ARTICLE
Protein transduction therapy into cochleae via the round window niche in guinea pigs

Hiroki Takeda1, Takaomi Kurioka2, Taku Kaitsuka1, Kazuhito Tomizawa1, Takeshi Matsunobu1, Farzana Hakim3, Kunio Mizutari2, Toru Miwa1, Takao Yamada1, Momoko Ise1, Akihiro Shiotani2, Eiji Yumoto1 and Ryosei Minoda1

Cell-penetrating peptides (CPPs) are short sequences of amino acids that facilitate the penetration of conjugated cargoes across mammalian cell membranes, and as such, they may provide a safe and effective method for drug delivery to the inner ear. Simple polyarginine peptides have been shown to induce significantly higher cell penetration rates among CPPs. Herein, we show that a peptide consisting of nine arginines ("9R") effectively delivered enhanced green fluorescent protein (EGFP) into guinea pig cochleae via the round window niche without causing any deterioration in auditory function. A second application, 24 hours after the first, prolonged the presence of EGFP. To assess the feasibility of protein transduction using 9R-CPPs via the round window, we used "X-linked inhibitor of apoptosis protein" (XIAP) bonded to a 9R peptide (XIAP-9R). XIAP-9R treatment prior to acoustic trauma significantly reduced putative hearing loss and the number of apoptotic hair cells loss in the cochleae. Thus, the topical application of molecules fused to 9R-CPPs may be a simple and promising strategy for treating inner ear diseases.

Molecular Therapy — Methods & Clinical Development (2016) 3, 16055; doi:10.1038/mtm.2016.55; published online 17 August 2016

INTRODUCTION
Treatment modalities for human sensorineural hearing loss have not yet been fully established. A major obstacle for this is a lack of an effective and safe delivery method for therapeutic drugs or molecules into cochleae, because mammalian inner ears have unique and complex anatomical structures: a perilymphatic space; and, an endolymphatic space where both auditory and vestibular sensory cells exist. The cochlea of the inner ear, which contains auditory sensory cells, is one of the most critical structures in the auditory pathway. The cochlea consists of three fluid filled compartments: the scala tympani; the scala vestibule; and the scala media (Figure 1a). The surface of the hearing sensory organ is covered by endolymph that fills the scala media. The inner ear is isolated from the systemic circulation by the “blood-labyrinth barrier” and from the middle ear by the round window membrane (RWM). These complex structures make drug delivery into the inner ear difficult. Given this background, there are two routes to achieve inner ear drug delivery that are used clinically in humans: the systemic route and the intratympanic route. Systemic administration has been used clinically for glucocorticoid treatments of acute sensorineural hearing loss and Meniere’s disease. Intratympanic administration treatment appears to be superior to systemic administration. Systemic administration tends to require higher-dosage regimens because of the blood-labyrinth barrier and the extent of the systemic circulation. Besides such high-dose regimens can cause undesirable side effects. Contrarily, intratympanic drug administration may be able to avoid the adverse systemic effects thus generating therapeutic effects in the inner ear via the RWM which consists of three layers: an outer epithelium; a middle core of connective tissue; and an inner epithelium. The RWM is the membranous septum between the middle ear and the perilymphatic space in the cochleae in humans, monkeys, felines, and rodents. Because of the limited permeability nature of the RWM the development of treatment delivery strategies may be challenging. Permeability through the RWM can be affected by factors such as molecular size, configuration, concentration, liposolubility, electrical charge level, and membrane thickness. Regarding size, in vivo experiments have revealed that 1-μm microspheres can traverse chinchilla RWMs, whereas 3-μm spheres cannot. Lundman et al. demonstrated that the passage of endotoxins with a molecular weight >100,000 through a normal RWM was limited. Thus, lower molecular weight molecules pass through the RWM, while higher molecular weight molecules do not readily pass through the RWM. Indeed, intratympanic drug therapy utilizing lower molecular weight molecules has been performed successfully clinically, as is shown in the two following examples: aminoglycoside antibiotics (e.g., gentamicin: its molecular weight is 478) for the treatment of Meniere’s disease; and glucocorticoids (e.g., dexamethasone: its molecular weight is 392) for the treatment of sensorineural hearing loss. Although the impenetrable nature of the RWM for higher molecular weight molecules may be a defense system for the inner
ear, the lack of penetration by higher molecular weight molecules could simultaneously constitute a barrier to good therapeutic outcomes when intratympanic drug therapy is applied via the RWM for inner ear dysfunction.

The human immunodeficiency virus type 1 trans-activator of transcription (TAT) protein can enter cells upon addition to a culture medium.\(^1\)\(^1\)\(^2\) The transduction of TAT, which contains a high proportion of arginine and lysine residues, is responsible for the ability of the virus to be able to penetrate the plasma membrane.\(^1\)\(^3\)\(^-\)\(^2\)\(^2\)\(^3\) Considering this superior nature of polyarginine peptides, with an optimal length of 9–11 residues, or suppress gene expression.\(^1\)\(^6\) Among the various CPPs, arginine-rich peptides have been the most widely studied.\(^2\)\(^2\)\(^4\)\(^-\)\(^\)\(^6\)\(^1\)\(^7\)\(^-\)\(^9\) Simple polycationic peptides, with an optimal length of 9–11 residues, induce significantly higher cell penetration rates than TAT proteins do.\(^9\)\(^-\)\(^2\) Considering this superior nature of polycationic peptides, attachment of polycationic peptides to higher molecular weight therapeutic molecules should prove to be useful for facilitating the penetration of therapeutic molecules to therapeutic targets in the inner ear.

Apoptosis is programmed cell death that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells.\(^2\)\(^3\) This process is implicated in several inner ear disorders, including drug-induced, noise-induced, age-related, and genetic hearing loss conditions.\(^2\)\(^4\) Caspases, a family of cysteine proteases, are essential effector molecules for initiating apoptosis.\(^2\)\(^5\)\(^-\)\(^2\)\(^8\) “X-linked inhibitor of apoptosis protein” (XIAP; its molecular weight is 84,000), the most potent member of the inhibitors of apoptosis (IAP) family, interacts physically with the BIR1, BIR2, and BIR3 domains of caspases.\(^3\)\(^-\)\(^1\)\(^1\) XIAP also enhances the survival signal directly by forming a complex with TAB1/TAK1.\(^2\)\(^1\)\(^-\)\(^1\)\(^1\) XIAP also enhances the survival signal directly by forming a complex with TAB1/TAK1 via its BIR1 domain.\(^3\)\(^1\) XIAP has a protective effect against several types of inner ear damage. Tabuchi et al.\(^3\)\(^2\) reported on the protective effect of XIAP against gentamicin-induced hair cell damage in an in vitro study utilizing rat cochleae, while Wang et al.\(^3\)\(^3\)\(^-\)\(^3\)\(^4\) reported that XIAP overexpressed in mice exerted a protective effect against noise-induced and age-related hearing loss in cochlear hair cells and spiral ganglion cells (SGCs). Thus, XIAP appears to have protective effects against drug- and noise-induced hearing loss. Only one report though has examined the treatment efficacy of XIAP in vivo: Cooper et al.\(^3\)\(^5\) demonstrated
the successful protective treatment effect of XIAP against cisplatin-mediated ototoxicity using an adeno-associated viral vector that was injected into the scala tympani of the cochleae in rats. When considering the possible clinical applications of adeno-associated viral vector-based XIAP treatment for humans, the direct injection of viral vectors into the scala tympani, which is part of the perilymphatic space, may cause inner ear damage; moreover, the direct injection of therapeutic molecules into either the perilymphatic or the endolymphatic spaces of the cochleae have not been performed clinically because of this damage risk. Thus, the need exists for a simpler and safer strategy to administer XIAP into the cochleae. CPP-based XIAP treatment may be such a treatment modality. Additionally, this CPP-based XIAP strategy may be an important treatment modality for administering other therapeutic molecules for treating various inner ear diseases.

Herein, we first examined the safety and efficacy of protein transduction using a CPP consisting of nine arginines (9R-CPP) via the round window (RW) niche. Second, to prove the feasibility of 9R-CPP-based treatment via the RW, we performed 9R-CPP-based XIAP treatment against noise-induced hearing loss in guinea pigs in vivo.

RESULTS
Protein transduction in vitro
HEK293 cells were cultured in medium containing EGFP-9R or EGFP for 24 hours. In the cells, which were cultured with EGFP-9R, EGFP-9R protein was transduced into all of the cytoplasm and into some of the nuclei. In the cells, which were cultured with enhanced green fluorescent protein (EGFP) without 9R, EGFP was not transduced into any cells. Western blotting analyses revealed that the relative GFP intensity in the cells which were treated with EGFP-9R was 100%, whereas that in EGFP-treated cells was 0% (Supplementary Figure S1a,b). The EGFP protein expression levels at 24 hours after EGFP-9R treatment were equivalent to EGFP protein expression levels at 24 hours after plasmid transfection (Supplementary Figure S1a,b). A chronological study after EGFP-9R treatments revealed that EGFP protein expression levels after EGFP-9R treatments were highest at 12 hours and they still maintain some level of EGFP expression even at 72 hours after EGFP-9R treatments (Supplementary Figure S1c,d). Additionally, the higher concentrations (10 µmol/l) of EGFP-9R induced longer protein expression periods when compared with lower concentrations (1 µmol/l) (Supplementary Figure S2e). The EGFP protein expression level within the cells after EGFP-9R treatments was approximately similar in level to the EGFP protein level of 2% of the original (100%) EGFP-9R solution (Supplementary Figure S2a). It appears that 2% of the total amount of EGFP moved into the cells via EGFP-9R treatment. WST assay revealed that the viability of both HEK293 cells and MEFs were unaffected even at high dosages (Supplementary Figure S2c,d).

Protein transduction into the guinea pig cochlea in vivo
We performed a single-protein transduction study using the EGFP (s-EGFP Group) and the EGFP-9R (s-EGFP-9R Group) (Figure 1b–d) and a double-protein transduction study using the double EGFP-9R (d-EGFP-9R Group) (Figure 1e). For quantification of EGFP protein transfection, we utilized two strategies: western blot analysis and modified labeling index (mLI) analysis.

Western blot analyses were performed utilizing whole cochlea. The GFP transduction levels at 24 hours in the s-EGFP Group did not show any significant changes compared with those of the normal non-treated cochleae (referred to as 0 hours in Figure 2a,b). The GFP transduction levels in the s-EGFP-9R Group at 24 hours was significantly higher compared to those at 0 hours and also to those at 24 hours in the s-EGFP Group, while the GFP transduction levels in the s-EGFP-9R Group at 48 hours showed no significant differences compared to those at 0 hours and those at 24 hours in the s-EGFP Group. The GFP transduction levels at 48 hours in the d-EGFP-9R Group was significantly higher compared with those at 0 hours and those at 24 hours in the s-EGFP Group, while GFP transduction levels at 48 hours in the s-EGFP-9R Group indicated no significant changes compared with those at 0 hours and those at 24 hours in the s-EGFP Group (Figure 2). When we compared GFP transduction levels of the s-EGFP-9R and the d-EGFP-9R groups, at the same elapsed time points, after their final treatments; relative intensity at 24 hours in the s-EGFP-9R Group versus that at 48 hours in the d-EGFP-9R Group, no significant differences were detected. These results strongly suggest that a single EGFP-9R application at the RW niche induces significant protein transduction in the cochlea, and that the protein transduction is maintained for at least 24 hours after the placement. A double EGFP-9R application is effective at maintaining protein transduction levels for at least 48 hours.

To assess the localization of the transduced proteins in the cochlea, we performed mLI assessments. The mLIs of the treated cochlea in the s-EGFP-9R Group were significantly higher than those in the s-EGFP Group, in the SVs, OCs (inner hair cells (IHCs), outer hair cells (OHCs), and supporting cells; Supplementary Figure S4a,b), and SGCs at 12 and 24 hours (Figure 3a and Supplementary Figure S4b), while there were no significant differences between the mLIs in the s-EGFP Group and in the normal nontreated cochlea (referred to as 0 hours in Figure 3a). There were no significant differences between the mLI values of the treated-side cochlea at 72 hours in the s-EGFP-9R Group and the mLI at 0 hours (Figure 3a). These findings suggest that the mLIs of the treated cochlea in the s-EGFP-9R Group returned to the baseline level by 72 hours. Additionally, when we compared the mLI at each time point in the s-EGFP-9R Group, the values at 48 hours were significantly lower than those in the SVs at 24 hours, and the mLIs at 72 hours were significantly lower than those in the SGCs at 12 hours and those in the SVs, OCs, and SGCs at 24 hours (Figure 3a).

We assessed the mLI at each turn of the cochlea after 24 hours in the s-EGFP-9R Group. The mLIs of the SVs and SGCs at the basal turns in the s-EGFP-9R Group were significantly higher than those at the apical turns, and the mLIs of the SVs at the middle turns were significantly higher than those at the apical turns (Figure 3b). Thus, the mLIs in the s-EGFP-9R Group returned to the baseline level by 72 hours. Additionally, when we compared the mLI at each time point in the SVs, OCs, and SGCs at 48 hours (Figure 3a).

Regarding the double-protein transduction study, the mLIs of the treated cochlea in the SVs at 48 hours in the d-EGFP-9R Group were significantly higher than those in the SVs at 48 hours in the s-EGFP-9R Group or those at 0 hours (Figure 3a). The mLIs of the treated cochlea in the SGCs at 72 hours in the d-EGFP-9R Group were also significantly higher than those at 72 hours in the SGCs of the s-EGFP-9R Group (Figure 3a). Next, we compared the mLIs in the s-EGFP-9R Group and in the d-EGFP-9R Group at the same elapsed time points after their final treatments. Consequently, the mLIs at 48 and 72 hours in the d-EGFP-9R Group were not significantly different from those at 24 and 48 hours in the s-EGFP-9R Group, respectively. These findings suggest that a double EGFP-9R application is effective at reproducing protein transduction levels of the first application.
Figure 2 Quantification of protein transduction levels via western blot. (a) GFP and β-actin protein expression was detected utilizing western blotting in the s-EGFP-9R and d-EGFP-9R groups. (b) Quantification of GFP protein transduction levels was performed utilizing the relative intensity. The relative intensity in the s-EGFP-9R Group at 24 hours was significantly higher than that at 0 hours and at 24 hours in the s-EGFP group. The EGFP transduction level at 48 hours in the d-EGFP-9R Group was significantly higher compared with that at 0 hours and that at 24 hours in the s-EGFP group, while EGFP transduction level at 48 hours in the s-EGFP-9R Group indicates no significant change compared with that at 0 hours and at 24 hours in the s-EGFP group.

Figure 3 Quantification of protein transduction levels via mLI. (a) The mLIs in the s-EGFP, s-EGFP-9R and d-EGFP-9R groups are shown. The mLIs of the treated-side cochlea in the s-EGFP-9R Group peaked at 24 hours, decreased over time, and became approximately equal to that in 0 hours by 72 hours. (b) The mLIs at each turn at 24 hours in the s-EGFP-9R Group are shown. The mLI in the SV and SGC at the lower turns was generally higher, versus at the higher turns. Each n = 5. *P < 0.05; **P < 0.01.
Thickness of the RWM
At 24 hours in the s-EGFP-9R Group, the thicknesses of the RWMs of the treated-side cochleae were significantly greater than those of the nontreated side cochleae (Figure 4). The increase in RWM thickness may have affected the slight decrease in protein transduction after the second transduction.

Impact of protein transduction on inner ear function and morphology
To assess the impact of protein transduction on inner ear function, we assessed auditory thresholds, the number of HCs, and the number of SGCs at 28 days in the s-EGFP and s-EGFP-9R Groups. ABR testing revealed that there was no significant change in the auditory thresholds at 4, 12, or 20kHz between the s-EGFP and s-EGFP-9R Groups (Supplementary Figure S5). Both groups had a normal surface morphology in the OCs (Supplementary Figure S6a). There were no statistically significant differences in the hair cells and SGCs numbers between the groups (Supplementary Figure S6b and S7).

Protein transduction of XIAP or XIAP-9R in vivo
Western blot analyses were performed periodically after protein application (Figure 5a). The quantification indicated that the transduction level in the s-XIAP-9R Group increased, with a peak at 12 hours after application (Figure 5b). The transduction level of XIAP in the s-XIAP Group did not show any significant changes.

Protective effect of XIAP-9R treatment against noise-induced hearing loss
To examine the feasibility of 9R-CPP-based treatment via the RW niche, we performed XIAP-9R treatment to protect against noise-induced hearing loss. The auditory thresholds in the s-XIAP-9R Group at 14 days after noise exposure were significantly better than those in the Saline Group at 32kHz, whereas no significant differences were found between the s-XIAP and saline groups (Figure 6).

Effects of XIAP-9R treatment on hair cell loss
To clarify the morphological effects of the s-XIAP-9R Group, we counted the number of OHCs at 14 days after noise exposure. A surface morphological study revealed that the OHCs loss rate at the base of the cochlea, which corresponds to 32kHz in the s-XIAP-9R Group, decreased significantly, compared to that in the saline Group, while there was no significant difference in the OHCs loss rate between the s-XIAP and saline Groups (Figure 7a).
Effects of XIAP-9R treatment on noise-induced apoptosis

A TdT-mediated dUTP nick end labeling (TUNEL) assay was used to assess the mechanism of cochlear HC loss and the protective effect of XIAP-9R against acoustic trauma. Previous studies have shown that TUNEL-positive nuclei were observable as early as 1 hour after noise exposure and that they were observed continuously up to 72 hours after noise exposure. Therefore, we performed TUNEL labeling for HCs and supporting cells of the OC in the basal turns of the cochlea in each group at 48 hours after noise exposure (Figure 7b). TUNEL-positive nuclei were clearly apparent in the region of the OHCs in the saline Group. There were fewer TUNEL-positive nuclei in the s-XIAP and s-XIAP-9R Groups (Figure 7b). Subsequently, a quantitative analysis revealed that the number of TUNEL-positive OHC nuclei decreased significantly in the s-XIAP-9R Group, but not in the s-XIAP Group, compared with that in the saline Group (Figure 7c). These results suggest that XIAP-9R treatment against noise-induced cochlear damage had a protective effect via the inhibition of apoptosis.
To further examine the mechanism of the antiapoptotic effects of XIAP in noise exposure in the cochleae, we examined cleaved caspase-3 in the cochleae at 24 hours after noise exposure. It has been reported that cleaved caspase-3 is detectable in the cochleae from immediately after noise exposure up to 2 days after noise exposure, and it is therefore a prime target for the antiapoptotic effects of XIAP. Cleaved caspase-3-positive OHCs were found more frequently in the basal parts of the OCs than in the middle and apical turns; this is consistent with the finding that apoptotic OHCs were found most frequently at the basal turns after noise exposure (data not shown). Cleaved caspase-3-positive cells were found less frequently in the s-XIAP-9R Group than in the s-XIAP Group or in the saline Groups. The number of cleaved caspase-3-positive OHCs in the s-XIAP-9R Group was significantly lower than in the saline group (Figure 7d,e). These results suggest that an apoptotic mechanism, via caspase-3, was suppressed by the XIAP-9R treatment.

**DISCUSSION**

Western blot analyses revealed that a single EGFP-9R application at the RW niche induced significant protein transduction in the cochleae. The protein transduction in the whole cochleae was maintained for at least 24 hours after the application when compared with the nontreated cochlea, whereas, a single EGFP application did not induce significant protein transduction. In the XIAP-9R study, the western blot analysis data similarly revealed that XIAP transduction was maintained for at least 24 hours after the treatment. Double EGFP-9R applications were effective at maintaining protein transduction levels for at least 48 hours after the first applications. To assess the localization of the transduced proteins in the cochlea after 9R-CPP-mediated protein transfer via the RWM, we utilized mLI assessment, which is a "photoshop-based image analysis method," which has been utilized for assessing protein expression levels in *in vivo* and *in vitro* experiments. Consequently, we found that protein transduction signals were significantly detectable in all assessed locations (the SV, SGC, and OC regions) until 24 hours after the EGFP-9R application, when compared with nontreated cochleae. While at 48 hours after the EGFP-9R applications, significant protein transduction levels were detectable in the SV and SGC regions in the cochleae. In the double application study, significant protein transduction signals were detectable in the SVs at 48 hours after the initial applications, when compared with the nontreated cochleae. Thus, sequential changes of the protein transduction levels found in our mLI analysis in the EGFP-9R experiment were similar to those found in the western blot analysis; in both analyses, we could detect significant protein transduction signals in the cochleae for at least 24 hours after the single EGFP-9R treatments, and for at least 48 hours after the double EGFP-9R treatments, but according to the results, there were some minor differences in the sequential changes of the protein transduction levels between the western blot analysis data and the mLI analysis data, (for example, at 48 hours after the single EGFP-9R application, significant protein transduction signals were not detectable in western blot analysis, but significant signals were detectable in two of the three locations in the cochleae with mLI analysis), we think that these differences reflect differences in the target samples: whole cochleae in the western blot analyses and selected locations in the cochleae in the mLI analyses. We therefore maintain that protein transduction via the RWM utilizing 9R-CPP is a proven, valid, and reliable protein transduction strategy into the cochleae.

Our *in vitro* studies revealed that the EGFP protein expression levels at 24 hours after plasmid transfection via cationic lipid-mediated transfection. Recently, Zuris *et al.* have reported successful protein transfection into the cochlear tissues via cationic lipid mediated transfection. Although they injected target proteins and cationic lipid into the scala media, the direct injection into the cochlea has a potential risk to cause inner ear damage. Additionally, Zuris *et al.* have found cytotoxic effects in both *in vitro* and *in vivo* studies when utilizing the higher concentration of cationic lipid. Contrarily, 9R-CPP-based protein transfection did not show any cytotoxic effects in either HEK293 cells or MEFs even under at higher concentrations of 9R-CPP. Additionally, in *in vivo* studies, 9R-CPP-based protein transfection did not show any cytotoxic effects at either the highest concentration levels of EGFP-9R or XIAP-9R, which were available to us. Thus, when considering clinical applications of protein transduction into the cochleae, 9R-CPP-based protein transfection appears to be superior to cationic lipid-mediated transfection because of the lack of cytotoxicity with 9R-CPP-based transfections.

There are two known routes for the diffusion of substances from the tympanic cavity, the normal air space surrounding the bony cochlea, to the perilymphatic space in the cochlea of rodents. One is via the RWM and the other is via the bony wall of the cochlea. It has been reported that gentamicin and steroid hormones placed on the RW niche of guinea pigs are distributed into the perilymphatic space of the cochlea. The concentration at the basal turn of the cochlea was 1,000-fold higher than at the apical turn. This suggests that substances placed on the RW niche pass through the RWM and diffuse into the perilymphatic space from the basal turn to the apical turn. In guinea pigs, the lateral bony wall of the cochlear apex is thinner than at the basal turn. Additionally, a lacuno-canalicul system has been described in the cochlear walls of both humans and mice. These anatomical structures in guinea pigs appear to induce a higher concentration of a substance at the apical turn when the tympanic cavity is filled with the substance. The existence of the two routes (via the RWM and via the bony wall of the cochlea) may have contributed to the distribution of the substances used in the present study. The EGFP intensities at the basal turns in the cochleae were significantly higher than at the apical turns after placement of an EGFP-9R-soaked gelatin sponge. Considering our distribution pattern of EGFP intensity, we concluded that EGFP-9R probably diffuses mainly via the RWM and becomes distributed in the perilymphatic space in the cochlea, rather than via the cochlear bony wall.

Although the general mechanism by which CPPs enter cells has yet to be determined, it has been suggested that they use direct penetration and endocytosis for cellular internalization. Additionally, CPPs appear to use paracellular pathways to pass through the mucosa and brain endothelial cells. In the present study, EGFP-9R and XIAP-9R, which were placed at the RW niche, probably reached the perilymphatic space using these cellular and paracellular pathways. Generally, it is known that substances which exist in the perilymphatic space in the scala tympani reach the modiolus, the Rosenthal canals and the OC by passing successively through the canaliculi perforantes. It is also known that there is communication between the scala tympani and the scala vestibuli via the spiral ligament and the stria vascularis. In our experiments, EGFP-9R and XIAP-9R, which were placed at the RW niche, may have extended to each part in the cochlea via these routes.

To assess the feasibility of 9R-CPP-based treatment via the RW niche, we performed a protection study using XIAP-9R against noise-induced hearing loss in guinea pigs *in vivo*. XIAP-9R applied...
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Topically to the RWM successfully prevented auditory deterioration caused by noise-induced cochlear damage at higher frequencies. The presumed mechanism underlying this protective effect is that 9R-CPP coupled to XIAP facilitated XIAP permeability across the RWM and diffusion into the cochlear tissues, including OHCs; subsequently, 9R-XIAP achieved a protective effect against noise-induced hearing loss by suppressing the caspase-3 pathway. Considering this, 9R-XIAP treatment via the RW niche could be a simple and promising treatment modality for decreasing cochlear damage in sensorineural hearing loss caused by an apoptotic mechanism. The finding that XIAP-9R showed significant protective effects at higher frequencies but a limited protective effect at lower frequencies may limit the clinical application of XIAP-9R treatment. This finding may have been caused by differences in the XIAP distribution pattern dependent upon each specific cochlear turn; in the s-EGFP-9R Group, the EGFP intensity at lower turns showed significantly higher EGFP transduction. The concentration of XIAP-9R was 0.1 mg/ml, while the concentration of EGFP-9R was 4 mg/ml. Application of such a lower concentration of XIAP-9R at the RW niche should have lowered the local concentration of XIAP in the cochlea, especially at the higher turns. Tünemann et al.55 has also revealed that the transduction ability of arginines can be affected not only by their concentration, but also by the number of consecutive residues. Considering these points, optimization of the concentration levels and the number of consecutive arginine residues may increase the efficacy of transduction, and this in turn should increase the therapeutic efficacy of cargos coupled with the CPP. Furthermore, in the noise-induced hearing loss model used in the present study, the protective effect was lower at lower frequencies than at higher frequencies. Our model produced auditory deterioration primarily at higher frequencies and less so at lower frequencies. This difference in the auditory deterioration pattern may explain our finding of a reduced protective effect at lower frequencies in XIAP-9R-treated guinea pigs. To further explore this issue, we need to optimize the conditions for CPPs and to evaluate the effects of CPP treatment on other inner ear damage models, including models that produce auditory deterioration at lower frequencies.

A single 9R-CPP-based protein transduction via the RW niche exerts more efficient protein transduction compared with protein transduction without 9R-CPP for at least 24 hours after treatment. When we look at time points of the peaks of protein expression levels in our studies, there were some differences: 24 hours in in vivo mL study and 12 hours in WB results of in vivo study and in XIAP study. We think that this difference probably comes from difference of assessing methods (mL versus WB) and location of the samples (second turns of the cochlea versus whole cochlea). In either case, 12 to 24 hours of expression periods after a single 9R-CPP-based protein transduction may limit the usefulness of this method. There are two possible solutions for this issue. Our in vitro study revealed that a higher concentration level of 9R-CPP induced longer protein expression periods. In our in vivo study, we utilized the highest concentration of 9R-CPPs which we had available in our institution, though, if we might have used a higher concentration of 9R-CPPs, probably we could have achieved longer expression periods. Additionally, repeated applications may also help to extend expression periods. In our study, following an additional EGFP-9R application at 24 hours after the first, EGFP protein transduction levels at 48 hours after the first application were significantly higher compared to those in the controls. Additionally, regarding the EGFP protein transduction levels at the same elapsed time after one versus two applications, no significant differences were observed between the s-EGFP and s-EGFP-9R groups. These findings indicate that multiple consecutive applications through the RW niche are effective in terms of extending the expression duration. However, when we compared EGFP protein transduction levels at the same elapsed time between single EGFP-9R application and double EGFP-9R application in detail, the EGFP protein transduction level at 48 hours in the d-EGFP-9R group was somewhat lower than that at 24 hours in the s-EGFP-9R group. This slight decrease in EGFP protein transduction level may be caused by thickening of the RWM: the thickness of the RWM at 24 hours in the treated cochlea was significantly greater than that in the nontreated cochlea. The thickening of the RWM might be a limiting factor in transduction efficacy in more multiple consecutive applications. This thickening phenomenon might have been caused by the gelatin sponge,67 which was used as a carrier of the EGFP-9R. If so, the use of other materials instead of gelatin sponges, or the application of 9R-CPP-conjugated cargos without other materials, may diminish the thickening of the RWM. Consequently, these strategies may increase the transduction efficacy by repeated applications.

Many studies have shown that CPPs are efficient carriers of bioactive cargos, including proteins, peptides, and oligonucleotides. Although the most common application of CPPs is the transduction of proteins and peptides, other applications include CPP-mediated oligonucleotide delivery.62,63 The non-specific nature of CPP-mediated delivery may be a drawback when considering clinical applications. Laakkonen et al.62 reported that LyP-1 peptide, a CPP, induced strong accumulation in primary MDAMB-435 breast cancer xenografts after intravenous peptide injections. Tan et al.65 reported that a CPP conjugated with an anti-HER-2/neu peptide mimetic (AHNP), an ErbB2 extracellular domain-binding peptide, preferentially targeted ErbB2-overexpressing in breast cancer cells. The Matsushita and Kemp et al.66 reported that the addition of a nuclear localization signal (NLS) resulted in delivery of the peptide exclusively to the nuclear compartment of the cells. Thus, the attachment of such homing peptides or other targeting motifs to CPPs may increase retention in specific tissues, cell types, or intracellular locations.

We utilized one octave band noise centered at 4 kHz at 116 dB sound pressure level (SPL) for 2 hours to generate a noise-induced hearing loss model. This noise exposure level induced deterioration of the auditory thresholds, mainly at high frequencies. It has been well known that there are differences in the frequencies of noise and the location of subsequent cochlear damage. Hair cell loss at the basal turns seem to be independent of the noise frequency band used in exposure.67 The upward spread of cochlear damage with respect to the exposure spectrum is typical of an acoustic injury.68 It has also been well known that the hearing impairment level depends on the sound intensity, the types of sound, and the susceptibility of the animals. Thus, the relationship between the noise frequency and the location of the subsequent damage is reasonable and logical.

When considering clinical applications of CPP mediated treatments, in humans, it is relatively easy to access the RW niche via the tympanic membrane, and repeated applications via the tympanic membrane are also feasible. Although we used gelatin sponges as a carrier for EGFP-9R in the current study, we suggest that the repeated application of 9R-CPP-conjugated cargos via eardrops without using a carrier through an incised tympanic membrane could be a clinically applicable method.

In summary, we demonstrated that the use of a 9R-CPP via the RW niche is an effective and safe method for protein transduction

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into guinea pig cochleae. We also demonstrated some effectiveness of repeated applications. Additionally, we showed that 9R-CPP-based treatment is feasible, using XIAP-9R in guinea pigs. Thus, we believe that 9R-CPP-based transduction via the RW niche is a promising potential method for the delivery of therapeutically relevant molecules into the cochlea in humans.

**MATERIALS AND METHODS**

**Ethics statement**

All animal experiments were approved by the Committee on the Use and Care of Animals at Kumamoto University and the National Defense Medical College. They were performed according to accepted veterinary standards.

**Recombinant proteins**

EGFP and EGFP fused to 9R (EGFP-9R) were prepared as described previously. Briefly, the constructed plasmids were transfected into BL21 DE3 Escherichia coli cells, and then protein expression was induced by 0.2 mmol/l isopropyl 1-thio-β-D-galactopyranoside. The proteins were purified using TALON resin (Clontech, Mountain View, CA) and stored at −80°C after dialysis against phosphate-buffered saline (PBS). For the purification of recombinant XIAP and XIAP fused to 9R (XIAP-9R), mouse full-length Xiap cDNA was amplified utilizing polymerase chain reaction using appropriate linker primers and subcloned into the EcoRI-Xhol sites of PET21a (+) (Novagen, Madison, WI) or the BamHI-EcoRI sites of PET21a (+), fused with the 9R sequence at the carboxyl terminus. Similarly, the proteins were generated and stored.

**Experimental protocol in vivo**

Regarding the EGFP and EGFP-9R experiments, we used Hartley guinea pigs, weighing 250–300 g each (Kyudo, Saga, Japan). The EGFP and EGFP-9R experiments involved three groups: single EGFP application (s-EGFP group); single EGFP-9R application (s-EGFP-9R group); and, double EGFP-9R applications (d-EGFP-9R group), in which the second application was performed 24 hours after the first. EGFP and EGFP-9R applications were achieved in the following manner. Guinea pigs were anesthetized via the intraperitoneal administration of 10 mg/kg of xylazine (Bayer; Shawnee Mission, KS) and 40 mg/kg of ketamine (Daichisankyo, Tokyo, Japan) in 0.9% NaCl. The animals underwent a postauricular incision, and then the mastoid bulae were opened. Subsequently, a gelatin sponge of 5–10 mm³ (Astellas Pharma, Tokyo, Japan) that was soaked in 5 μl of EGFP (24.4 mg/ml) or EGFP-9R (4.0 mg/ml) was placed on the RW niche of the right ear, followed by the intraperitoneal administration of 20 mg/kg of ketamine and 40 mg/kg of xylazine (Daichisankyko, Tokyo, Japan). The holes in the mastoid bullae were sealed with the Ecori-Xhol sites of PET21a (+) (Novagen, Madison, WI) or the BamHI-EcoRI sites of PET21a (+), fused with the 9R sequence at the carboxyl terminus. Similarly, the proteins were generated and stored.

In the s-EGFP and s-EGFP-9R Groups, whole cochleae were harvested and fixed in 4% PFA (Wako, Osaka, Japan) for 12 hours at 4°C. The inner ears were decalcified in disodium ethylenediaminetetraacetic acid (EDTA) for 14 days. The cochleae were embedded in optimal cutting temperature (OCT) medium (Sakura Finetek Japan, Tokyo, Japan) and sectioned serially at a thickness of 6 μm utilizing cryostat (GMI, Bellevue, WA). The cryosectioned slices were fixed with 4% PFA and blocked with 0.1% Triton-X (IBI Scientific, Kapp Court Peosta, IA) in PBS, and then incubated with Hoechst 33258 Dye (Molecular Probes) for 30 seconds for nuclear staining. During each process, the tissues were washed three times for 5 minutes each with PBS. The samples were examined under a BX-9000 fluorescence microscope (Keyence, Osaka, Japan), and the images were captured and stored on a computer. We used the mLI to evaluate the EGFP protein transduction levels in each cryosectioned slice of the cochlea. The original labeling index method was reported by Lehr. The mLI method used is described as follows: each image was captured at a resolution of 1,360 × 1,024 pixels at the same photographic exposure condition; tissue-free areas outside of the bony cochlea were then selected using the Magic Wand tool in Adobe Photoshop (Adobe Systems, San Jose, CA); and, the tolerance level was then adjusted to be able to select the entire outside areas of the bony cochlea. Thus, the determined tissue-free areas were used as the background. Subsequently, each stained area in the organ of Corti (OC), SGCs, and stria vascularis (SV) was determined similarly. These images were converted to 8-bit grayscale images. The optical densities of the background areas and each stained area were assessed using the histogram tool in Adobe Photoshop; consequently, the mean staining intensity and the number of pixels in each area were determined. The final staining intensity was calculated as the difference between the mean staining intensity and the mean background intensity. Next, the staining ratios were calculated as the ratios of the number of pixels in each stained area to that in the entire image. These measuring processes of mLI were performed in a blinded manner.

**Noise exposure**

Guinea pigs were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg) and medetomidine (1.0 mg/kg). They were exposed to one octave band noise centered at 4 kHz at 116 dB sound pressure level (SPL) for 2 hours in a ventilated sound exposure chamber. The sound chamber was fitted with speakers (Model 2380A; JBL, Northridge, CA) driven by a noise generator (DANAC-31; Dana Labs, Tokyo, Japan) and a power amplifier (D-45; Crown International, Elkhart, IN). Sound levels were calibrated at Type 6224 precision sound level meter; Aco Instruments, Tokyo, Japan) at multiple locations within the sound chamber to ensure uniformity of the stimulus.

**Auditory thresholds**

Auditory thresholds were measured using the ABR (System 3; Tucker-Davis Technologies, Alachua, FL). The animals were anesthetized by the intraperitoneal administration of xylazine and ketamine. Electrodes were placed beneath the pinna of the treated ear and at the vertex just below the surface of the skin, and then the ground electrode was placed under the contralateral ear. An average of 1,024 sweeps was calculated for 4, 8, and 12 kHz in
the s-EGFP and s-EGFP-9R Groups, and for 4, 8, 16, and 32 dB in the s-XIAP and s-XIAP-9R Groups. The stimulus levels near the threshold were varied in 5-dB steps and the threshold was defined as the lowest level at which waves in the ABR could be clearly detected by visual inspection. In the s-EGFP and s-EGFP-9R Groups, the hearing thresholds were measured at 28 days after protein transduction. In the s-XIAP and s-XIAP-9R Groups, the ABR was measured 1 day before noise exposure and on 14 days after noise exposure.

Morphological analyses

In the s-EGFP and s-EGFP-9R groups, the thicknesses of the RWM at 24 hours after treatment were assessed. The bilateral cochleae were serially cryosectioned to a thickness of 8 μm in each group. Nomarski images of the whole RWM were captured using a BZ-9000 microscope (Keyence, Osaka, Japan) and then they were stored on a computer at a density of 96 dpi. The center part of the RWM was cut out from the captured RWM images. Subsequently, the lengths and areas of the RWM in the cut-off images were measured utilizing ImageJ software (NIH, Bethesda, MD). The values obtained by dividing the areas by the length were utilized for statistical analyses.

For hair cell counting, in the s-EGFP and s-EGFP-9R Groups, the cochleae were removed from the temporal bone 28 days after treatment. After fixation overnight at 4 °C, the bony capsules and lateral walls of the cochleae were removed. The OCs were incubated with Texas Red-X phalloidin (Molecular Probes) for 30 minutes after blocking with 0.3% Triton-X in PBS for 10 minutes. During the blocking process, the tissues were washed and punctured three times each with PBS. The surface images of the OCs were captured and stored on a computer. The numbers of IHCs and OHCs in 300 μm lengths of the OCs in each turn were counted, and the survival rate of the hair cells (HCs) was measured.

In the s-XIAP, s-XIAP-9R, and saline groups, similarly, OCs were harvested on 14 days after noise exposure. After immersion in the fixative agent overnight at 4 °C, the temporal bones were decalcified in 0.1 M EDTA (pH 7.4) containing 5% sucrose and stirred at 4 °C for 2 days. After decalcification, the cochlea was microdissected. The OCs were incubated with primary antibodies overnight at 4 °C after blocking with 0.1% Triton-X in PBS supplemented with 5% goat serum for 1 hour. During each process, the tissues were washed three times for 20 minutes each with PBS. Primary antibodies were detected using secondary antibodies and viewed under confocal fluorescence microscopy (Nikon CI, Japan). Hair cells were identified using anti-myosin VIa antibody (Proteus Biosciences, San Diego, CA) as the primary antibody and Alexa 350 (Invitrogen, Carlsbad, CA) as the secondary antibody. ImageJ software (NIH) was used to measure the total length of the cochlear whole mounts, and a cochlear frequency map was computed to precisely localize IHCs based on frequency-specific regions. ImageJ plugin (http://www.masseyeandear. org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-histology-resources/imagej-plugin-for-cochlear-frequency-mapping-in-whole-mounts) that translates cochlear position into specific and a cochlear frequency map was computed to precisely localize IHCs based

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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