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Enhancement of Trans-Tympanic Drug Delivery by Pharmacological Induction of Inflammation

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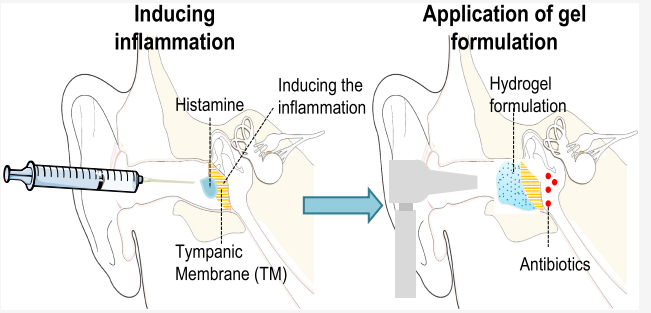
ABSTRACT: Drug delivery directly across the tympanic membrane (TM) could eliminate systemic exposure to antibiotics prescribed for otitis media, the most common reason for pediatricians to prescribe antibiotics. Here, we hypothesized that inducing inflammation of the TM could enhance drug flux across the TM. We demonstrated that the flux of ciprofloxacin across the TM was greatly increased by treatment with the proinflammatory agent histamine. That enhancement was blocked by concurrent treatment with blockers of histamine receptor 1. Treatment of the TM with histamine was able to enhance drug flux sufficiently to eradicate otitis media in vivo in chinchillas, but only if the histamine was applied prior to treatment with antibiotics.



KEYWORDS: *trans-tympanic permeation, otitis media, drug delivery, inflammation, histamine*

1. INTRODUCTION

Otitis media (OM) is among the most common causes of pediatric medical visits in the United States.[1,2] By 3 years of age, 80% of children have experienced at least one episode of OM with the peak incidence occurring between 6 and 12 months of life.[3] It is estimated that there are nearly 2.2 million new cases of OM in the United States and 300 million cases worldwide annually.[4] The disease is usually triggered by nasopharyngeal colonization by otopathogens such as nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, with subsequent invasion of the middle ear.[5,6] According to the US Centers for Disease Control and Prevention, oral antibiotics are recommended for treating OM for patients aged over 6 months. Oral antibiotics can cause adverse effects such as diarrhea, vomiting, or skin rash,[7] which can limit treatment. Failure of treatment in children can lead to recurrent OM and chronic OM, with increased risk of hearing loss and speech delay.[8,9] Moreover, these frequent administrations of antibiotics are believed to contribute to the emergence of antibiotic-resistant bacteria.[10] A drug delivery system that could place drugs directly into the middle ear through the tympanic membrane (TM) would bypass the systemic circulation and would obviate all of these undesirable effects. Our group previously developed such a hydrogel drug delivery system where the use of chemical permeation enhancers (CPEs) facilitated a sustained flux of high concentrations of antibiotics into the middle ear.[11,12]

The TM is a trilaminar membrane that separates the external ear and the middle ear cavity.[13] It is about 0.8−1 cm in diameter and 40−120 mm thick.[14] It is composed of three layers. The outermost layer, a stratified squamous keratinized epithelium continuous with the epidermis of the external auditory canal,[15,16] is an impenetrable barrier to drug delivery to the middle ear.[17] In OM, the TM is approximately 5-fold thicker due to inflammation. Nonetheless, inflamed TM have ∼5 times the permeability of pristine ones.[18] We have hypothesized that inflammation itself increased the permeability of the TM to drugs, and that the resulting increase in flux could be induced by application of a proinflammatory agent such as histamine. Histamine is a proinflammation agent produced primarily in the granules of tissue mast cells.[19,20] Inflammation increases skin vascular and epithelial paracellular permeability.[21−24] Inflammation disrupts the apical junctions of the epithelial barrier, increasing epithelial permeability.[25] Inflammation can increase flux across skin,[26] which is structurally similar to the TM, and other structures. For example, transport of dexamethasone across the round window membrane into perilymph (inner ear) was increased by histamine.[27] Here, we demonstrated that transtympanic drug permeation was enhanced by histamine, and that the local

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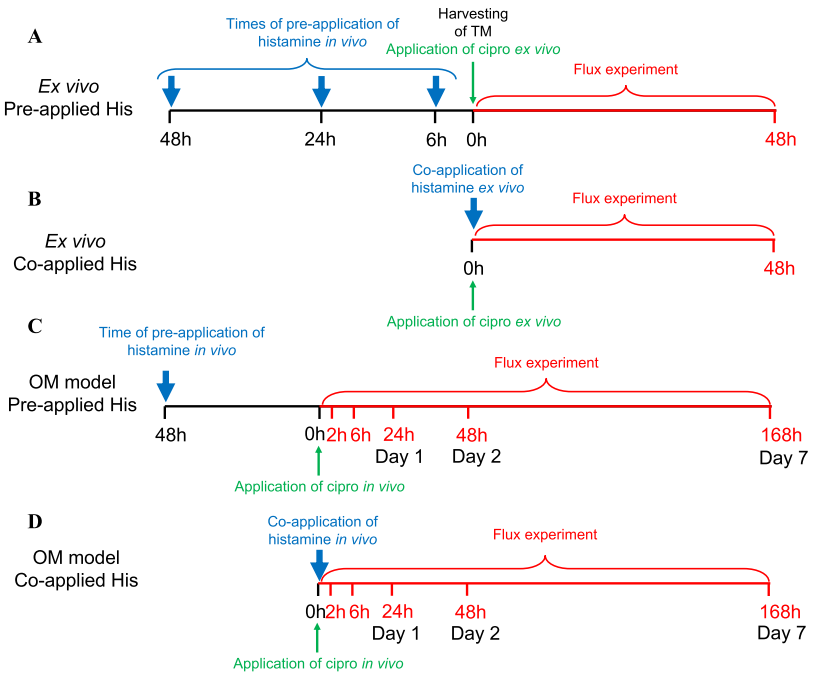

Figure 1. Experimental schemas of (A, B) ex vivo and (C, D) in vivo studies.

application of small doses did not lead to systemic distribution or toxicity.

2. EXPERIMENTAL SECTION

2.1. Materials. Pharmaceutical-grade histamine, loratadine, diphenhydramine, and ciprofloxacin hydrochloride were used as received from Sigma-Aldrich (St. Louis, MO). US pharmaceutical grade Kolliphor P 407 microprilled (poloxamer 407) was used as received from BASF (Florham Park, NJ).

2.2. Hydrogel Formation. 18% (w/v) P407 hydrogel formulations were made by dissolution in deionized water. Ciprofloxacin, CPEs, and histamine were added subsequently and maintained at 4 °C with gentle stirring.

2.3. Ex Vivo Permeation Experiment. The cross-TM permeation rate of ciprofloxacin was determined with auditory bullae harvested from healthy chinchillas. 200 μL of all formulations (containing 8 mg ciprofloxacin) were applied into the bullae at 37 °C so as to be deposited onto the TMs. Permeation of ciprofloxacin across the TM into the receiving chamber was quantified at pre-determined intervals, using LC-MS. In experiments where histamine was pre-applied, the ex vivo experiment was preceded (6, 24, or 48 h prior to harvesting of the bullae) by application of 0.5% histamine solution onto their TMs via a soft catheter. In some experiments, the H1 blockers loratadine or diphenhydramine where co-administered with the histamine. Details regarding TM harvesting, TM electrical resistance measurements (to document integrity), and other aspects of the ex vivo permeation experiments have been reported.[28]

2.4. *NTHi* OM Model and Pharmacokinetics. All animal procedures were performed using isoflurane/oxygen anesthesia in accordance with approved IACUC protocols at Boston Children's Hospital. Baseline plasma samples for drug levels were obtained through the cephalic sinus. *NTHi* isolates were inoculated into brain−heart infusion medium at 37 °C for 12−18 h in a shaking incubator (150−250 rpm) and grown to the mid log phase. The liquid culture was diluted in Hank's balanced salt solution (HBSS), and about 25−75 colony forming units (CFUs) in 100 mL were inoculated directly into the middle ear bulla under aseptic conditions. Once middle ear infection was confirmed using methods described previously,[11,12] the chinchillas were treated with 200 μL of test formulations applied via a soft catheter directly on to the TM through the outer ear canal under anesthesia. Prior to application of ciprofloxacin-containing formulations and 2, 6, 24, 48, and 168 h after, ∼100 μL of middle ear fluid was collected from the superior bulla (i.e., not through the TM) through a small incision with an 18-gauge angiocatheter connected to an empty tuberculin syringe. The fluid (10 μL) was diluted 1:10 in HBSS then inoculated onto blood agar plates for bacterial identification and quantification. Blood samples were obtained at the same times. Middle ear fluid and blood samples were obtained under anesthesia. Ciprofloxacin concentrations were quantified by LC-MS.

2.5. Histopathology. After the last collection time point, animals were sacrificed, TMs were excised and immediately fixed in 10% neutral buffered formalin. TMs were sectioned (5 μm thick) and stained with hematoxylin and eosin (H&E) by iHisto INC (Salem, MA). All stained specimens were evaluated under light microscopy.

2.6. Statistical Analysis. Results are reported as averages with standard deviations, and the differences among treatments were assessed by 2-tailed unpaired Student' *t* tests. Two-tailed $P < 0.05$ and $P < 0.01$ was considered statistically significant and highly statistically significant, respectively. These analyses were carried out using statistical analysis software (SPSS, IBM Corporation, Armonk, NY).

3. RESULTS

3.1. Ex Vivo Efficacy. TMs were harvested, and the flux of ciprofloxacin across them was assessed by placing a drug solution on the outer aspect of the TM and measuring drug accumulation on the other side (Figure 1A). In separate animals, a single dose of 0.5% histamine solution was applied

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to the TM of healthy chinchillas at 6, 24, or 48h prior to harvesting of the intact TMs (Figure 1A) for studies of drug flux ex vivo. (In such experiments, the histamine was "pre-applied" as compared to being applied at the same time as the drug.) Histamine pre-application led to marked thickening of the TM with infiltration of inflammatory cells and edema. (Figure 2). Pre-application of histamine greatly increased the

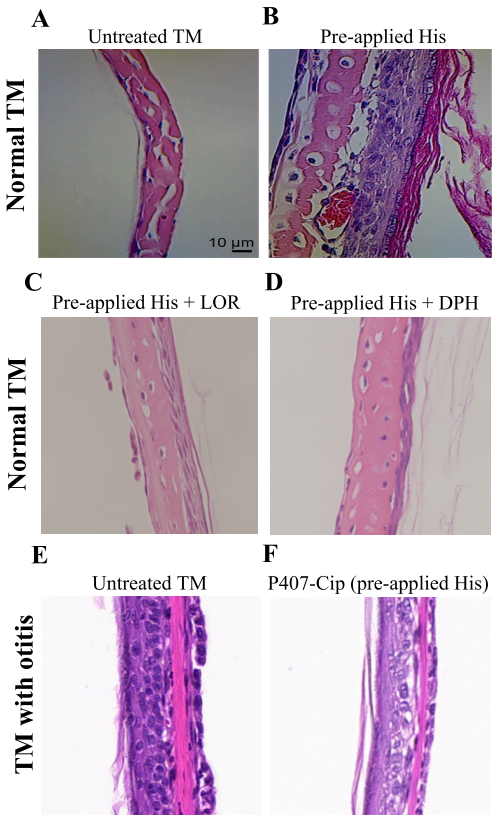

Figure 2. Representative H&E-stained sections of TM cross sections. (A) Healthy TM. (B–D) Healthy TM with pre-applied histamine (pre-applied His) alone (B) or with loratadine (LOR; C) or diphenhydramine (DPH; D). H&E-stained sections of TM after 7 days of otitis media infection without treatment (E) or with after pre-treating with histamine followed by administration of P407-Cip (F).

trans-tympanic flux of ciprofloxacin (4% Cip; Figure 3A). The flux of ciprofloxacin increased with longer periods of preapplication of histamine. Pre-application of histamine for 48 h increased the flux of ciprofloxacin 4.5-fold ($P < 0.01$). This enhancement of flux was also seen when histamine was applied before ciprofloxacin contained in a hydrogel was placed on the TM (18% P407–4% Cip; Figure 3B). Incorporation into a hydrogel reduced ciprofloxacin flux ~1.6-fold, but this reduction was not statistically significant ($P > 0.05$). Here also, the flux of ciprofloxacin was greatly increased by pre-applied histamine and was greater with longer periods of preapplication. 48 h of pre-application of histamine increased the flux of ciprofloxacin by 6.7-fold ($P < 0.01$).

We had hypothesized that the increase in flux would be due to inflammation. An alternate explanation could be that histamine was enhancing flux through a direct effect on the TM itself, i.e., acting as a CPE. Co-application of CPEs has been shown to increase the trans-tympanic flux of ciprofloxacin.[28] To determine whether histamine was acting as a permeation enhancer, we measured ciprofloxacin flux across TMs ex vivo as above, but where histamine was applied to TMs at the same time as ciprofloxacin (Figure 3C). The simultaneous and ex vivo application would obviate the impact of inflammation on flux. Co-applied histamine had little or no effect on the flux of ciprofloxacin, whether the drug was free or in a hydrogel ($P > 0.05$). To facilitate comparison of the various formulations, we calculated the ratio of their cumulative flux across the TM ex vivo at 48 h to the flux from the drug alone (Figure 3D). Pre-application of histamine resulted in a greater flux than in any other group.

To further define the mechanism of action of histamine by demonstrating the molecular specificity of its effects, histamine pre-treatments were codelivered with the H1 blockers 1.25% loratadine or 0.5% diphenhydramine (Figure 4). The H1 receptor mediates the inflammatory effects of histamine.[29,30] Both H1 blockers blocked the effect of histamine pretreatment on ciprofloxacin flux ($P < 0.01$). The H1 blockers also prevented the inflammation caused by histamine (Figure 2). The TM thickness was reduced ($P < 0.05$) from 65 ± 10 μm (pre-applied with histamine) to 30 ± 6 μm when histamine was co-applied with one of the H1 blockers, still thicker ($P < 0.05$) than normal (14 ± 3 μm).

3.2. In Vivo Efficacy. We have previously shown that bacterial OM can be eradicated by a hydrogel-based system from which the flux of ciprofloxacin could be enhanced incorporated CPEs.[18] We studied whether the increase in flux by histamine could achieve the same goal, performing the same function as CPEs. OM was established in chinchillas by direct inoculation of bacteria (Nontypeable *Haemophilus influenzae*, *NTHi*) into the middle ear through the bulla on day 0 (Figure 1C,D). On day 3, a 4% Cip in solution or in an 18% P407 gel was placed on the TM of all animals. In some animals, 0.5% histamine was applied on day 1 (i.e., 48 h before application of the formulation). In others, 0.5% histamine was incorporated into the formulation (i.e., it was co-applied).

In animals treated with 4% Cip with or without P407, ciprofloxacin levels in middle ear fluid were relatively low (Figure 5A) and did not lower bacterial counts (CFUs) in the middle ear fluid (MEF; Figure 5B) or clear infections completely (Figure 5C). In animals treated with 4% Cip in P407, pre-application of histamine caused a 4-fold increase in the peak MEF ciprofloxacin level ($P < 0.01$) compared with 18% P407–4% Cip treatment without pre-application of histamine. The peak level, 186.2 μg/mL, was at least 370-fold greater than the minimum inhibitory concentration (MIC) of ciprofloxacin for *NTHi* (0.1–0.5 μg/mL). The increase in MEF ciprofloxacin levels was sustained; on day 7, the level was ~45 μg/mL, ~90 times the MIC for *NTHi*. In this group, infection was eradicated within a day. The increase in MEF ciprofloxacin level was not seen in animals where the histamine was co-applied with the gel, and while the CFUs in MEF were reduced, infection was not eradicated. Histamine, whether applied before or with 4% Cip solution, had little effect ($P > 0.05$) on MEF drug levels; infection was not eradicated in either of those groups. This is consistent with our prior observation that a hydrogel is needed to maintain the hydrogel in contact with the TM in order to attain adequate flux.[18,28]

Despite the very elevated drug level in MEFs, ciprofloxacin was undetectable in plasma samples obtained from the transverse sinus of animals treated with pre-applied histamine and 4% Cip in P407 (Table 1).

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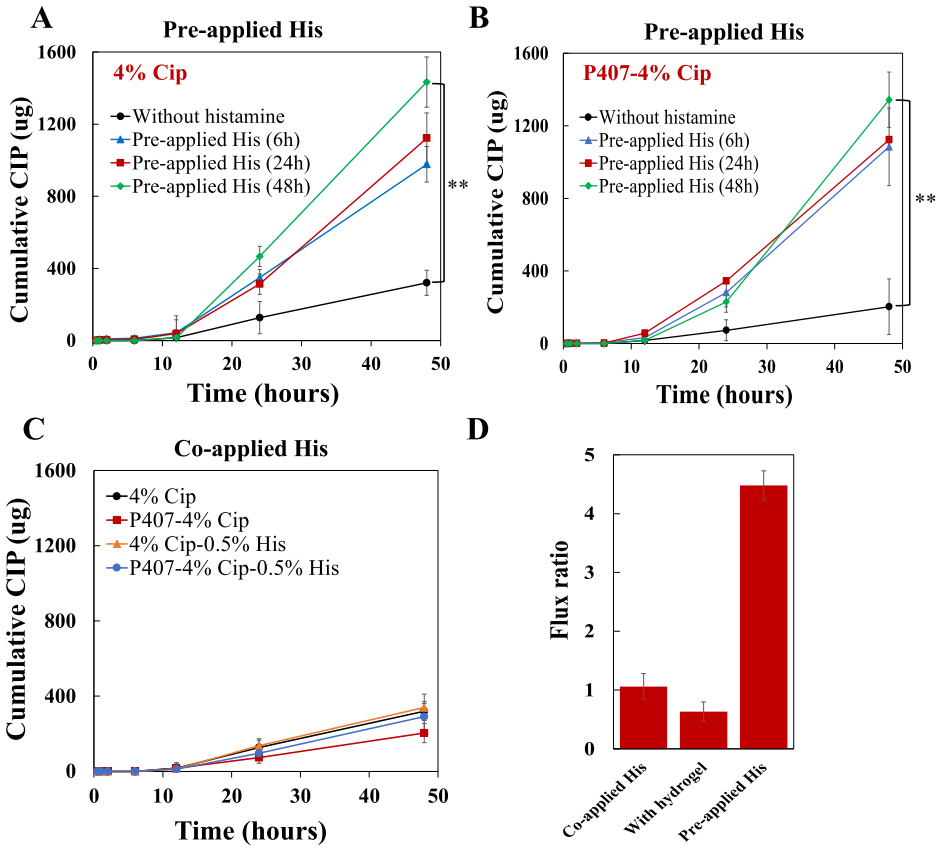

Figure 3. Ex vivo ciprofloxacin flux across the TM. (A) Flux of 4% Cip with and without various durations of pre-treatment with 0.5% histamine (n = 5) (preapplied His). (B) Flux from P407−4% Cip with and without various durations of pre-treatment with 0.5% histamine (n = 5) (pre-applied His). (C) Ciprofloxacin flux from formulations with or without simultaneous application of histamine (n = 5) (coapplied His). (D) Ratio of the cumulative drug flux at 48 h from various associated treatments to the flux from 4% Cip. Co-applied His: 0.5% histamine coapplied with 4% Cip, With hydrogel: 4% Cip applied in P407, Pre-applied His: 0.5% histamine applied 48 h before 4% Cip treatment. Data are means ± SD. **, $P < 0.01$ for the comparison of the Cip flux at 48 h from formulations with and without pre-application of histamine 48 h prior to ex vivo studies.

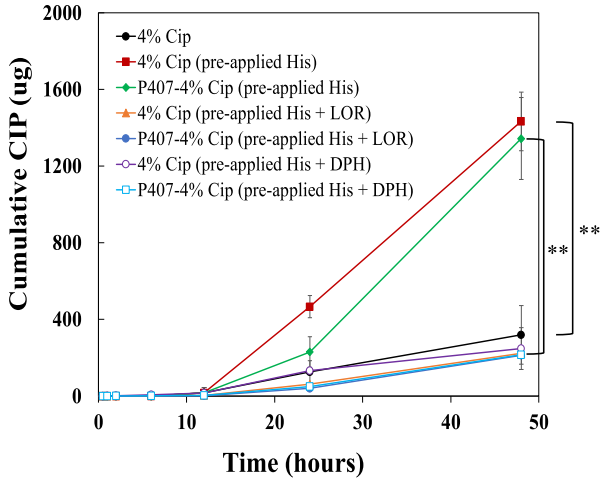

Figure 4. Ex vivo ciprofloxacin flux across TM with or without pre-treating the TM (48 h before flux studies began) with 0.5% histamine (pre-applied His) alone or with loratadine (LOR, 1.25%), or diphenhydramine (DPH, 0.5%). Data are means ± SD (n = 5). **, $P < 0.01$ for the comparison of the Cip flux at 48 h from P407−4% Cip or 4% Cip with pre-application of histamine to the same flux with coapplication of LOR or DPH.

3.3. In Vivo Biocompatibility. The TMs of animals with OM were much thicker (57 ± 8 μm) than those from healthy animals (14 ± 3 μm) ($P < 0.01$; Figure 2). In animals treated with pre-applied histamine and % Cip in P407, the TM thickness was reduced to 42 ± 6 μm compared with untreated TMs ($P > 0.05$), still thicker than normal ($P < 0.01$). This persistent thickening could be due to a sustained effect of histamine.

4. DISCUSSION

Pre-treatment with histamine markedly increased trans-tympanic drug flux, resulting in clearance of middle ear infection. This supports the view that inflammation *per se* could contribute to the flux of drugs across the infected TM seen with other trans-tympanic systems developed for the treatment of OM.[11,18] This increase in flux occurred despite a marked thickening of the TM.

An alternative explanation for the effect of histamine on flux could have been that histamine itself increased drug flux across the TM by a mechanism unrelated to its proinflammatory activity, such as by acting as a CPE. We have previously shown that CPEs can greatly increase flux across the TM when co-applied with a drug of interest.[11,18,31] Here, histamine enhanced trans-tympanic flux when given prior to administration of ciprofloxacin, allowing inflammation to develop, and not when co-applied, suggesting that the mechanism was not by a CPE-like effect. Furthermore, the fact that histamine-reduced flux across the TM was reduced by H1-blockers suggests that histamine acted through a known molecular

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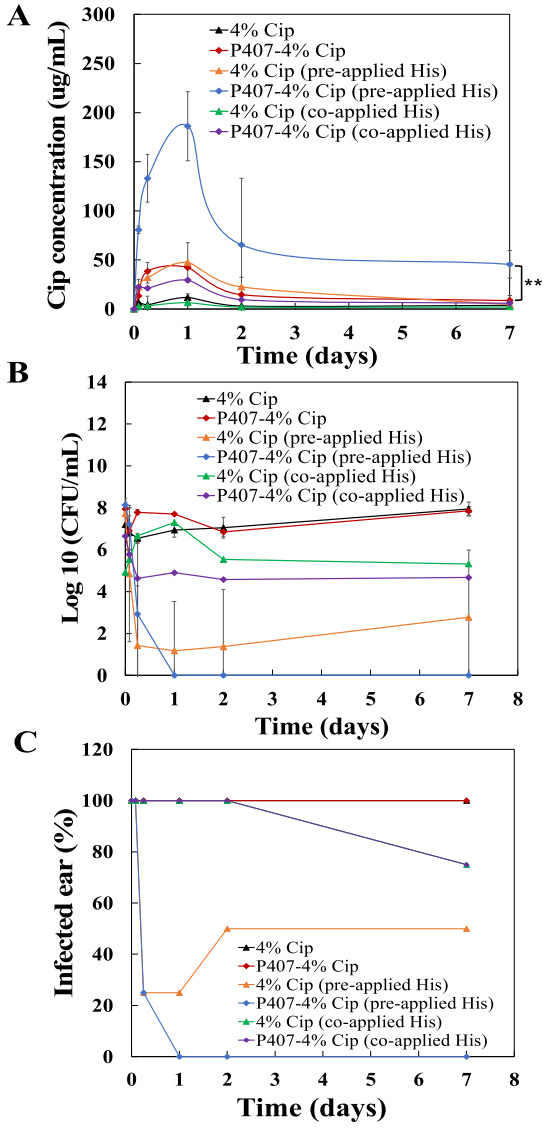

Figure 5. Efficacy of formulations. Time course of (A) concentration of ciprofloxacin (Cip) and (B) bacterial CFUs in middle ear fluid. (C) Percentage of animals with OM (defined as nonzero CFU values in their middle ear fluid aspirates) in chinchillas with OM from *NTHi* treated with different formulations. Data are means ± SD (n = 5). **, $P < 0.01$ for the comparison of the Cip flux at day 7 from P407−4% Cip with and without pre-application of histamine.

Table 1. Blood Ciprofloxacin Levels in Animals with OM Treated with Histamine 48 h before P407-4% Cip[a]

time	concentration of ciprofloxacin in plasma (μg)
0	not detected
2 h	not detected
6 h	not detected
1 day	not detected
2 days	not detected
7 days	not detected

[a]Drug levels were determined by LC-MS at pre-determined intervals after gel application. The detection limit for ciprofloxacin by LC-MS was 25 ng/mL.

mechanism that causes inflammation and not a nonspecific CPE-like effect. Interestingly, co-application of histamine with ciprofloxacin, which was not effective in increasing flux here, has been reported to increase flux of dexamethasone across the round window,[27] even though they used a lower concentration of histamine (0.1%) This could be due to difference in barrier structure or drug.

Middle ear drug levels remained elevated throughout the experiment (7 days). The flux level at day 7 was comparable to that in studies where flux was increased by CPEs and the same concentration of ciprofloxacin was used.[11] With histamine, the elevated flux was presumably due to sustained inflammation from the histamine. This view is supported by the fact that the TMs were still thickened and had inflammatory cells at 7 days. With CPEs, the TMs had returned to a normal appearance by day 7.[11]

Pre-treatment with histamine enabled eradication of otitis media with ciprofloxacin, showing that this approach can be used to treat middle ear diseases. However, the fact that histamine only was effective when pre-applied presents a practical limitation in the treatment of acute OM: Patients would not be expected to present for treatment 2 days in advance. However, it could still be helpful in therapeutic failures of oral antibiotics (due to the very high middle ear fluid levels achieved) or in chronic otitis media. We also note that taking oral anti-inflammatory agents (e.g., as analgesics) could mitigate the response to topical histamine.

Despite supratherapeutic drug levels in the middle ear, there was no systemic drug distribution detected in the blood samples. Avoiding systemic exposure is important in order to minimize systemic side effects such as rashes and diarrhea and to avoid the development of antibiotic resistance.[32−34] In the particular case of antibiotics such as fluoroquinolones, there is the concern that systemic exposure could have musculoskeletal effects.[35] Note that local administration, and the consequent absence of systemic administration, enables the use of ciprofloxacin in ear drops in children in approved products such as CiproDex ear drops.

Delivery of 1% histamine directly into the middle ear and onto the round window has been reported to be safe.[36] We used one-half that concentration of histamine in this study, but placed it on the outer surface of the tympanic membrane (i.e., outside the middle ear). If the flux of histamine across the TM is comparable to that of ciprofloxacin (>100-fold reduction in concentration), then it is very unlikely that there would be any deleterious effects.

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Author Contributions

[§]Z.Z. and X.L. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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