Peptides Rapidly Transport Antibiotic Across the Intact Tympanic Membrane to Treat a Middle Ear Infection

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Article

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Abstract

The tympanic membrane (TM) forms an impermeable barrier to medical therapies for middle ear (ME) diseases like otitis media. By screening a phage-displayed peptide library, we have previously discovered rare peptides that mediate active transport of cargo across the intact membrane of animals and humans. Since the M13 filamentous bacteriophage on which the peptides are expressed are large (nearly 1 μm in length), this offers the possibility of noninvasively delivering drugs, large drug packages or gene therapy to the ME. To evaluate this possibility, EDC chemistry was employed to covalently attach amoxicillin or neomycin molecules to phage bearing a trans-TM peptide, as a model for large drug packages. Eight hours after application of antibiotic-phage to the TM of infected rats, ME bacterial titers were substantially reduced compared to untreated animals. As a control, antibiotic was linked to wild-type phage, not bearing any peptide, and application to the TM did not affect ME bacteria. The results support the ability of rare peptides to actively deliver pharmacologically relevant amounts of drugs through the intact TM and into the ME. Moreover, since bacteriophage engineered to express peptides are viral vectors, the trans-TM peptides could also transport other viral vectors into the ME.

INTRODUCTION

Middle ear (ME) diseases include otitis media (OM), which affects up to 90% of children [1, 2] and is a chronic/recurrent condition in 10–15% [3, 4]. According to the American Academy for Pediatrics, over 5 million AOM cases occur annually in US children, resulting in >10 million antibiotic prescriptions, ~30 million visits for medical care [5] and 650,000 surgeries per year [6]. It is the most common condition treated with antibiotics, and increasing incidence of antibiotic resistance among the organisms responsible for AOM is a cause for concern [5]. Cholesteatoma is another aggressive middle ear disease in which epidermal cells invade the ME and exhibit destructive growth that can erode middle and inner ear structures, leading to hearing loss or even complete deafness [7].

OM is primarily treated with systemic antibiotics or, in more refractory cases, installation of pressure equalization tubes into a surgical opening in the tympanic membrane (TM) and/or adenoidectomy [8]. While these therapies are often effective, they have potential side effects. The use of systemic antibiotics for such a common disease contributes to the development of antibiotic resistant bacterial strains throughout the body [9, 10]. Due to effects on the gut microbiome they can also produce gastric distress [10], which can be serious in infants [12]. Tympanostomy tubes can lead to TM scarring or tympanosclerosis [13]. Cholesteatoma must be surgically removed, but complete resection is frequently impossible, leading to a high risk of recurrence requiring additional surgery [14]. Drug treatments to inhibit cholesteatoma growth could be valuable, but tissue growth inhibitors delivered systemically would likely have significant side effects.

The local delivery of pharmacotherapy to the ME would be a useful alternative to systemic drugs, reducing side effects and antibiotic-resistant bacteria at other sites [15]. However, the TM is an impermeable structure. Surgically breaching the TM is currently required for local drug delivery to the ME.

Previous experimental studies have used tissue permeants [16] or magnetically driven nanoparticles [17] to transport drugs across the TM. However, no non-invasive methods have yet been approved for safe and effective for human use.

Many epithelial barriers have mechanisms for the active transport of macromolecules across their cells [18, 19]. To determine whether this might also be true for the TM, we used the technique of phage display to search for peptides that would be able to cross the membrane. This method, first introduced in 1985 [20], has been used in numerous biological systems to identify peptides and other molecules with specific properties, including drug delivery. The technique employs large combinatorial libraries of bacteriophage, each expressing a random peptide on its surface (Fig. 1). The libraries are typically screened through multiple rounds of biopanning for specific structural and/or functional characteristics [19]. Utilization of phage display does not require prior understanding of structure or interacting ligand prerequisites. It can be based on biological activity outcome to isolate a peptide sequence that will interact with the desired biologic target process. We chose to apply this method on the TM [19] aiming to identify a cohort of peptide that can target the TM and enter the ME. Repeated sequential screening of the library for trans-TM transport can led to collapse of a naïve library expressing a very large number of peptides to a very small number of clones sequences that can enter the ME.

We screened a 12-mer phage library (PhD-12™, New England Biolabs) containing M13 bacteriophage each expressing one of 10^{10} peptides, as described previously [21]. We tested two screening methods. In the first, we applied the library to the TM for two hours, amplified the ME contents in phage host bacteria, and re-applied the resultant library to the TM, for a total of three rounds. For the second method, we employed sequential screening, first for peptides that bound to the TM, then for those that were internalized, and finally for those that penetrated the TM. For each strategy we sequenced the inserts of 30 phage from the final round. The first strategy resulted in library collapse to four peptides (TMT1-4), with one related peptide (TMT5) found on a repeat screen. The second strategy resulted in sixteen peptide sequences, of which four (BPT1-4) were recovered more than once in our sequenced sample. In all, the nine peptide phage all crossed the TM more efficiently than wild-type (WT) phage without a peptide, although a range of transport efficiencies was observed (Fig. 2). Peptide phage movement across the TM was oxygen and temperature dependent, suggesting active transport. Peptides linked to a DNA template were transported at rates similar to those for peptide phage, indicating that phage is not required for transport [22]. Subsequent structural [23] and inhibitor studies revealed that transport occurred by the process of transcytosis [24]. Finally, an *in vitro* assay was used to demonstrate peptide-mediated transport across the intact human TM [22].

These studies identified peptides with the capacity to actively cross the TM carrying large (~ 1 μm) phage or DNA cargo. The trans-TM peptides identified are not similar to known cell- or tissue-penetrating peptides. Trans-TM transport offers the possibility of efficiently delivering large-molecular-weight drugs, gene therapy vectors and other cargo for the treatment of ME disease. The purpose of the present investigation was to determine whether or not peptide-mediated trans-TM transport could carry

pharmacologically meaningful quantities of a drug across the intact membrane and influence ME disease.

RESULTS

Peptides transport a viral expression vector through the TM

The M13 bacteriophage used to construct the phage library is a bacterial virus that specifically infects *E. coli*. The bacteriophage is employed as a viral vector, to induce expression of a modified version of the filamentous phage protein pIII by the bacterial host during phage reproduction. The modified pIII is then assembled within the bacterium onto the surface of the phage progeny. Five copies of the modified pIII are located at one end of the phage progeny Each pIII has an additional 12 amino acids added to the carboxy terminal, which is the free end of the pIII filament. As noted above, from 10^{10} random peptides expressed in the library, we characterized nine that are transported at levels significantly higher than phage without a peptide. The most efficient of these peptides was recovered from the ME at titers 10,000 times higher than those observed with WT phage (Fig. 2). Recovery of WT phage from the ME, which averaged around 10 phage particles, seemed most likely to consist of contamination rather than trans-TM transport.

Activity of antibiotics linked to peptide or phage

The minimal bactericidal concentration of amoxicillin linked to the synthetic TMT3 peptide, at 10 µg/ml, was equivalent to that of the free antibiotic. In contrast, ciprofloxacin linked to the TMT3 peptide showed reduced activity (1 µg/ml versus 100 ng/ml for free antibiotic) presumably due to steric hindrance (Fig. 3).

The Antibiotics, neomycin and amoxicillin cross-linked to bacteriophage expressing TMT3 retained bactericidal activity but at a reduced level compared to antibiotic alone, also presumably due to steric hindrance (Fig. 4). In contrast, ciprofloxacin linked to phage completely lost activity. Presumably, active sites necessary for ciprofloxacin activity, such as the fluorine that endows quinolones with useful bactericidal properties and/or or the extra carbon atom that dramatically enhances ciprofloxacin activity over that of norfloxacin [25], were blocked or substantially altered by the linkage chemistry.

Antibiotic linked to a trans-TM peptide failed to reduce ME bacterial load

When either amoxicillin or ciprofloxacin linked to TMT3 peptide were applied to the TMs of rats in which the ME had been inoculated with NTHi, no reduction in ME bacterial load was observed after 4 hours of incubation. (Fig. 5). The lack of activity was comparable to that observed when free antibiotic was applied to the TM. This suggested that transport of higher numbers of antibiotic molecules and/or longer dwell times on the TM might be required.

Multiple antibiotic molecules linked to TMT3 phage reduced ME bacterial infection

EDC chemistry links several hundred neomycin molecules to M13 phage [26] and presumably links a similar number of amoxicillin molecules (Fig. 6). We infected rat MEs with NTHi. When amoxicillin or neomycin linked to either TMT3 or BPT3 phage was then applied to their TMs for 8 hours, a significant reduction in ME bacterial titers was observed by comparison to infected MEs that were untreated. As expected, ciprofloxacin linked to TMT3 phage had no effect (Fig. 7). Application of WT phage linked to neomycin also did not reduce ME titers.

DISCUSSION

Summary

We previously observed active transport through the intact TM, mediated by rare peptides either expressed on the surface of M13 bacteriophage or as free peptides linked to a DNA reporter [21, 22]. To test the ability of peptides to transport a drug, individual antibiotic molecules were linked to a peptide. Alternatively, large numbers of antibiotic molecules were linked to phage bearing a peptide as a model drug package. Single antibiotic molecules linked to trans-TM peptide and applied to the TM did not reach the ME at therapeutic concentrations. However, multiple antibiotic molecules attached to trans-TM bacteriophage achieved therapeutic ME levels within hours of application of to the TM.

Delivery of antibiotic to the ME

The results achieved with antibiotic linkage to peptide phage demonstrate that trans-TM peptides can mediate delivery of large numbers of drugs to the ME lumen within hours, quantities sufficient to be pharmacologically effective. Obviously, the use of phage as a delivery substrate is not practical for clinical use. Bacteriophage can stimulate intracellular innate immune receptors. They also been shown to enter mammalian cell nuclei and alter gene expression or even nuclear DNA [27]. Neither use of neomycin, used initially because of the published chemistry for linkage [26], is appropriate for OM therapy, since this antibiotic may enter the inner ear via the round window membrane, and is ototoxic to inner ear sensory cells. However, the reported experiments provide an important proof of concept for the delivery of clinically relevant drug packages across the intact TM. Alternative drug packaging methods, such as drug nanoparticles labeled with trans-TM peptides, should be feasible and would be appropriate for clinical use.

Peptides transport a viral expression vector through the TM

The M13 bacteriophage used to construct the phage library is a bacterial virus that specifically infects *E. coli*. In the peptide phage library, bacteriophage are employed as viral vectors to induce expression of a modified version of the filamentous phage protein pIII by the bacterial host, which is then assembled into

the phage progeny. The discovery of peptide phage that transit the TM establishes that trans-TM peptides are capable of delivering a viral expression vector, one that is much larger than an adenovirus or adeno-associated virus vector, across the intact TM. Future treatment of genetic middle ear disorders, e.g. [28, 29, 30], using gene therapy could be achieved via the attachment of trans-TM peptides to clinically appropriate viral vectors.

Therapeutic implications of direct, active transport across the TM

The isolation of peptides capable of actively transporting large particles (as noted above, M13 phage are approximately 1 μm in length) across the TM provides a potential targeting mechanism for the delivery of therapies into the ME. This could include drug packages, gene therapy vectors or even bactericidal phage. As noted above, the most common current therapy for OM is systemic antibiotics, with a number of problematic side effects. Local application of antimicrobials would eliminate exposure of bacteria throughout the body, reducing the risk of antibiotic resistant strains. Gastric distress would also be eliminated. While local delivery has advantages, in children it currently requires surgical intervention involving general anesthesia to penetrate the TM while avoiding damage to the delicate structures of the ossicular chain and inner ear. A noninvasive delivery mechanism would significantly enhance the practicality of local delivery of antibiotics for OM.

The simplicity of noninvasive local drug delivery would also be beneficial in those developing countries for which limited access to advanced clinical care means that OM is undertreated [31]. According to the WHO, undertreated OM causes an estimated 28,000 deaths/year due to meningitis, and is responsible for half of the world's burden of handicapping hearing loss, making it the world's leading cause [32, 33]. Widespread antimicrobial resistance has led to resurgence of interest in the treatment of bacterial infections potentially using in lytic bacteriophage [34, 35, 36]. Bacteriophage therapy has been effectively applied to resolve longstanding otitis externa due to antibiotic-resistant *Pseudomonas* infection [37]. Bacteriophage-based therapies approach has particular utility in that the phage can evolve in step with bacteria [38] reducing the trade-off in development of bacteria resistant to phage [39]. Bacteriophage have also been shown to infect bacteria in biofilms [40], which are thought to contribute to OM chronicity [41]. The ease with which bacteriophage can be genetically engineered [42] suggests that induction of trans-TM peptide expression in phage lytic for OM bacteria could be practical, and would provide a novel noninvasive OM therapy. Finally, drugs to limit cholesteatoma growth and recurrence could also be delivered across the intact TM, to reduce the need for revision surgeries.

Future directions

A number of important questions regarding the transport of peptides across the TM remain unanswered. Major issues for potential therapeutic applications are whether peptides have any negative effects on the ME or inner ear. We recently determined that direct exposure of the ME to phages bearing peptides TMT1 through TMT4 does not produce ME inflammation or impact hearing thresholds over the course of 4 days

[22]. More detailed and longer-term safety studies will help to determine the potential clinical potential of active trans-TM transport. Since this study was performed in rats, whether antibiotics can be delivered in effective quantities through the human TM also needs to be evaluated.

MATERIALS AND METHODS

Animals

Experiments were performed using 60–90 day old Sprague Dawley rats (Envigo). All animal studies were performed to in accordance with the National Institutes of Health standards for the Care and Use of Animals in Research guidelines, and were approved by the Institutional Animal Use and Care Committee of the San Diego VA Healthcare System. The study is reported in accordance with ARRIVE guidelines.

Infection of the ME

Rats were anesthetized with rodent cocktail (4.0 mg/kg xylazine, 0.75 mg/kg Acepromazine, and 40.0 mg/kg ketamine i.p.). The ME bulla was approached ventrally from a midline incision in the neck. A small opening in the bullar bone was made with a 25g needle, and $5x10^3$ PFUs of nontypeable *Haemophilus influenzae* (NTHi) were injected through a 28g needle in 50 µl of saline, using an angle that would not damage the tympanic membrane.

Preparation of bacteriophage

Tetracycline resistant *E. coli* ER2738 carrier bacteria (New England Biolabs) was used to amplify M13 filamentous phage, allowing blue/white screening using IPTG/X-gal plates for all the subsequent titration steps. One-liter cultures of *E. coli* ER2738 bacteria were infected with phage and grown for 5.5 hours. The bacteria were removed by centrifugation, and phage were then precipitated from the supernatant by the addition of 20% (wt/vol) polyethylene glycol 8000 with 2.5 M NaCl, followed by two rounds of centrifugation as described previously [21, 22]. The final phage pellet was suspended in sterile PBS at a concentration of 10^{10} PFU/µL and stored at 4°C.

Linkage of antibiotic to trans-TM peptide

The trans-TM peptide TMT3 [21], covalently linked to either amoxicillin or ciprofloxacin, was commercially synthesized (Biosynthesis, Inc., Louisville, TX). Linkage was to the carboxy terminus of the peptide (the end which is linked to phage protein pIII in peptide-phage) allowing the presumed active end of the peptide to remain free. Antibacterial activity of the conjugate was assessed by incubation with NTHi growing in liquid culture and was compared to the unconjugated antibiotic.

Linkage of antibiotic to trans-TM bacteriophage

To conjugate antibiotic to bacteriophage expressing a trans-TM peptide we employed 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, ThermoFisher Scientific) as a linking agent. EDC can covalently bind amoxicillin, ciprofloxacin or neomycin to the pVIII bacteriophage protein (Fig. 6).

Neomycin was chosen because an EDC procedure for linkage to the phage major coat protein pVIII had previously been developed by Yacoby et al. [26], and shown to result in the conjugation of several hundred antibiotic molecules per individual phage. The phage pVIII protein, of which 2,700 copies are expressed along the length of the phage capsid [43], contains three carboxylic amino acids (Glu2, Asp4 and Asp5) and five free amine groups (Lys8, Lys39, Lys43, Lys44, Lys48) that can be conjugated by application of EDC chemistry. Following their published protocol [25], two peptide-phage with high trans-TM transport characteristics, TMT3 and BPT3 [20], were conjugated with neomycin. TMT3 phage was similarly conjugated with amoxicillin or ciprofloxacin.

For the conjugation, 0.4 mg of EDC reagent was added to the phage (in a 5 mL volume) one hour prior to addition of amoxicillin or neomycin, to allow binding of the appropriate EDC moiety to phage (Terminal COOH, Asp, and Glu). In the case of ciprofloxacin, EDC reagent was added to ciprofloxacin first, to allow binding of the carboxylic acid side group. Neomycin, amoxicillin or ciprofloxacin were dissolved in 1.0 mL of conjugation buffer (0.1M Sodium citrate buffer pH 5, 0.75 M NaCl) at a final concentration of 2.5mM. This antibiotic mixture was then added to the EDC-phage mixture and incubated at room temperature for 2.0 hours in a rotary mixer. The targeted drug-carrying phage were separated from the reactants by two dialysis steps for 16 hours against 1,000 volumes of sterile phosphate buffered saline. The antibacterial activity of each antibiotic-phage conjugate was then evaluated by incubation with NTHi in liquid culture, and compared to the activity of unbound antibiotic.

The phage concentration of the preparation was determined by measuring the absorption at 269 nm and 320 nm using Nanodrop (ThermoFisher) and then using phage concentration calculator: http://www.abdesignlabs.com/technical-resources/phage-calculator/.

Bacterial growth curves

A stock solution of NTHi (5 mL) was grown, in Brain Heart Infusion (BHI) media enriched with 5% Fildes (BD Diagnostic Systems), overnight at 37°C with shaking. Next day a growth curve was started by addition of 100 µL of NTHi stock culture to 1 mL tube of fresh BHI media plus 100 µL of antibiotic solution or antibiotic-phage to be test and growth was followed by monitoring the OD_{600} every hour for the first 8 hours and then 24h later. Measurements were carried out in triplicate for each condition and bacterial growth analyzed using GraphPad Prism 9.0 software.

Application of antibiotic linked to peptide or phage to the TM in vivo

Immediately after NTHi inoculation of the ME, TMT3 peptide linked to amoxicillin (AMX) or to ciprofloxacin (CIP) was applied to the TM in saline at 10^3 times the minimal effective dose, as established previously by incubation with NTHi in liquid culture, and allowed to remain on the TM for 4 hours. The concentration of AMX-TMT3 peptide was 10 mg/mL while concentration of CIP-TMT3 peptide was 1 mg/mL. The ME contents were then removed and NTHi tittered onto chocolate agar plates.

TMT3 phage covalently linked to amoxicillin or neomycin was similarly applied to the TM of infected MEs at 10^3 times the minimal effective dose, and incubated for 8 hours. NTHi titer of the ME contents was then performed. NTHi-infected control animals received either antibiotic alone, or neomycin conjugated to wild-type M13 phage not bearing a peptide, respectively. The phage titer was 10^8 PFU/μL for all constructs applied.

Declarations

DISCLOSURE

From 2008–2022, Dr. Allen F. Ryan was an advisor to Otonomy Inc., which developed slow-release drug treatments for the ear. This relationship has been approved by the UCSD Committee on Conflict of Interest, and the company played no part in the research presented here.

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Data Availability

The datasets used and/or analyzed during the current study available from the corresponding author on request.

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Figures

Fig. 1

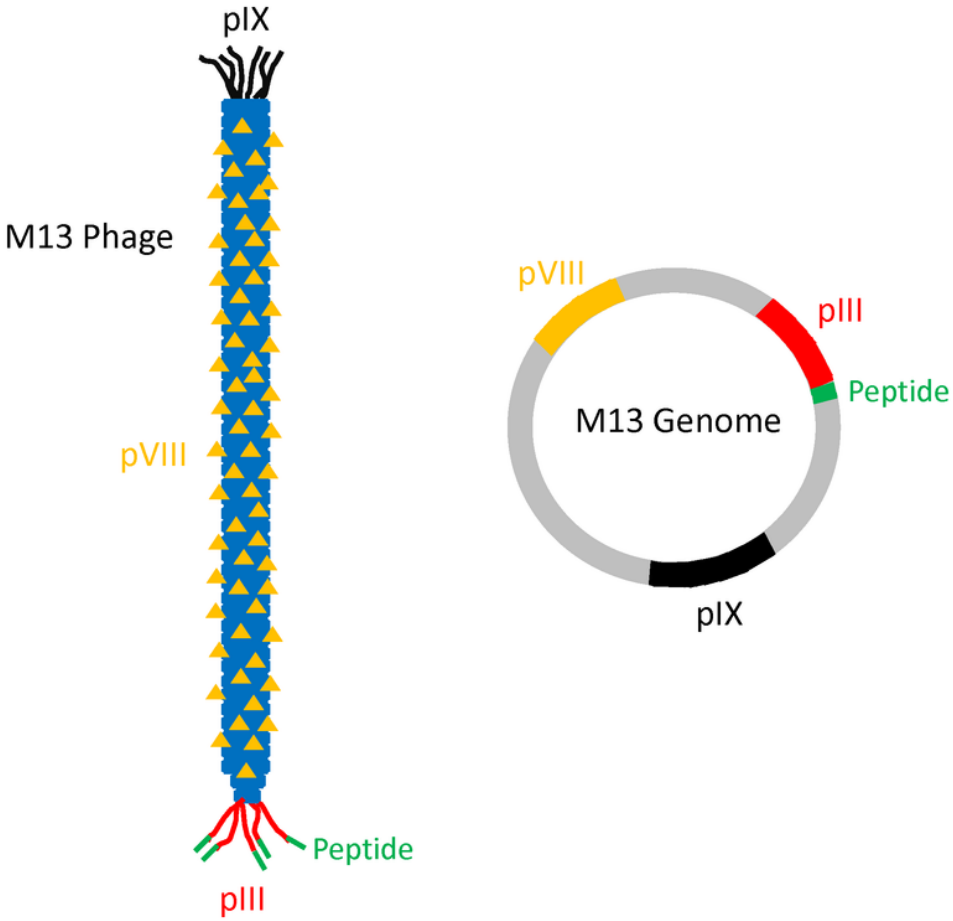

Figure 1

Schematic representation of M13 phage engineered to express a peptide at the free ends of the pIII filaments. Each phage has only five PIII molecules. In contrast, the body of the phage, shown substantially shortened here for display purposes, contains thousands of PVIII molecules on its surface.

Fig. 2

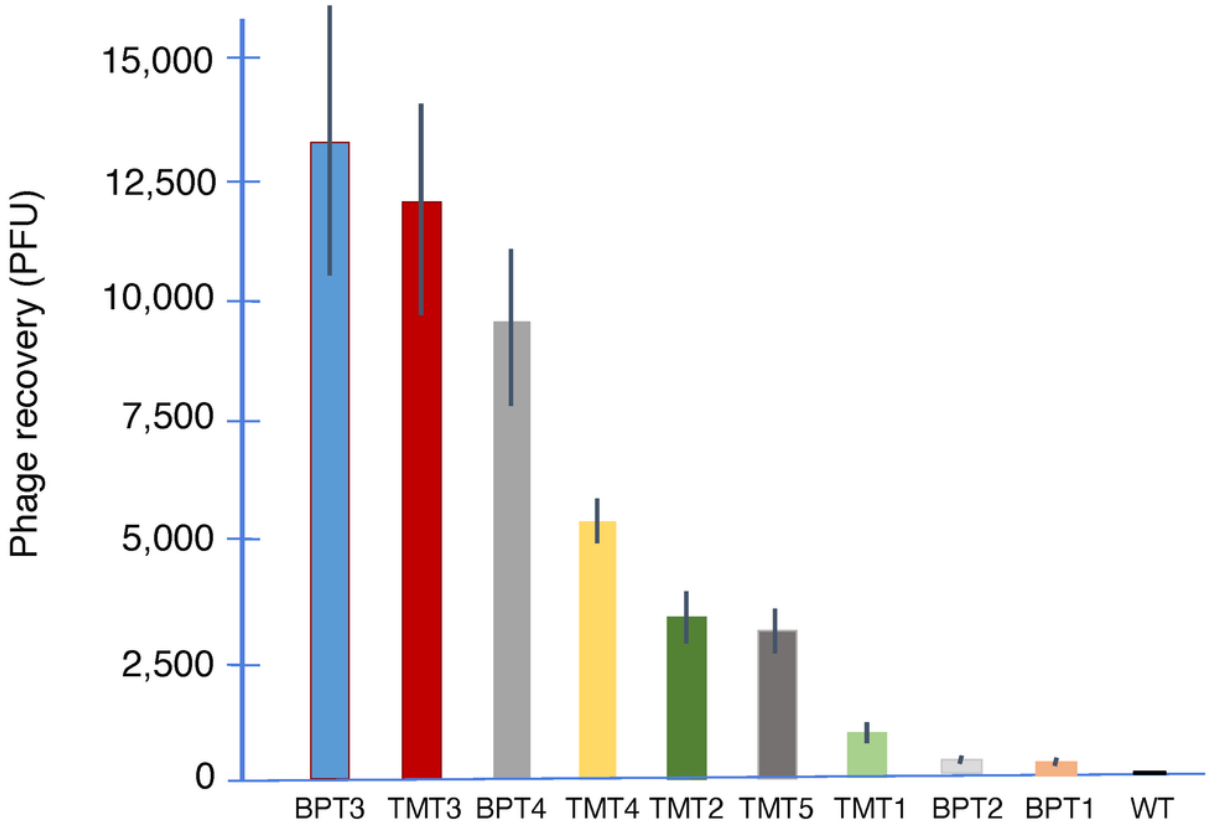

Figure 2

Phage recovery from the ME for nine trans-TM peptide phage, reflecting a range of peptide transport rates (adapted from [21]). The present study employed the TMT3 and BPT3 peptides and peptide phage.

Fig. 3

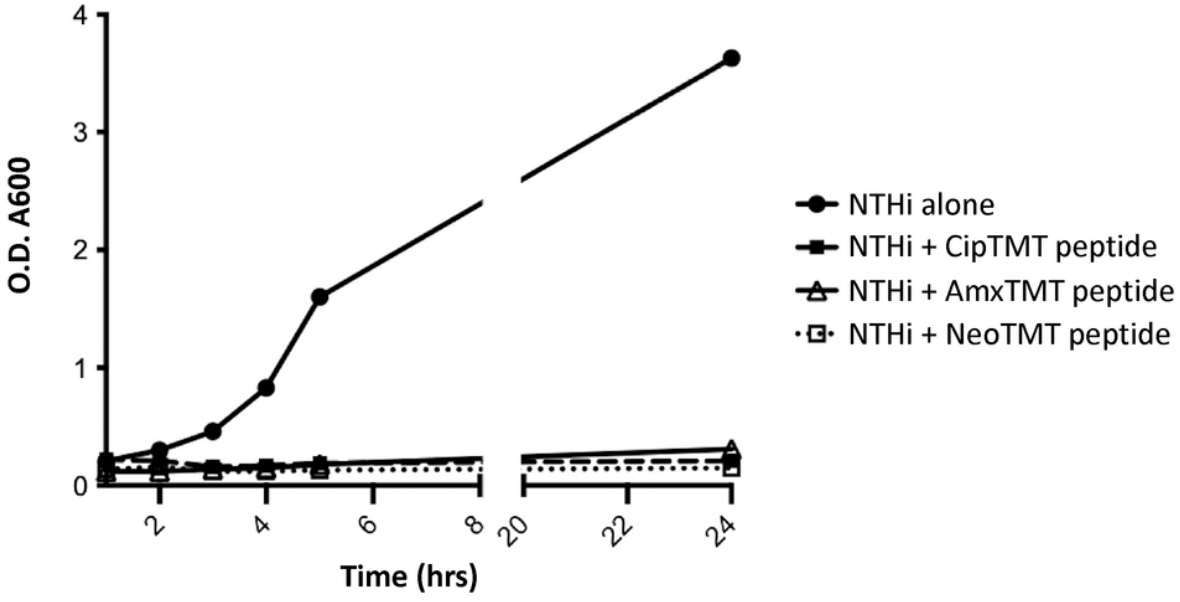

Figure 3

Linkage to peptide does not affect amoxicillin activity against NTHi in liquid culture. Recovery of NTHi after 8 hours in liquid culture media with amoxicillin or amoxicillin linked to TMT3 peptide.

Fig. 4

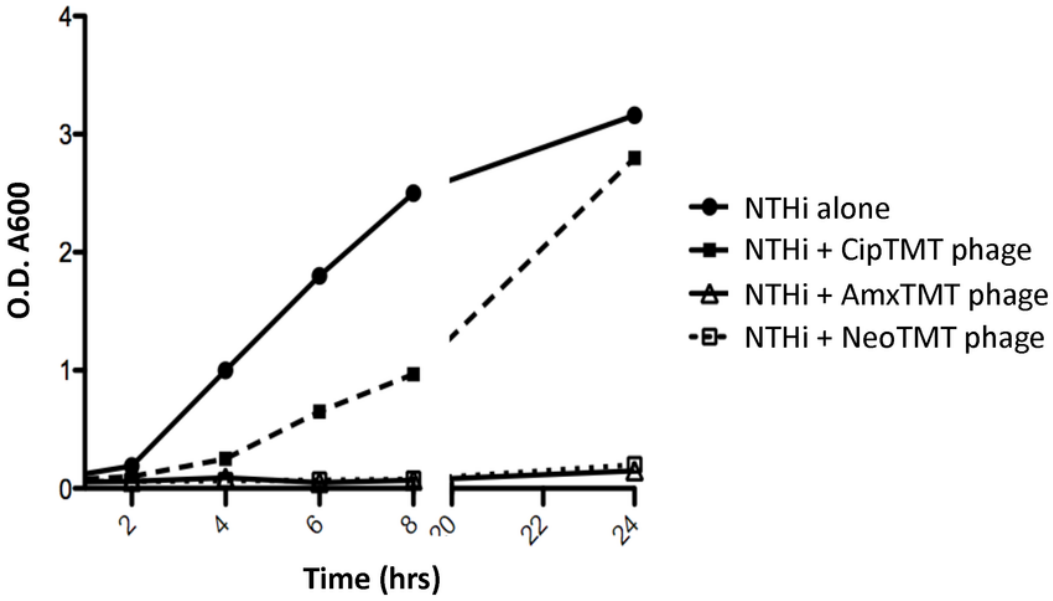

Figure 4

Neomycin and amoxicillin molecules crosslinked to bacteriophage retained anti-bacterial activity, but ciprofloxacin showed reduced activity.

Fig. 5

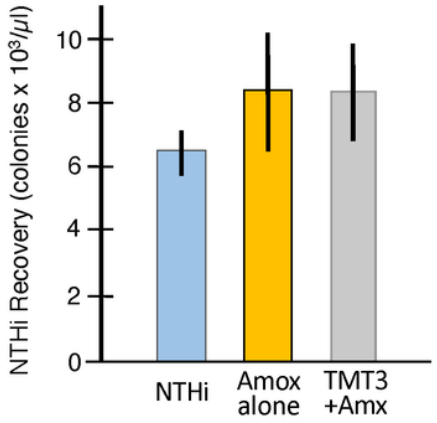

Figure 5

Antibiotics linked to a trans-TM peptide applied to the TM of an infected ear had no effect on NTHi titers.

Fig. 7

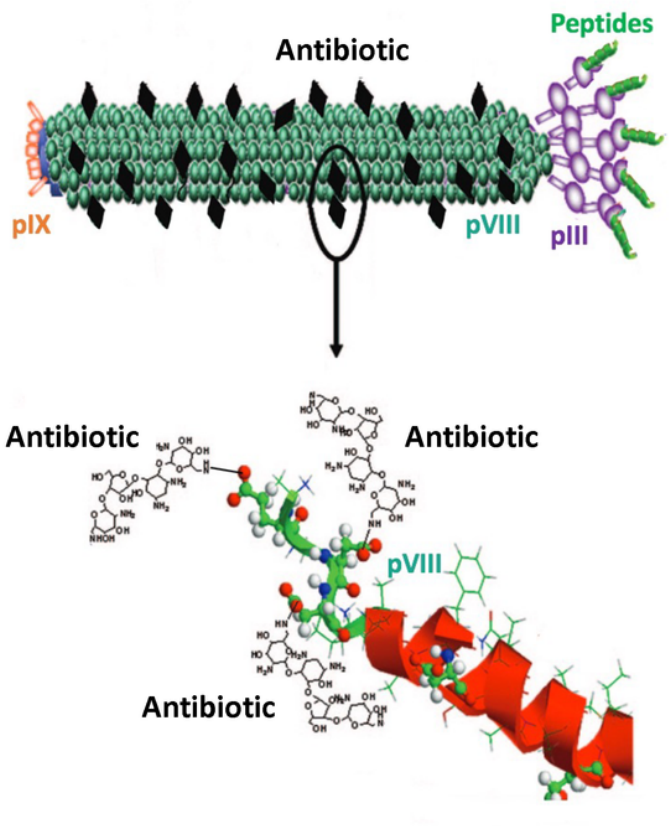

Figure 6

Schematic diagram: Linkage of antibiotic to phage using EDC mediated coupling.

Fig. 6

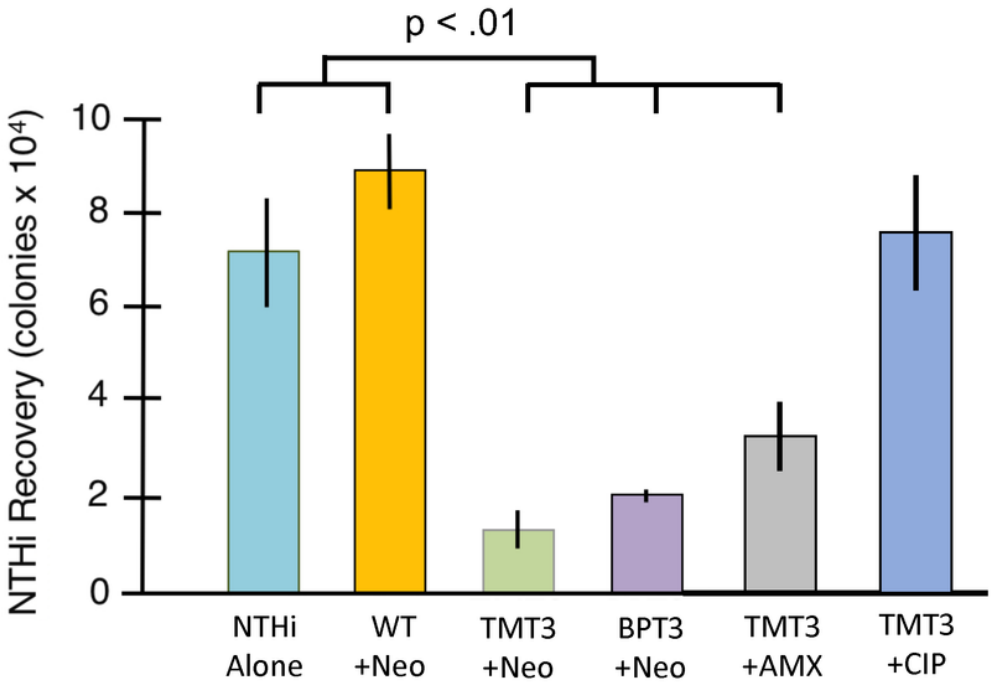

Figure 7

Application of neomycin or amoxicillin linked to a trans-TM phage on the TM reduced bacterial titers in an infected ME, while ciprofloxacin-TMT3 phage did not.