carvedilol with the aim to identify the minimal concentration required to provide full protection. When tested at 5 μM, carvedilol provided partial protection, with OHC survival differing significantly from that in both the control and gentamicin-only treated cultures (p < 0.001 in both cases), suggesting that this concentration is at the limit of its protective efficacy (Figure 2C). When tested at 10 and 20 μM, carvedilol provided complete protection against the gentamicin-induced loss of OHCs (p < 0.001) (Figure 2D,E, respectively). Higher concentrations of carvedilol (≥30 μM) proved to be generally cytotoxic, with widespread cell death observed (Figure 2F).

When carvedilol was tested alone, no OHC death was observed at either 10 or 20 μM, with hair cell numbers similar to those observed in controls (Figure 3A–D). Some degree of

![Figure 3](image)

Figure 3. Carvedilol disrupts mechanosensory hair bundles at 20 μM and is generally cytotoxic in vitro at ≥30 μM. Cochlear cultures were exposed for 48 h to 0.5% DMSO (A,E), 5 μM gentamicin + 0.5% DMSO (B), and escalating concentrations of carvedilol: 10 (C), 20 (D,F), 30 (G), and 40 μM (H). Scale bar is 10 (F) and 50 μm (H). Quantification of hair cell survival in a 300 μm long segment of the mid-basal region of cochlear cultures is reported in (I).

inner hair cell (IHC) damage and disruption to hair bundle morphology in both IHCs and OHCs can be observed when tested at 20 μM both alone and in combination with 5 μM gentamicin (Figures 2E and 3D). Figure 3E,F shows confocal images of IHC stereociliary bundles in control conditions (Figure 3E) and after exposure to 20 μM carvedilol (Figure 3F), revealing the full extent of morphological disruption caused by carvedilol.

At concentrations ≥30 μM, carvedilol is generally cytotoxic to all cell types in cochlear cultures, both alone and in the presence of 5 μM gentamicin (Figures 2F and 3G,H). Figure 3I summarizes the quantification of OHC survival through analysis of the mid-basal region of cochlear cultures for both carvedilol alone and also coexposed with gentamicin.

**Carvedilol Exerts Its Otoprotective Effect by Acting as a Permeant Blocker of the Hair Cell’s MET Channel.** Despite a narrow therapeutic window in vitro, we decided to investigate the potential mechanism by which carvedilol...
provides its protective effect. It is well established that AGs can enter hair cells via their MET channels and that block of this channel reduces or prevents their entry into the cells, thereby protecting them from any toxicity resulting from intracellular accumulation. To determine whether carvedilol protects via an interaction with the MET channel, we recorded MET currents from OHCs both before and during extracellular superfusion of carvedilol at concentrations of 1, 3, and 10 μM.

Figure 4A shows an example of the MET currents recorded before (black), during (red), and after (blue) exposure to carvedilol (10 μM) at membrane potentials ranging from −164 to +96 mV. Carvedilol reduces the size of MET currents at all membrane potentials, with this reduction particularly pronounced at intermediate and depolarized potentials. Upon re-exposure to the control solution, the currents recover, indicating a reversible block of the channel. The voltage-dependent block and subsequent recovery of the currents can also be clearly seen from the current–voltage relationships as shown in Figure 4B.

Average normalized current–voltage relationships derived from all cells recorded from at the three different concentrations tested, normalized to the maximum control current recorded at +96 mV for each cell, demonstrated the increase in the block with increasing compound concentration and the voltage-dependence of the block, with the strongest block observed at the intermediate and depolarized potentials (Figure 5A).

Some recovery of the currents can be seen at the extreme depolarized potentials (+96 mV), with even more pronounced recovery at the extreme hyperpolarized potentials (−164 mV). This recovery with hyperpolarization is more evident from the average fractional block curves showing the current during carvedilol superfusion relative to the control current at each membrane potential (Figure 5B). The maximum block is seen at the intermediate membrane potentials for each concentration of carvedilol with some recovery at depolarized potentials and even greater recovery at the extreme hyperpolarized potentials. This recovery at the hyperpolarized potentials is indicative of a permeant blocker with the
compound, positively charged at physiological pH (calculated 
\( pK_a = 8.7 \)), being drawn into the cell by the electrical driving 
force and therefore reducing the block of the channel. Curves 
are fitted to a two barrier—one binding site model of the MET 
channel permeation pathway. Dose−response curves for the 
extracellular block of the MET channels by carvedilol were 
generated at each membrane potential and fitted with eq 1 
described in the Experimental Section. The dose−response 
curves derived from the currents at −164, +16, and +96 mV 
are shown in Figure 5C. The \( K_D \) values range from 6.3 \( \mu \text{M} \) at −164 mV to 0.1 \( \mu \text{M} \) at +16 mV, close to the potential at which 
the block was strongest (+12.8 mV). The \( K_D \) at −84 mV was 
2.0 \( \mu \text{M} \), lower than that previously reported for the AG 
dihydrostreptomycin (DHS), which was found to have a \( K_D \) of 
7.0 \( \mu \text{M} \) at −84 mV in 1.3 mM extracellular Ca\(^{2+}\), indicating 
that carvedilol is a relatively high affinity blocker of the MET 
channel. Figure 5D reports the \( K_D \) and Hill coefficient values 
for each dose−response curve showing the strongest 
interaction near +16 mV. The Hill coefficients ranged from 
0.5 to 1.3, suggesting that two molecules may interact with the 
channel, potentially showing negative cooperativity.\(^{26}\)

These results demonstrate that carvedilol is a relatively high-affinity permeant blocker of the MET channel and consistently 
protects OHCs from AGs damage at 10 and 20 \( \mu \text{M} \). However, 
in vitro, it appears to be cytotoxic at higher concentrations.

**Chemistry.** Driven by these results, we decided to 
investigate the potential of carvedilol as the chemical starting 
point for future drug development. We aimed at enhancing its 
protective effect in mouse cochlear cultures, increasing its 
block of the MET channel current and reducing the 
cytotoxicity observed in vitro. We decided to investigate its 
structure by modifying the carbazole moiety, the anisole ring, 
and the \( \beta \)-hydroxyl amino group. We first synthesized 
compound 2 by substituting the carbazole with anisole. The

\[ \text{Scheme 1}^{a} \]

\[
\begin{align*}
    &\text{OCH}_3 &\text{OCH}_3 &\text{OCH}_3 \\
    &\text{Br} &\text{N} &\text{OCH}_3 \\
    &\text{OCH}_3 &\text{OCH}_3 &\text{OCH}_3 \\
    &\text{OCH}_3 &\text{OCH}_3 &\text{OCH}_3 \\
\end{align*}
\]

\[ \text{Reagents and conditions: (i) 2-(bromomethyl)oxirane, anhydrous K}_2\text{CO}_3, \text{DMF, 70 °C, 6 h; (ii) 2-(2-methoxyphenoxy)ethanamine, ethylene glycol dimethyl ether, 80 °C, 6 h; (iii) 1,2-dibromoethane, NaOH, water, reflux, 3 h; (iv) 2-(2-methoxyphenoxy)ethanamine, TEA, THF, 65 °C, 6 h.} \]

\[ \text{Scheme 2}^{a} \]

\[
\begin{align*}
    &\text{O} &\text{N} &\text{OCH}_3 \\
    &\text{N} &\text{OCH}_3 \\
    &\text{O} &\text{N} &\text{OCH}_3 \\
    &\text{N} &\text{OCH}_3 \\
\end{align*}
\]

\[ \text{Reagents and conditions: (i) 2-(bromomethyl)oxirane, NaOH, water, DMSO, 45 °C, 16 h; (ii) 2-phenoxyethanamine, ethylene glycol dimethyl ether, 80 °C, 24 h; (iii) butan-1-amine, ethylene glycol dimethyl ether, 80 °C, sealed tube, 24 h; (iv) phenylpiperazine, ethanol, 65 °C, 16 h; (v) 4, ethylene glycol dimethyl ether, 80 °C, sealed tube, 48 h.} \]
synthesis involved the preparation of compound 1 by coupling 2-methoxyphenol and 2-(bromomethyl)oxirane followed by epoxide ring opening with commercially available 2-(2-methoxyphenoxy)ethanamine in ethylene glycol dimethyl ether at 80 °C, providing the desired compound in moderate yield (Scheme 1).

In parallel, to simplify the structure of carvedilol and likely removing its interactions with adrenergic receptors, we investigated the requirement for the β-hydroxyl amino group by evaluating compound 4, which was prepared by coupling 2-(2-methoxyphenoxy)ethanamine and 3, which was previously made by reacting 2-methoxyphenol with 1,2-dibromoethane.

We then investigated the role of the anisole ring by simplifying to a simple phenol and an aliphatic chain; the role of the basic center by inserting a cyclic tertiary amine (phenyl-piperazinyl) and a tertiary amine bearing two 2-(2-methoxyphenoxy)ethanamine moieties. These compounds are prepared starting from the common intermediate, which is prepared by reacting 4-hydroxycarbazole with 2-(bromomethyl)oxirane in good yield. The epoxide ring is then opened by the appropriate amine using ethylene glycol dimethyl ether as solvent for 6, 7, and 9 in moderate to good yield, while in the case of 8, ethanol was used as solvent, affording the desired compound in 80% yield (Scheme 2).
We then investigated the role of the hydroxyl group by preparation of 13, the carbazole-amine linker 14, removing the β-hydroxyl amine by linking the carbazole and the anisole rings via a six carbon chain 15 and removing the basic center by the introduction of an amidic bond 19. Based on the biological data obtained with compounds 9 and 13, we combined these modifications to make compound 16. For the synthesis, 4-hydroxycarbazole was reacted with either 1,3-dibromopropane, 1,4-dibromobutane, or 1,6-dibromohexane to give compounds 10–12, respectively. Compounds 10 and 11 were coupled with 2-(2-methoxyphenoxy)ethanamine, yielding 13 and 14, while compound 12 was coupled with 2-methoxynaphenol to give 15 in moderate yield. Compound 16 was obtained by coupling 10 with 4. For the preparation of the amide analogue 19, 4-hydroxycarbazole was reacted with ethyl chloroacetate to afford ester 17, which was hydrolyzed with aqueous sodium hydroxide providing acid 18. Coupling with 2-(2-methoxyphenoxy)ethanamine was performed under standard amide coupling conditions using 1-hydroxybenzotriazole (HOBt) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) affording the desired compound 19 in good yield (Scheme 3).

We then investigated two isomers of carvedilol: 2-hydroxy carbefedilol derivative 21, which was obtained by opening the epoxide ring of compound 20 with 2-(2-methoxyphenoxy)ethanamine, providing the desired compound 21 in moderate yield (Scheme 4); and the 1-carbazole isomer by directly linking the side chain to the nitrogen of the carbazole ring. First, carbazole reacted with epichlorohydrin to yield compound 22 followed by epoxide ring opening using 2-phenylethylamine, obtaining compound 23 in 49% yield.

Finally, to improve the solubility of compound 9, we designed compound 25 in which one of the 2-methoxyphenoxy group was substituted with a morpholine ring. Compound 3 was reacted with commercially available 4-(2-aminoethyl)-morpholine to obtain compound 24 which was then coupled with 5 to give the desired compound 25 in 29% yield.

**Carvedilol Derivatives: Protective Abilities and MET Channel Block.** The newly synthesized compounds were screened to assess their protective ability against 5 μM gentamicin using mouse cochlear cultures initially at 20 μM, a concentration at which carvedilol provided full protection and was not cytotoxic. Any compounds that showed full or partial protection were then screened at lower concentrations to establish their minimal protective concentration. We initially designed and synthesized 6 compounds aimed at evaluating the chemical moiety essential for the interaction with the MET channel, and these include modification at the carbazole (2 and 4) and anisole (6 and 7) rings, the removal of the hydroxyl group (13), and the introduction of a second 2-phenoxyethyl moiety to form a tertiary amine (9). When tested at 20 μM (Figure 6), compounds 2 and 4 lacking the carbazole moiety provided no protection (Figure 6C,D), while compound 6 with a phenyl group instead of the anisole proved to be generally cytotoxic (Figure 6E). Compound 9, bearing two 2-methoxyphenoxymethyl moieties, offered only partial protection (Figure 6G), while compounds 7 and 13 consistently protected against OHC loss (Figure 6F,H). As a reference, control cochlear cultures exposed to 0.5% dimethyl sulfoxide (DMSO, Figure 6A) and 5 μM gentamicin (Figure 6B) as well as quantification for both controls and cultures exposed to compounds (Figure 6I) are included.

The three derivatives (7, 9, and 13) that showed partial or full protection at 20 μM were subsequently tested at 10 and 5 μM against 5 μM gentamicin. Only compound 13 provided full protection at 5 μM (as well as at 10 μM), showing an improvement compared to the parent compound carvedilol which was only partially protective at 5 μM (Figure 7A–E). As stated before, in the control condition, some 110 OHCs were present in each cochlear segment, reducing to 15 OHCs following exposure to 5 μM gentamicin. Additional exposure to 5 μM carvedilol increased the average number of surviving OHCs to 66 (54% protection), whereas 5 μM compound 13 resulted in 99 surviving OHCs (88% protection) (Figure 7E).

When tested alone, 13 showed similar toxicity characteristics to carvedilol, proving toxic to OHCs at concentrations ≥ 30 μM and affecting the IHC and OHC bundle morphology at 20 μM. Contrary to carvedilol, 13 was not toxic at 30 μM when tested together with 5 μM gentamicin, but it also did affect the IHC and OHC bundle morphology (data not shown).

In conjunction with assessing the protective abilities of these six compounds, potential interactions with the MET channel were investigated by recording MET currents from OHCs before and during exposure to 10 μM of each compound. Figure 8 shows the fractional block of the currents during compound exposure relative to the control currents at each membrane potential, revealing that two compounds 4

**Figure 6. Otoprotective effect of compounds 2, 4, 6, 7, 9, and 13 in cochlear cultures against 5 μM gentamicin.** (A) Control culture exposed to 0.5% DMSO for 48 h. (B) Culture exposed to 5 μM gentamicin + 0.5% DMSO for 48 h. (C−H) Cultures exposed to 5 μM gentamicin for 48 h + 20 μM: (C) 2, (D) 4, (E) 6, (F) 7, (G) 9, and (H) 13. Scale bar is 50 μm. (I) Quantification of OHC survival for the control and compound exposed cultures.
(magenta) and 2 (blue) have limited interaction with the channel at this concentration.

The poor interaction of compounds 2 and 4 with the MET channel corresponded to the lack of protection observed for these compounds at 20 μM, providing further evidence that the protection offered by carvedilol may come through a block of the MET channel. In addition, these results indicate the need for the carbazole moiety to allow the interaction with the channel. Compound 9 (bright green), which offered only a partial protection at 20 μM, showed approximately 40% block of the MET current at all membrane potentials with no release at extreme hyperpolarized potentials, suggesting this compound may act as a non-permeant blocker. Compounds 6 (red) and 7 (orange) were strong MET channel blockers at 10 μM with a blocking profile similar to carvedilol giving approximately 50–60% block of the current at −164 mV, the most relevant physiological potential. Interestingly, compound 13 (dark green) was the most effective MET channel blocker of this series, providing almost 100% block of the MET current at all membrane potentials. This result is also in accordance with its protective effect, as 13 showed protection of the OHCs from gentamicin damage at concentrations down to 5 μM. This result again suggests that the protection observed is due to a block of the MET channels, reducing the entry of gentamicin into the cells. Compounds 6, 7, and 13 appear to be permeant blockers of the MET channel, indicated by the release of the block at the extreme hyperpolarized potentials, and in the case of compound 13, the release of the block was less pronounced than with carvedilol itself.

A further seven compounds (8, 14, 15, 19, 21, 23, and 25) were then designed and synthesized in an attempt to improve protective abilities and physicochemical properties. Of these, 14, 15, 21, and 23 were generally cytotoxic when tested at 20 μM against 5 μM gentamicin (Figure 9D,E,H,I, respectively), 19 and 8 offered no protection (Figure 9C,G, respectively), and 25 was consistently protective (Figure 9J). When tested at 10 μM, 25 was protective in 2 out of 4 screens but offered no protection at 5 μM (data not shown). MET channel interactions were subsequently investigated for derivative 25 that showed protection at 20 μM and partial protection at 10
μM. The resulting fractional block plot reveals that at a concentration of 10 μM, 25 blocks the MET channel at all membrane potentials (Figure 10). However, the degree of the block is far less than that observed for carvedilol and 13 suggesting this derivative has a reduced affinity for the channel.

Combining the desired characteristics of a nonpermeant MET channel blocker (9) with a high-affinity blocker (13), we designed, synthesized, and tested a further derivative (16). This compound was found fully protective at 20 μM against 5 μM gentamicin on only one out of three occasions (Figure 9F), with no protection in the other two trials. The lack of consistency with the data was probably caused by the poor solubility of the compound. When tested at 10 μM, compound 16 did not offer any protection (data not shown). When tested in our electrophysiology assay, compound 16 showed limited interaction with the MET channel at both 3 and 10 μM (data not shown).

Assessing the Protective Effect and MET Channel Properties of Adrenergic Receptor and Calcium Channel Blockers. We then proceeded to investigate whether the adrenergic (α and β) receptors, primary pharmacological targets of carvedilol, play a role in its otoprotective abilities. We tested the nonselective β-blocker propranolol 26, the selective β₁-blocker CGP20712 27, the selective α₁-blocker naftopidil 28, and the nonselective adrenergic blocker and calcium channel-blocker verapamil 29 (Figure 11).

Compounds 26, 27, and 29 did not offer any protection against 5 μM gentamicin when tested at 20 μM (Figure 13C,D,F, respectively), while 28 proved to be partially effective under the same conditions (Figure 12E,G). However, when the test concentration was lowered to 10 μM, compound 28 did not protect cochlear culture hair cells against gentamicin damage (data not shown).

We subsequently investigated the affinity of the partially protective compound 28 for the MET channel. This compound did not interact strongly with the MET channel which is revealed from the fractional block plot shown in Figure 13.

Electrophysiological Properties of Compound 13: A Strong Permeant MET Channel Blocker. The cochlear culture protection assay revealed that carvedilol derivative 13, at a concentration of 5 μM, displayed a consistent protective effect against 5 μM gentamicin. When tested on its own,
compound 13 appears to be cytotoxic at 30 μM. However, when tested in the presence of 5 μM gentamicin, compound 13 is not toxic, suggesting a competitive interaction at the level of the MET channel that may effectively reduce the cytotoxic effects of both compounds. In order to compare MET channel interactions between compound 13 and carvedilol, MET currents were recorded before and during 13 exposure at 1, 3, and 10 μM and the resulting current–voltage relationships, fractional block curves, and dose–response curves generated (Figure 14).

From the average normalized current–voltage relationships (Figure 14A) and the fitted average fractional block curves (Figure 14B) it can be seen that the block of the channel by 13 is very similar to that of carvedilol, with the maximum block seen at the intermediate potentials and release of the block at extreme depolarized and hyperpolarized potentials, indicating that this compound is also a permeant blocker of the MET channel. Dose–response curves were generated, derived from the currents at each membrane potential, and fitted with eq 1 (see Experimental Section). Figure 14C shows the curves derived from the currents at −164, +16, and +96 mV, where the $K_D$ values were found to be 4.6, 1.0, and 1.7 μM, respectively. The $K_D$ at −84 mV (2.4 μM) is similar to that of carvedilol (2.0 μM), suggesting that both compounds are relatively high affinity blockers of the MET channel at a potential close to the resting potential in vitro. The Hill coefficients ranged from 1.1 to 2.0, suggesting there may be two or more binding sites within the channel for 13 (Figure 14D).

Kinetics of MET Channel Block for Carvedilol and Compound 13. One further property of the MET channel interaction that was investigated for both carvedilol and 13 was the kinetics of the block, to determine whether or not these compounds are open-channel blockers, similar to berbamine and d-tubocurarine, or can reside in the closed channel, similar to the permeant MET channel blocker FM1-43. The time course of the block is revealed by applying large force steps to the hair bundles both before and during exposure to the compound and recording the resulting currents. Such currents can be seen before and during exposure to carvedilol (1 and 3 μM; Figure 15A,B) and 13 (1 and 3 μM; Figure 15C,D). From a holding potential of −84 mV, channel opening results in rapidly activating inward currents in all conditions. During the step, the currents show minimal adaptation in control conditions and an exponential decline during both carvedilol and 13 exposure. This suggests that both compounds are open-channel blockers, accessing their binding site once the channel has opened. Time constants were measured from the current decline and found to be 18.0 ± 5.0 ms (1 μM carvedilol; $n = 3$); 9.6 ± 0.4 ms (3 μM carvedilol; $n = 4$); 9.9 ± 0.9 ms (1 μM 13; $n = 3$); and 6.0 ± 1.6 ms (3 μM 13; $n = 3$).

![Figure 13](image1.png)  
**Figure 13.** Fractional block plot for compound 28. The size of the current during exposure to the compound relative to the control current at each membrane potential reveals that compound 28 has a low affinity for the MET channel.

![Figure 14](image2.png)  
**Figure 14.** Compound 13 acts as a permeant blocker of the MET channel, with similar blocking properties to carvedilol. (A) Average normalized current–voltage relationships for the peak MET currents recorded before and during exposure to 1, 3, and 10 μM 13. (B) Fractional block curves for compound 13 tested at 1, 3, and 10 μM reveal that it is a permeant MET channel blocker. Fitting parameters are $\Delta E = −2.22 kT$; $E_c = −21.3 kT$; $\delta_1 = 0.71$; $z = 1.0$; $\delta_3 = 0.71$; Hill coefficient 1.4. Maximum block occurs at +33.9 mV. (C) Dose–response curves derived from the currents recorded at −164, +16, and +96 mV and fit with eq 1. −164 mV: $K_D = 4.6 \mu M$, Hill coefficient 1.6; +16 mV: $K_D = 1.0 \mu M$, Hill coefficient 2.0; +96 mV: $K_D = 1.7 \mu M$, Hill coefficient 1.4. (D) Half-blocking concentration and Hill coefficient as a function of membrane potential.
Figure 15. Kinetics of MET channel block mediated by carvedilol and 13 reveals that both act as open-channel blockers. (A–D) Currents resulting from a mechanical step delivered by the fluid jet (±40 V driver voltage, DV shown above each trace), from a holding potential of −84 mV, before (black trace) and during (red trace) superfusion of (A) 1 μM carvedilol, (B) 3 μM carvedilol, (C) 1 μM 13, and (D) 3 μM 13. Currents (averaged from 10 repetitions) before and during compound exposure have been scaled and superimposed. The currents during compound superfusion were fitted with single exponentials (A) τ = 19.3 ms, (B) τ = 8.6 ms, (C) τ = 10.1 ms, and (D) τ = 6.9 ms. From these time constants, entry rates of the drug molecules into the hair cells were calculated (see the Experimental Section), and their energy profiles for permeation through the MET channel pore were determined (Figure 16). Both compounds bind much stronger (free energy E_b < −11.5 kT) to the binding site in the channel pore than DHS (E_b = −8.27 kT at 1.3 mM extracellular Ca^{2+}) and the drugs D-tubocurarine (−12.0 kT) and berbamine (−12.0 kT) that we evaluated before. A consequence of this is that, unlike the other compounds, the maximum block for these monovalent cations (at physiological pH) occurs at positive membrane potentials (Figures S5B and 14B). Moreover, their permeation through the MET channels is considerably slower than DHS. For example, with 1 μM of compound, 80 MET channels with an open probability of 0.1 and a membrane potential of −150 mV, the entry rates into the OHCs are 165 molecules/s for carvedilol and 125 molecules/s for 13, compared with some 1130 molecules/s for DHS. For higher concentrations, the entry rates started to saturate, so they never approach those for DHS (e.g., for 100 μM, rates were 1078 molecules/s for carvedilol, 998 molecules/s for 13, and 11 460 molecules/s for DHS).

Carvedilol and Compound 13 Reduce GTTR Loading into Hair Cells. To further assess whether carvedilol and 13 protect sensory hair cells against AG damage by preventing the entry of the antibiotics into cells, a fluorescent gentamicin analogue (gentamicin Texas Red: GTTR) was used to enable quantification of gentamicin uptake. Pre-incubation with either 1% DMSO, 100 μM carvedilol or 100 μM 13 for 5 min prior to 0.2 μM GTTR application resulted in significantly reduced loading of GTTR in the presence of both carvedilol and 13 relative to the DMSO control (p < 0.001 in both cases) (Figure 17). A significant difference was not observed between the GTTR loading in the presence of carvedilol or 13. These findings further suggest that both carvedilol and 13 protect against AG damage by competitively blocking the MET channel and thereby preventing AG entry into hair cells, minimizing accumulation and consequent apoptosis induction.

Comparison of Protection and Toxicity of Carvedilol with Compound 13 in Zebrafish Larvae. In order to compare the protective effect of 13 to that of carvedilol in vivo, 4 days post fertilization (dpf) zebrafish larvae were treated with either neomycin or gentamicin in the presence of each compound, and the number of remaining hair cells was assessed (Figure S1A–D). Dose–response curves for carvedilol and compound 13 were constructed, and EC_{50}s (the effective concentration at which 50% of hair cells survive) were derived. The EC_{50}s for carvedilol and 13 protection against neomycin damage were 2.47 and 2.08 μM, respectively. The EC_{50} for carvedilol protection against gentamicin damage was 10.95 μM, whilst for compound 13, it was 10.81 μM. The protective effect of these compounds therefore extends to other AGs in addition to gentamicin.

We then assessed the toxicity of carvedilol and compound 13 by treating 3 dpf zebrafish larvae with each at 30 and 100 μM for 48 h (see the Supporting Information Methods). At a concentration of 100 μM, carvedilol killed the larvae in two out of three trials, whilst it was not toxic at 30 μM. With compound 13, neither concentration was toxic for the larvae. Larvae treated with 100 μM carvedilol had slowed or no circulation, while those treated with 30 μM of carvedilol or either concentration of compound 13 had no obvious defects in circulation. Treated larvae were startled and assayed for movement. As expected, larvae treated with 100 μM carvedilol showed reduced movement, whilst those treated with 100 μM of compound 13 showed increased movement compared to the control.

Assessing Effects of Carvedilol and Compound 13 on the Antimicrobial Efficacy of Gentamicin. Carvedilol and compound 13 were tested in a bacterial cell viability assay to identify if either of these compounds decreased the antimicrobial activity of gentamicin. The minimum inhibitory
compounds. (B) In a control culture pretreated with 1% DMSO before the addition of 0.2 μM GTTR, compared to cultures pretreated with 100 μM carvedilol or 13. Both compounds significantly reduced GTTR loading (p < 0.001). No significant difference in reduction was seen between the two compounds. (B–D) Representative fluorescence image from which intensity values were measured and a DIC image for (B,B') the control (C,C') carvedilol and (D,D') 13. Asterisks indicate the first row of OHCs, from which fluorescence intensity values were taken. N = 30 cells, with 10 cells analyzed from three separate experiments. Scale bar is 50 μm.

**DISCUSSION AND CONCLUSIONS**

The nonselective α- and β-adrenergic blocker carvedilol was reported to provide protection against AG damage in a whole zebrafish larval model study. Here, we investigated its potential using a mammalian system of AG-induced toxicity, mouse cochlear cultures, and zebrafish larvae. In parallel, we investigated its potential molecular target by studying its electrophysiological profile. First, we determined that carvedilol (10 and 20 μM) was able to fully protect cochlear cultures against the damage caused by exposure to 5 μM gentamicin for 48 h. Unfortunately, carvedilol proved to be cytotoxic at higher concentrations (≥30 μM) and caused severe damage to the mechanosensory hair bundles when tested at 20 μM, both alone and together with gentamicin. Despite this, we decided to investigate further the mode of protection of carvedilol by studying its potential interaction with the MET channel, which is the main entry route of AGs into hair cells. From our experiments, carvedilol proved to be a relatively high-affinity, permeant, and reversible blocker of the MET current, and at least the same protective effect against gentamicin damage compared to carvedilol. These data show that the hydroxyl group is not needed for the interaction with the channel, and we can postulate that the enhanced block of the channel may be derived by an increased basicity of the nitrogen, with a calculated pKᵢ for compound 13 and carvedilol being 9.3 and 8.7, respectively, which may lead to a stronger interaction with the channel. To our surprise, the extension of just one extra carbon in the alkyl chain spacer between the carbazole and the basic center, compound 14, proved to be detrimental as the compound was found to be more toxic than the parent compound. The role of the basic center was investigated by substituting the nitrogen with a carbon (15) or by making the nitrogen nonbasic (19). Neither of these compounds had any protective effect, supporting the need for a basic center that is positively charged at physiological pH for the protective effect. Also unsuccessful was the conversion of the secondary amine into a tertiary cyclic amine, as compound 8 did not have any protective effect against gentamicin damage. In compound 9, we inserted a second ethyl-anisole moiety, and it was found to interact tightly with the channel and to act as a nonpermeant blocker as noted by the lack of the release of the block at the extreme hyperpolarized (−164 mV) potential, probably due to some new interactions between the channel and the second anisole moiety. However, the level of the block of the MET current was reduced if compared to carvedilol, and it was only partially protective.

We then investigated two isomers of carvedilol moving the side chain to the 2-position (21) or linking it to the nitrogen in the carbazole ring (23); both compounds were found to be toxic to the cochlear cultures. Driven by the results obtained with compounds 13 (improved protective effect and block of the MET current) and 9 (nonpermeant MET channel blocker), we designed compound 16, which lacks the hydroxyl group as in compound 13 and has the second ethyl-anisole as in compound 9. Compound 16 proved to be only partially protective, but we noticed a lack of consistency during the assays which is probably due to the poor solubility of this compound in the biological media, preventing any meaningful interpretation of the results. Whilst we solved the solubility issue for this compound by substituting the second ethyl-
anisole moiety with an ethyl morpholine 25, this did not translate into an increased protective effect. We then investigated other adrenergic and calcium blockers, with compounds 26, 27, and 29 (propranolol, CGP20712, and verapamil, respectively) not showing any protective effect, and 28 (naftopidil) only offering partial protection at 20 μM. Interestingly, 28 is the only compound that showed some protection but it does not have the carbazole moiety and bears a naphthol ring. Based on our previous findings showing D-tubocurarine as a potent blocker of the MET channel current (K_D = 2.2 μM) without showing any toxicity at a higher concentration (50 μM),20 the cytotoxic effect observed with some of these compounds is unlikely to be related to their ability to block the MET channel and is probably series related. A hit-to-lead optimization campaign will be focused on increasing the protective effect, reducing the toxicity, and addressing any adrenergic effect of these compounds. In addition, there will be the possibility of formulating the potential otoprotective agent to allow administration into the inner ear via transtympanic injection.

From a mechanistic point of view, we investigated in more detail the interaction of 13, the most protective derivative, with the MET channel. This compound showed a very similar interaction with the channel to carvedilol, having a K_D of 4.6 μM at a potential of −164 mV. Kinetics of the MET channel block showed that both carvedilol and 13 are open-channel blockers, suggesting that they are able to interact with the channel only when it is open. As shown in the energy profiles graph, both carvedilol and 13 bind tighter to the negatively charged vestibule of the MET channel compared to the AG DHS with a longer time consequently spent inside the channel. This result reflects on the rate of entry into the cells for these compounds, which is considerably lower (10 fold) compared to DHS. This stronger interaction with the MET channel may be behind the protective effect observed by carvedilol and 13 which hinders the interaction of AG with the channel and as a consequence reducing its entry into the hair cells. The stronger protection of 13 (E_fl = −21.3kT) compared to carvedilol may be due to its binding inside the channel pore more strongly than carvedilol (E_fl = −15.4kT) (Figure 16). Both carvedilol and compound 13 were able to block the loading of GTTR into the hair cells, further supporting the notion that the protective effect of both compounds is due to their block of the MET channel and the prevention of AG uptake into hair cells.

Finally, to exclude a protective effect specific to gentamicin, we compared the protection of carvedilol and compound 13 against neomycin using hair cells in the lateral line organs of zebrafish larvae. Both compounds were protective against neomycin damage at a concentration of ≥12.5 μM, and found to be slightly less effective when tested against gentamicin, providing full protection at a concentration ≥ 25 μM. In addition, neither carvedilol nor compound 13 interfered with gentamicin antimicrobial activity.

In conclusion, we established that carvedilol is able to protect cochlear cultures from AG-induced damage, although it is also cytotoxic in vitro at higher concentrations. We have established a clear SAR identifying the need for a carbazole moiety, a basic center, and preferentially an anisole moiety. The toxicity observed in vitro with cochlear cultures may not be an issue as carvedilol is widely used in the clinic and is not associated with hearing loss. Furthermore, carvedilol and its derivatives did not show a toxic effect in vivo with zebrafish larvae at 30 μM. Although the “therapeutic window” is narrow in vitro, our current data show that carvedilol and its derivatives are a valid chemical starting point for the future development of drugs that will prevent AGs induced ototoxicity, and that the MET channel is a potential target for such compounds.

**EXPERIMENTAL SECTION**

All commercial reagents were purchased from Sigma-Aldrich, Alfa Aesar, Apollo Scientific, Fluorochem or Tokyo Chemical Industry and of the highest available purity. Unless otherwise stated, chemicals were used as supplied without further purification. Anhydrous solvents were purchased from Acros (AcroSeal) or Sigma-Aldrich (SureSeal) and were stored under nitrogen. Petroleum ether refers to the fraction with a boiling point between 40 and 60 °C. Thin-layer chromatography: precoated aluminum-backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short- and long-wave UV light (254 and 366 nm). Flash column chromatography was carried out using commercial prepacked columns from Biotage, Isco, Grace, or filled with Merck silica gel 60 (40–63 μm) or C18 silica on an ISCO Combiflash Rf or a Biotage Isolera Prime. HPLC purification was performed on an Agilent 1100 series HPLC spectrometer, using a Phenomenex Luna 10 μm C18 150 mm x 15 mm column, eluted using water and acetonitrile at 15 mL/min and detected at 254 nm.

Proton nuclear magnetic resonance spectra were recorded at 500 or 600 MHz on a Varian VNMRS 500 MHz or Varian VNMRS 600 MHz spectrometers, respectively (at 30 °C), using residual isotopic solvent (CDCl₃, δ = 7.27 ppm, DMSO δ = 2.50 ppm) as an internal reference. Chemical shifts are quoted in parts per million (ppm). Coupling constants (J) are recorded in hertz (Hz). The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), and dt (doublet of triplet). Carbon nuclear magnetic resonance spectra were recorded at 125 or 151 MHz on Varian 500 or 600 MHz spectrometers, respectively, and are proton-decoupled, using residual isotopic solvent (CDCl₃ δ = 77.00 ppm, DMSO δ = 39.52 ppm) as an internal reference.

LCMS data were recorded on a Waters 2695 HPLC using a Waters 2487 UV detector and a Thermo LCQ ESI-MS. Samples were eluted through a Phenomenex Luna 3 μm C18 50 mm × 4.6 mm column, using water and acetonitrile acidified by 0.1% formic acid at 1 mL/min and detected at 254 nm. The following methods were used: method 1: water (+0.1% formic acid)/acetonitrile (+0.1% formic acid) = from 65/35 to 10/90 in 3.5 min, then isocratic 10/90 0.4 min, then from 10/90 to 65/35 in 0.1 min; method 2: water (+0.1% formic acid)/acetonitrile (+0.1% formic acid) = from 70/30 to 10/90 in 5 min, then isocratic 10/90 1.0 min, then from 10/90 to 70/30 in 0.5 min, and then isocratic 70/30 for 0.5 min.

LCMS (MDAP): LCMS data were recorded on a Shimatzu Prominence Series coupled to a LCMS-2020 ESI and APIQ mass spectrometer. Samples were eluted through a Phenomenex Gemini 5 μm C18 110A 250 mm × 4.6 mm column, using water and acetonitrile acidified by 0.1% formic acid at 1 mL/min and detected at 254 nm. The following method, marked as method 3, was used: water (+0.1% formic acid)/acetonitrile (+0.1% formic acid) = isocratic 95/5 1 min, then from 95/5 to 5/95 in 20 min, then isocratic 5/95 for 4 min, and then from 5/95 to 70/30 in 5 min.

Physicochemical properties were calculated using MarvinSketch 16.8.1.5.0 by ChemAxon (https://www.chemaxon.com).

Compound purity was assured by a combination of high-field multinuclear NMR (H, C) and HP LC/MS; purity by the later was always >95%.

**Chemistry. Synthesis of 2-((2-Methoxyphenoxo)methyl)oxirane (I).** A mixture of 2-methoxyphenol (1.0 g, 8.00 mmol), 2- (bromomethyl)oxirane (0.69 mL, 6.06 mmol), and anhydrous K₂CO₃ (2.23 g, 16.11 mmol) in dimethylformamide (DMF, 5 mL) was stirred at 70 °C for 6 h. After cooling, the solvent was evaporated under reduced pressure, the residue was dissolved in water, and the aqueous phase was extracted with ethyl acetate. The organic phase
was washed with saturated NaHCO₃ solution, dried over MgSO₄, and concentrated. The residue was then purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 100/100) to give 1 as a yellow gum which crystallized at room temperature to give 0.90 g, 62%.1H NMR (DMSO 600 MHz): δ 7.61 (t, J = 7.8 Hz, 1H), 7.44 (d, J = 8.0 Hz, 1H), 7.32 (d, J = 8.2, 7.1 Hz, 1H), 7.28 (t, J = 8.0 Hz, 1H), 7.17–7.11 (m, 1H), 7.08 (d, J = 8.0 Hz, 1H), 6.68 (d, J = 7.9 Hz, 1H), 6.52 (dd, J = 6.5, 4.5 Hz, 1H), 2.92 (t, J = 4.7 Hz, 1H), 2.83 (dd, J = 5.1, 2.7 Hz, 1H).13C NMR (DMSO 151 MHz): δ 154.93, 141.56, 139.39, 126.89, 125.10, 122.72, 122.00, 119.09, 111.93, 110.91, 104.68, 101.14, 69.21, 50.38, 44.26. LCMS: method 2: RT: 3.65 min; M + H+: 240.16.

Synthesis of 1-(9H-Carbazol-4-yl)-3-(2-phenoxyethylaminoo)-prop-2-ol (6). A solution of 2-phenoxyethanamine (0.22 mL, 1.67 mmol) in ethylene glycol dimethyl ether (0.75 mL) was heated at 80 °C for further 1 h. After cooling, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 100/100) to give the desired compound which was further purified by reverse phase HPLC gradient elution of water/acetonitrile = 95:5 to 100:0 in 20 min to give 6 as an off-white solid (0.085 g, 54%). 1H NMR (DMSO 600 MHz): δ 12.11 (s, 1H), 8.20 (d, J = 7.8 Hz, 1H), 7.42 (d, J = 8.0 Hz, 1H), 7.34–7.28 (m, 1H), 7.28–7.24 (m, 1H), 7.10 (g, J = 7.4 Hz, 1H), 7.05 (g, J = 8.0 Hz, 1H), 6.89 (dd, J = 7.9, 6.3 Hz, 3H), 6.66 (d, J = 7.9 Hz, 1H), 5.13 (d, J = 4.9 Hz, 1H), 4.19–4.11 (m, 2H), 4.11–4.06 (m, 1H), 4.01 (t, J = 5.6, 2H), 2.93 (td, J = 5.5, 2H), 2.81 (dd, J = 11.9, 6.8 Hz, 1H), 2.01 (s, 3H).13C NMR (DMSO 151 MHz): δ 159.02, 155.40, 141.53, 139.34, 129.88, 126.92, 124.94, 122.90, 122.17, 119.00, 114.86, 112.00, 110.77, 104.25, 100.87, 70.69, 68.91, 67.86, 53.08, 48.90. LCMS: method 2: RT: 1.53 min; M + H+: 377.19.

Synthesis of 1-(Butylamino)-3-(9H-carbazol-4-yloxy)propan-2-ol (7). A solution of butan-1-amine (0.50 mL, 5.02 mmol) in ethylene glycol dimethyl ether (0.50 mL) was heated at 80 °C for 16 h. The reaction solvent was then removed under reduced pressure, and the residue was left overnight, continuing the reaction with no solvent. The residue was then purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 100/100) to give 7 as a yellow gum, which was further purified by reverse phase HPLC gradient elution of water/acetonitrile = 95:5 to 100:0 in 20 min to give 7 as a pale yellow solid (0.055 g, 35%). 1H NMR (DMSO 600 MHz): δ 7.14 (s, 1H), 8.31 (d, J = 7.7 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 7.35–7.30 (m, 1H), 7.28 (t, J = 8.0 Hz, 1H), 7.12 (t, J = 7.4 Hz, 1H), 7.06 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 7.9 Hz, 1H), 5.04 (br s, 1H), 4.18–4.11 (m, 2H), 4.09–4.04 (m, 1H), 2.83 (dd, J = 11.9, 4.8 Hz, 1H), 2.73 (dd, J = 11.9, 6.8 Hz, 1H), 2.55 (dt, J = 6.9, 2.8 Hz, 2H), 1.47–1.35 (m, 2H), 1.35–1.25 (m, 2H), 0.85 (t, J = 7.3 Hz, 3H).13C NMR (DMSO 126 MHz): δ 155.47, 141.59, 139.40, 126.90, 124.94, 122.90, 122.23, 118.94, 110.78, 109.99, 104.25, 100.95, 71.05, 68.84, 53.19, 49.61, 32.28, 20.39, 14.36. LCMS: method 2: RT: 0.69 min; M + H+: 313.35.

Synthesis of 1-(9H-Carbazol-4-yl)-3-(4-(phenylpiperazin-1-yl)-prop-2-ol (8). A mixture of 5 (0.20 g, 0.84 mmol) and phenylpiperazine (0.13 mL, 0.84 mmol) in ethanol (25 mL) was stirred at 65 °C for 16 h. The reaction solvent was then removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 100/100) to give 8 as a colorless oil which crystallized on standing (0.28 g, 80%).1H NMR (CDCl3 500 MHz): δ 8.29 (d, J = 7.8 Hz, 1H), 7.89 (s, J = 11.9 Hz), 7.74–7.77 (m, 1H), 7.71–7.67 (m, 1H), 7.62–7.59 (m, 1H), 7.58 (t, J = 8.0 Hz, 1H), 6.95 (d, J = 8.1, 2H), 6.88 (t, J = 7.3 Hz, 1H), 6.69 (d, J = 8.0 Hz, 1H), 4.42–4.49 (m, 2H), 4.28–4.23 (m, 1H), 3.32–3.19 (m, 1H), 2.95–2.87 (m, 1H), 2.73–2.65 (m, 2H).13C NMR (CDCl3 126 MHz): δ 151.17, 150.82, 144.11, 138.69, 129.13, 126.66, 125.01, 122.93, 119.88, 116.97, 117.73, 116.15.
Synthesis of 1-Bis[2-(2-methoxyphenoxy)ethyl]aminio]-3-(9H-carbazol-4-yl)oxy)prop-2-ol (9). A solution of 4 (0.13 g, 0.42 mmol) in ethylene glycol dimethyl ether (0.50 mL) was heated in a sealed tube at 80 °C. A solution of 5 (0.10 g, 0.42 mmol) in ethylene glycol dimethyl ether (0.50 mL) was added dropwise, and the reaction was heated at 80 °C for 5 h. Then, 4 (0.13 g, 0.42 mmol) was added, and the reaction mixture was stirred at 80 °C for 24 h. Then, a solution of 4 (0.07 g, 0.21 mmol) in ethylene glycol dimethyl ether (0.50 mL) was added, and the reaction mixture was stirred at 80 °C for further 18 h. After cooling, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 20/100) to give the desired compound as a yellow oil which was triturated with methanol/aceton to give 9 as a colorless solid (0.12 g, 52%).

Synthesis of 3-(9H-Carbazol-4-yl)oxy)-N-[2-(2-methoxyphenoxy)ethyl]butan-1-amine (14). A solution of 11 (0.10 g, 0.33 mmol), 2-(2-methoxyphenoxy)ethanamine (0.13 g, 0.79 mmol) and KOH (0.046 g, 0.82 mmol) were added, and the stirring was continued for further 3 h. The solvent was then removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 20/100) to give the desired compound as a yellow oil which was triturated with methanol/aceton to give 14 as a colorless solid (0.12 g, 32%).

Synthesis of 3-(9H-Carbazol-4-yl)oxy)-N-[2-(2-methoxyphenoxy)ethyl]pyrrol-1-amine (15). A solution of 12 (0.10 g, 0.29 mmol), 2-(2-methoxyphenoxy)ethanol (0.16 mL, 0.58 mmol), and KOH (0.03 g, 0.58 mmol) in acetonitrile (8 mL) was stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 50/50) to give 15 as a colorless solid (0.05 g, 59%).
The residue was triturated with ethyl acetate to give a colorless solid (4.8 mg, 3%). ¹H NMR (CDCl₃, 500 MHz): δ 8.29 (d, J = 7.8 Hz, 1H), 8.07 (s, 1H), 7.47–7.33 (m, 2H), 7.30 (t, J = 8.0 Hz, 1H), 7.18 (t, J = 7.4 Hz, 1H), 7.03 (d, J = 8.1 Hz, 1H), 6.92–6.83 (m, 3H), 6.83–6.73 (m, 4H), 6.65 (d, J = 8.0 Hz, 1H), 4.32 (t, J = 6.0 Hz, 2H), 4.16–4.09 (m, 4H), 3.80 (s, 5H), 3.12 (t, J = 6.4 Hz, 4H), 3.03 (t, J = 6.9 Hz, 2H), 2.20 (p, J = 6.5 Hz, 2H). ¹³C NMR (CDCl₃, 126 MHz): δ 159.00, 155.63, 151.71, 146.04, 136.37, 129.83, 126.69, 124.81, 122.91, 120.96, 120.82, 119.65, 113.71, 117.14, 109.98, 109.86, 103.25, 100.88, 67.43, 65.68, 55.81, 53.88. LCMS: method 3, RT = 14.01; M + H⁺: 541.30.

**Synthesis of Ethyl 2-(9H-Carbazol-4-yloxy)acetate (17).** A mixture of 4-hydroxy carbazole (1.90 g, 10.38 mmol), ethyl chloroacetate (1.11 mL, 10.38 mmol), and K₂CO₃ (1.43 g, 10.38 mmol) in acetone (150 mL) was stirred at 56 °C for 4 h. The residue was triturated with ethyl acetate and hexanes to yield a colorless solid (0.13 g, 9%). ¹H NMR (CDCl₃ 500 MHz): δ 11.08 (s, 1H), 7.96 (d, J = 7.7 Hz, 1H), 7.93 (d, J = 8.5 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.29–7.23 (m, 1H), 7.11–7.06 (m, 1H), 6.69–6.92 (m, 3H), 6.89–6.82 (m, 2H), 6.76 (dd, J = 8.5, 2.2 Hz, 1H), 5.14–5.03 (m, 1H), 4.03–3.98 (m, 1H), 3.79–3.95 (m, 2H), 3.72 (s, 4H), 2.90 (t, J = 5.9 Hz, 2H), 2.79 (dd, J = 11.9, 3.9 Hz, 1H), 2.69 (dd, J = 11.8, 6.4 Hz, 1H). ¹³C NMR (CDCl₃ 151 MHz): δ 158.31, 149.59, 148.50, 141.48, 140.16, 124.56, 123.09, 121.47, 121.32, 116.19, 116.99, 118.96, 116.62, 114.01, 112.61, 111.04, 108.85, 95.53, 71.39, 68.77, 55.89, 52.93, 48.94; LCMS: method 3, RT = 12.59; M + H⁺: 407.15.

**Synthesis of 9-(Oxiran-2-ylmethyl)carbazole (22).** KOH (0.20 g, 3.59 mmol) was added to a solution of 9H-carbazole (0.50 g, 2.99 mmol) in acetonitrile (5 mL) and the reaction mixture was stirred at room temperature for 1 h. Then, the reaction mixture was cooled in an ice bath, and 2-(bromomethyl)oxirane (0.64 mL, 7.48 mmol) was added dropwise. After the reaction mixture was stirred at room temperature for 20 h. After this period, the reaction mixture was partitioned between ethyl acetate and water, and the organic layer was washed with water and brine, dried over MgSO₄, filtered, and concentrated. The residue was triturated with hexane and recrystallized from ethyl acetate/hexanes to yield a colorless solid (0.48 g, 68%) and used in the next step with no further purification. ¹H NMR (CDCl₃, 500 MHz): δ 8.11 (dt, J = 7.8, 1.0 Hz, 2H), 7.53–7.45 (m, 4H), 7.31–7.24 (m, 2H), 7.27 (s, 2H), 4.64 (dd, J = 15.9, 3.4 Hz, 1H), 4.42 (dd, J = 15.9, 4.8 Hz, 1H), 3.38–3.35 (m, 1H), 2.82 (s, J = 4.4 Hz, 1H), 2.54–2.51 (m, 4H), 1.48–1.45 (m, 2H), 1.12 (s, 3H), 1.03 (d, J = 6.5 Hz, 2H). LCMS: method 2, RT = 3.03 min; M + H⁺: 224.15.

**Synthesis of 1-(9H-Carbazol-2-yloxy)-(2-phenylethyl)aniline-2-propen-2-ol (23).** A solution of 22 (0.10 g, 0.43 mmol) and phenethylamine (0.05 mL, 0.43 mmol) in ethanol (5 mL) was stirred at 65 °C for 16 h. Then, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography gradient elution of petroleum ether/ethyl acetate (100/0 to 95/5). The compound obtained was further purified by flash column chromatography gradient elution of DCM/methanol (100/0 to 90/10) to give 23 as a colorless solid (0.076 g, 33%). ¹H NMR (DMSO 600 MHz): δ 11.08 (s, 1H), 7.96 (d, J = 7.7 Hz, 1H), 7.93 (d, J = 8.5 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.29–7.23 (m, 1H), 7.11–7.06 (m, 1H), 6.69–6.92 (m, 3H), 6.89–6.82 (m, 2H), 6.76 (dd, J = 8.5, 2.2 Hz, 1H), 5.14–5.03 (m, 1H), 4.03–3.98 (m, 1H), 3.79–3.95 (m, 2H), 3.72 (s, 4H), 2.90 (t, J = 5.9 Hz, 2H), 2.79 (dd, J = 11.9, 3.9 Hz, 1H), 2.69 (dd, J = 11.8, 6.4 Hz, 1H). ¹³C NMR (DMSO 151 MHz): δ 158.31, 149.59, 148.50, 141.48, 140.16, 124.56, 123.09, 121.47, 121.32, 116.19, 116.99, 118.96, 116.62, 114.01, 112.61, 111.04, 108.85, 95.53, 71.39, 68.77, 55.89, 52.93, 48.94; LCMS: method 3, RT = 12.59; M + H⁺: 407.15.
Bio-One 627161) and incubated for 48 h at 37 °C in an incubator in the presence of 1 mL RCM/DMEM-F12 (1:4) (H0887) (HBHBSS). Cochleae were removed from the bony labyrinth and explanted onto collagen-coated (Corning 354236) coverslips and left to adhere to the collagen for 24 h at 37 °C. Subsequent dissections were performed in Hanks buffed salt solution (HBSS; Thermo Shandon 14025050) buffered with 10 mM Hepes (Sigma H0887) (HBHBSS). Cochleae were removed from the bony labyrinth and explanted onto collagen-coated (Corning 354236) coverslips and immersed in rat cochlear culture media (RCM—93% DMEM-F12, 7% fetal bovine serum and 10 μg mL−1 ampicillin), sealed in Maximow slide assemblies, and left to adhere to the collagen for 24 h at 37 °C. Mouse Cochlear Culture Preparation. Cochlear cultures were prepared from CD-1 mice as previously described by Russell and Richardson.18 In brief, P2 pups were killed by cervical dislocation and surface sterilized by three 1 min washes in 80% ethanol. Subsequent dissections were performed in Hanks buffed salt solution (HBSS) and during compound exposure at membrane potentials ranging from −164 to +96 mV. Cultures were elicited by stimulating the OHC hair bundles using a fluid jet from a pipette (tip diameter 8–10 μm) driven by a piezoelectric disc.20 Mechanical stimuli (filtered at 1.0 kHz, 8-pole Bessel) were applied as 45 Hz sinusoids with driver voltage amplitudes of ±40 V. Cultures were acquired using pClamp ( Molecular Devices) software and stored on computer for off-line analysis. For all recordings, series resistance compensation was applied (60–80%), and the average residual series resistance was calculated to be 1.37 ± 0.08 MΩ (n = 47). The average maximum MET current size was 1.50 ± 0.07 nA (n = 49), resulting in a maximum voltage drop across the residual series resistance of 2.1 mV, a value sufficiently small to not require any correction to quoted voltage values.

Electrophysiology on Mouse Cochlear Cultures. MET currents were recorded and analyzed using previously described methods.20 In brief, OHCs in organotypic cultures prepared from P2 CD-1 mice were studied, with recordings performed in cultures that had been maintained for 1–2 days in vitro. MET currents were recorded using the whole-cell configuration of the patch clamp technique both before and during compound exposure at membrane potentials ranging from −164 to +96 mV. Cultures were elicited by stimulating the OHC hair bundles using a fluid jet from a pipette (tip diameter 8–10 μm) driven by a piezoelectric disc.20 Mechanical stimuli (filtered at 1.0 kHz, 8-pole Bessel) were applied as 45 Hz sinusoids with driver voltage amplitudes of ±40 V. Cultures were acquired using pClamp ( Molecular Devices) software and stored on computer for off-line analysis. For all recordings, series resistance compensation was applied (60–80%), and the average residual series resistance was calculated to be 1.37 ± 0.08 MΩ (n = 47). The average maximum MET current size was 1.50 ± 0.07 nA (n = 49), resulting in a maximum voltage drop across the residual series resistance of 2.1 mV, a value sufficiently small to not require any correction to quoted voltage values.

Dose response curves were fitted with the equation

\[
\frac{1}{I_C} = \frac{1}{1 + ([B]/K_0)^n}
\]

where \(I_C\) is the control current in the absence of the compound, [B] is the concentration of the blocking compound, \(K_0\) is the half-blocking concentration, and \(n\) is the Hill coefficient. Permeation and block of the MET channel for carvedilol and 13 were quantified by fitting a two-barrier—one binding site model to the fractional block curves, as described in detail before.20 21 This model is similar to that used to describe block of the MET currents by DHH but modified to allow for Hill coefficients different from one.

**Block of GTTR Loading into Mouse Cochlear Culture Hair Cells.** Covessrips were removed from the Maximow slide assemblies, placed in a Perspex viewing chamber and immersed in 500 μL HBHBSS. Cultures were treated with either 100 μM carvédilol, 100 μM of the carvédilol derivative 13 or 1% DMSO as a control, with carvédilol and 13 being dissolved in this solvent. After 5 min incubation time at room temperature, GTTR was added at a final concentration of 0.2 μM; and incubation was continued for a further 10 min. The culture was washed three times with HBHBSS before live imaging on a Zeiss Axioplan2 microscope. A 60x water immersion lens was used to take images of both the apical and basal regions of the cochlea across a time range from 14 to 24 min post-GTTR application. Three repeats were conducted. For quantification, analysis was performed on images from the mid-basal region, 24 min post-GTTR application. Ten cells from the first row of OHCs central to the 1200 pixel image were analyzed, as shown by the asterisk in Figure 16, obtaining intensity values from a 40 × 40 pixel region of interest (ROI). Three background ROIs were measured, averaged, and subtracted from each individual cell value, which were then averaged and repeated across three trials. One background ROI was taken from nonsensory HC cellular space to account for any endocytic loading.

**Statistics.** All graphical representations display mean ± standard error of the mean. Numbers above bars denote the number of independent experimental replicates. One-way ANOVA was applied followed by Tukey’s multiple comparison test, assuming normal distribution of the data. For GTTR live imaging experiments, an unpaired t-test was used. Significance was set at *p = 0.05, **p < 0.01, and ***p < 0.001.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01325.

Molecular formula strings (CSV)

Protection of carvédilol and compound 13 in zebra fish lateral line; antmicrobial activity of gentamicin in the...
presence of carvedilol and compound 13; supplemental methods zebrafish animal husbandry and screening for otoprotection; bacterial growth conditions and antimicrobial drug susceptibility testing (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Medical Research Council (MR/K005561/1 to C.J.K., G.P.R., and S.E.W.). All data are provided in the Results and the Supporting Information of the paper and in the Experimental Section.

■ ABBREVIATIONS

MET, mechanoelectrical transducer; AG, aminoglycoside; ROS, reactive oxygen species; OHC, outer hair cell; IHC, inner hair cell; DMF, dimethylformamide; TEA, tryethylamine; ROS, reactive oxygen species; OHC, outer hair cell; IHC, inner hair cell; DMF, dimethylformamide; TEA, tryethylamine; HOBt, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-N,N-diisopropylcarbodiimide; DIFEA, N,N-diisopropyl-ethylamine; GTTR, gentamicin Texas red; SAR, structure–activity relationship; DHI, dihydrostreptomycin; THF, tetrahydrofuran; DMC, dichloromethane.

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