Published in final edited form as:

J Drug Target. 2018 February ; 26(2): 127–134. doi:10.1080/1061186X.2017.1347791.

Optimization of peptides that actively cross the tympanic membrane by random amino acid extension: A phage display study

Arwa Kurabi, Daniel Schaerer, Lisa Chang, Kwang Pak, and Allen F Ryan

Division of Otolaryngology, Department of Surgery, School of Medicine, University of California, San Diego 9500 Gilman Drive #0666, La Jolla CA 92093-0666 and the San Diego, VA Healthcare System 3350 Villa La Jolla Drive, San Diego, CA 92161 USA

Abstract

Local treatment of middle ear (ME) disease currently requires surgical penetration of the tympanic membrane (TM). We previously discovered 12-mer peptides that are actively transported across the intact TM, a process that could be used for noninvasive drug delivery into the ME. To optimize transport and provide further understanding of the peptides transport mechanism, we extended two of the candidate peptides by six additional amino acids at random, and screened the resulting 18-mers libraries on TMs of rats with active bacterial otitis media (OM) for transport efficiency using phage display. Six identified peptides were individually tested *in vivo* for trans-TM transport to verify the tissue specificity. Three exhibited enhanced transport compared to their parent 12-mer scaffold, with the best showing an **approximately 9-fold** increase. Sequence analysis revealed anchor residues and structural features associated with enhanced transport. This included the prominent display of conserved sequence motifs at the extended free ends of the predicted peptide structures.

Keywords

Drug delivery; otitis media; active transport; peptide structure; conserved motifs

Address for Correspondence: Arwa Kurabi, Ph.D., Division of Otolaryngology, 0666, UCSD School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0666, akurabi@ucsd.edu.

AK: 858-774-3274 (akurabi@ucsd.edu)

DS: 858-229-7497 (dschaerer@ucsd.edu)

LC: 510-648-8468 (lic012@ucsd.edu)

KP: 858-552-8585 x7077 (kpak@ucsd.edu)

AFR: 858-534-4659 (afryan@ucsd.edu)

Disclosure of interest

The authors report no conflicts of interest. However, Dr. Ryan is a cofounder of Otonomy, Inc., serves as a member of the Scientific Advisory Board, and holds an equity position in the company. This relationship has been approved by the UCSD Committee on Conflict of Interest. Otonomy, Inc. played no part in the research reported here.

Geolocation information

UCSD School of Medicine and VA Medical Center.

Latitude and Longitude: 32.875, -117.234

Introduction

Otitis media (OM) is an extremely common infection of childhood. OM causes more office visits, drug purchases and surgical procedures than any other pediatric condition [1]. More than 90% of children experience OM before age 5 [2,3], while recurrent OM (defined as more than six episodes) and chronic OM affect approximately 20% [4,5]. OM is estimated to cost the US \$3 billion per year for health care [6], and an additional \$3 billion/year in associated costs [7,8]. In 2009, OM was responsible for more than 700,000 US pediatric tympanostomy surgeries, which require general anesthesia [9].

OM produces a transient conductive hearing loss, and may also result in fibrosis and scarring that permanently damage the conductive apparatus of the middle ear (ME). In one study, 33% of teenagers exhibit visible tympanic membrane (TM) scarring due to prior OM [10]. Severe OM may also damage the inner ear, due to the transit of bacterial and/or inflammatory substances through the round window membrane, resulting in permanent hearing loss [11]. These effects on hearing occur during critical periods of language acquisition [12]. Although the long-term impacts of OM are somewhat controversial, chronic ME disease has been associated with deficiencies in speech perception [12], language acquisition [13,14], and learning [15,16].

The consequences of OM are far more serious in many developing countries. The World Health Organization estimates that undertreated OM causes 28,000 deaths annually, and is the primary etiology of 175 million cases of permanent hearing loss. This represents more than half the world's burden of significant hearing impairment [17]. This problem is especially acute in countries that lack ready access to adequate health care, including the presence of sufficient ENT surgeons to diagnose and treat serious cases of OM.

While antibiotics are no longer recommended for uncomplicated acute OM (AOM) or OM with effusion in children over two [18], treatment patterns have in fact shown little change since this recommendation [19]. Antibiotic therapy is recommended for young children [20], and for more complicated forms such as chronic suppurative OM [21]. Streptococcal vaccines have reduced OM due to the covered strains, but have increased incidence for other strains and microbes [22,23]. Ventilation tubes can be effective for recurrent or chronic OM [e.g. 24], but there is controversy about the long-term benefit and later TM abnormalities [25–27]. Access of systemically delivered antibiotics to the ME is also an issue. About 20% of children receiving systemic antibiotics for OM show no detectable levels in the tympanic cavity [28]. Systemic antibiotic therapy is also not without risk. There is a significant incidence of gastric distress and diarrhea [29]. In addition, the incidence of antibiotic-resistant microbes recovered from the MEs of OM patients is increasing [30]. Since the source of bacteria in the ME is the nasopharynx, this is likely exacerbated by systemic antibiotic exposure.

Local medical treatment would provide a means of reducing side effects and other potential problems with current therapies. However, the TM forms an impermeable barrier that must be surgically breached to deliver drugs to the ME. To explore the possibility of alternative ME delivery procedures, we recently used the technique of phage display to identify unique

peptides that can transport large (1 μ meter) phage particles across the intact TM of rats undergoing bacterial OM [31]. This transport is active and could form the basis for local drug delivery to the ME without the need for surgery or injections through the TM, both of which require general anesthesia in children. The peptides discovered were 12-mers in residues length. However, many studies have found peptide length to be correlated with biological activity. As an example, Grau-Campistany and colleagues [32] found that activity of an antimicrobial peptide consisting of 7-mer repeats was not observed for peptides of less than 17 amino acids in length, and increased with successive amino acid addition. We therefore sought to optimize transport by extending the peptides to 18-mers. Phage display techniques are powerful tools for identifying novel proteins and/or peptides that display functional biological characteristics [e.g. 33]. Phage peptide libraries are comprised of random DNA sequences that encode a very large number of diverse peptides, fused onto a gene encoding a phage surface protein. Each recombinant peptide is displayed on the surface of a distinct bacteriophage clone, where it can interact with target molecules or tissues. A biopanning selection process is employed to enrich the library for a desired biological property, typically employing multiple sequential rounds. Enriched phage species are then identified by sequencing the appropriate region of the phage genome. We again used the technique of phage display, adding random amino acids to lead trans-tympanic 12-mer peptides.

Materials and Methods

Animals.

Subjects were Sprague Dawley rats 60–90 days old. All experiments were performed to National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the VA San Diego Medical Center.

Induction of otitis media.

Rats were anesthetized (rodent cocktail) and the ME bulla exposed via a ventral midline incision in the neck. A small hole was made in the bullar bone with a 20gauge needle. 10^4 strain 3566 nontypeable *Haemophilus influenza* (NTHi) in 50 μ l of saline was then injected into the ME using a 30-gauge needle, taking care not to inset the needle into the lateral ME near the TM. This inoculation results in robust OM 48 hours later [34,35], which was confirmed by otoscopy.

Bacteriophage display.

Transtympanic 12-mer bacteriophage were previously isolated by screening a random library of phage engineered to express 10^9 random peptides on their surfaces, fused onto the M13 phage pIII protein. Screening resulted in the isolation of several peptides that could actively transit the TM [31]. Two peptides, one that exhibited very high rates of transport, TMT-3, and one that exhibited a lower rate, TMT-2, were chosen for optimization. Each contained one of two conserved sequence motifs noted in the 12-mer peptides from the prior screen. Two phage libraries were then constructed (Creative Biolabs, Shirley, NY) in which TMT-3 or TMT-2 formed the backbone on the M13KE RF vector. To the N-terminus of each bacteriophage 12-mer backbone, six amino acids were added at random using the NNK

synthesis strategy, resulting in 10^8 distinct 18-mers in each library. 50 μ l aliquots of each 18-mer phage library containing approximately 50 copies of each distinct 18-mer phage peptide were applied to the external surface of intact TMs of six rats, in which OM had been induced 48 hours earlier by the injection of strain 3566 NTHi through the bone of the ventral bulla, taking care not to damage the TM [35]. After a 15-minute incubation on the TM, the external ear canal was repeatedly rinsed with PBS to remove all bacteriophage. The rats were then sacrificed and the ME bullas harvested intact. The exterior surface of the bulla was then extensively rinsed to remove contaminating phage. The bulla was then opened, exposing the ME contents. Fluid was collected from the ME, diluted in PBS and bacteriophage were titered using *E. coli* ER2738 (New England Biolabs, Cambridge, MA). The phage samples were then pooled, amplified in *E. coli*, and the resulting library applied to the TMs of additional rats for two more rounds of library enrichment. After the third round, 30 individual phage plaques were selected at random and sequenced to identify the expressed peptide.

Determination of trans-TM transport rate.

Selected clones of sequenced 18-mer phage were amplified individually and 10^9 phage were applied to the TMs of previously infected rats as above, for 15 minutes or 1 hour. The ME contents were then harvested and titered to determine transport characteristics. TMT-2 and TMT-3 12-mer phage, as well as a wild-type (WT) phage (M13KE phage from New England Biolabs, Cambridge, MA) not bearing a peptide, were identically applied to TMs as controls.

Structural analysis of trans-TM peptides.

To explore the potential basis of trans-TM transport as well as differences in transport efficiency, we analyzed the structures of all six 18-mer peptides, as compared to the parent 12-mers. We sought to identify unique features associated with more or less efficient transport. To accomplish this, we evaluated the influence of amino acid composition using PepCalc software [36]; amino acid position and peptide primary structural features using Chemdraw (Perkin Elmer); and secondary and tertiary architecture using PEPFOLD 3.0 [37]. As the peptides occur on the distal end of the pIII proteins, for tertiary structure predictions we included the final six amino acids of the M13 pIII protein. This allowed us to model the effects of the pIII terminus on each expressed peptide, and to visualize the predicted structure that is likely presented to the TM.

BLAST analysis.

DNA reads were translated in all six reading frames and aligned to the pIII protein region that flank the N-terminal displayed peptides. Hereafter, the 12-mer and 18-mer peptides were aligned using BLASTP [38] to search the SWISS-PROT/UniProtKB and SPTreMBL databases. A BLAST search of all non-redundant protein sequences, and also a more restricted search using *Rattus* taxonomy, were performed. All other search parameters were set to default.

Molecular modeling.

Molecular modeling of the 12-mer and 18-mer peptides was performed using the PEPFOLD 3.0 server [37]. The initial structure of the peptides was predicted *in silico* from the amino acid sequences using 200 independent simulations, and sOPEP energy to sort the modeling clusters. The best model PDB files were visualized using PyMol software (<http://www.pymol.org/>). The surface representation and graphics were produced using the PyMol tool.

Results

18-mer library screening.

Three rounds of screening on infected TMs yielded two 18-mer libraries, TMT-2(18) and TMT-3(18). Titers of the libraries obtained after the third screening round, compared to that of the 12-mer parent phage after either 15 minutes or 1 hour incubation on the TMs of previously infected rats, are illustrated in Figure 1. After a 15-minute incubation on the TM, ME recovery of the TMT-2 12-mer was approximately 10-fold lower than that of the TMT-3 12-mer, as we have previously reported [39]. The TMT-2(18) library exhibited transport several times in excess of that observed for TMT-2 itself, while the TMT-3(18) library exhibited transport lower than that seen for TMT-3. No WT phage were recovered. After one hour on the TM, ME recovery of both the TMT-2 and TMT-3 12-mers increased several-fold, consistent with previous results over time for these phage [39]. However, the 18-mer libraries exhibited only modest increases at one hour.

18-mer library sequences.

When 30 clones of each library were sequenced, the TMT-2(18) library yielded highly diverse peptide sequences, as illustrated in Figure 2A. Only three clones were observed more than once in the sample: two were present in triplicate and one in duplicate. This failure of the library to collapse suggests that no one sequence exhibited a dominantly higher rate of transport than any other. In addition, the lack of sequence homology among the identified TMT-2(18) library demonstrates peptide binding promiscuity. As can be seen in Figure 2B, sequences from the TMT-3(18) library exhibited almost total collapse to one sequence providing evidence for specific enrichment. Of the 30 phage plaques sequenced, 27 showed an identical peptide sequence. Two additional sequences were also present.

Comparison of the rate of 18-mer phage peptide transport across the TM.

The three-phage species that were present three times each in the TMT-2(18) library sample were named TMT2(18)-1 to 3. The three-phage species present in the TMT-3(18) library sample were named TMT-3(18)-1 to 3. The six 18-mer phage were amplified and applied to the TMs of previously infected rats for 1 hour.

As can be seen in Figure 3, trans-TM transport of phage bearing peptides TMT-2(18)-1 was negligible, and similar to that of WT phage. TMT-2(18)-2 exhibited a **2.1-fold** enhancement of transport when compared to its parent TMT-2 12mer. TMT-2(18)-3 showed no transport enhancement.

Transport of phage bearing peptide TMT-3(18)-1 was **8.9-fold** higher than that of the TMT-3 12-mer. Phage expressing peptide TMT-3(18)-2 exhibited a **3-fold** enhancement, while the TMT-3(18)-3 phage exhibited a significant decrease.

Analysis of peptides amino acid sequences.

The PepCalc properties of the 18-mers are presented in Figure 4, which show some common features between the added 6-mers. In particular, the presence of additional basic amino acids in the 6-mers of TMT-3(18) 1–3, and the addition of aromatic residues at position 5 in the 6-mers of TMT-3(18) 1, TM-3(18)-2 and TMT2(18)-2 6-mers, stand out. We have previously noted that a central basic amino acid is present in several of our 12-mer trans-TM peptides [31,39], while aromatic residues are prominent in the TMT-2 12-mer.

The added 6-mers are arranged in order of measured recovery from the ME in Figure 5. In this orientation, it is clear that the two sequences with the highest levels of trans-TM transit, TMT-3(18)-1 and TMT-3(18)-2, share common features. At position 4 aliphatic residues are present, while at position 5, aromatic residues are observed. For TMT-2(18)-2, the only other 18-mer to exhibit increased transport due to the additional six amino acids, the aliphatic residue at position 4 and the aromatic residue at position 5 are also present. TMT-2(18)-3, which showed no enhancement of transport, showed only an aliphatic residue at position 4. TMT3(18)-3 which showed reduced transport compared to the parent 12-mer, showed no alignment to these features. TMT-2(18)-1, which completely lost trans-TM transport to become equivalent to WT phage, showed a unique string of asparagine residues at positions 3–5.

BLAST analysis of the sequences of all six 18-mers was performed. No sequence analogy to any cell- or tissue-penetrating peptides was detected. Similarly, no relationships to any proteins that might reasonably be expected to act as ligands for a cell surface or transmembrane protein were observed.

Structural analysis of 18-mer peptides.

Sequence analysis reveals the potential orientations of amino acid residues in a protein. PEPFOLD 3.0 was used to generate 3-D, least-energy structural predictions, which are displayed as surface models in Figure 6. The models are ordered by the degree of transport through the TM, with the peptide showing the highest ME recovery on the upper left, and that showing the lowest recovery on the lower right. For this figure, the final seven amino acids of the phage pIII protein have been included, and oriented similarly so that the display peptide structures can be better compared. In addition, a head-on view of each peptide is presented. We have previously described two motifs that are conserved in trans-TM peptides, ST(K/R)T and PxxP [39]. The TMT-3 12-mer and its derived 18-mers include the sequence STKT, while TMT-2 and its 18-mers include PKMP. These motifs are color-coded in the structural predictions.

It is clear from the modeling that TMT-2 and its daughter 18-mers have a primarily globular predicted structure, while TMT-3 and its derived 18-mers each form a more linear structure. However, the presentation of the above motifs differs between the various peptides. For

those with the highest trans-TM transport, the motifs are prominent when the peptide is viewed end-on. Those with lower rates tend to have the motifs located further from the free end of the peptide as displayed on the pIII filament. Location at the free end of the peptide would presumably allow better access to potential binding sites on the TM.

While this analysis provides some clues as to the potential for the trans-TM peptides to recognize binding partners, BLAST analysis of the 12-mer and 18-mer peptides did not reveal any obvious relationships to proteins that might reasonably be expected to be ligands or interacting partners with known extracellular or transmembrane host proteins. Therefore, the trans-TM transport mechanism remains unknown.

Discussion

In this study, we successfully identified a second generation of peptides, 18-mers that were particularly effective in penetrating the TM when compared to our previous 12-mer candidate sequences [31,39]. Our results demonstrate that increasing the length of the 12-mer trans-TM peptides by six amino acids has the potential to significantly increase transport rate. This was apparent at the individual 18-mer peptide level, although not so apparent in the library as a whole.

When we compared transit of the selected TMT-2(18) library to TMT-2 itself, there was a clear tendency for enhanced transport by the library, especially after a 15-minute exposure on the TM (Figure 1). This result suggested positive selection for 18-mers that were transported at higher rates than TMT-2, although the possibility of higher *E. coli* transfection/replication efficiency by phage nearing longer peptides must also be considered. In fact, only one of the three individual phage clones that were represented more than once in the sequenced postselection sample showed an increase in recovery from the ME when compared to the parent 12mer. This is consistent with the modest increase in transport shown by the library, as a whole.

In contrast, phage recovery for round three of the TMT-3(18) library was lower than that of TMT-3 itself. This would suggest little selection for phage variants that were transported at higher rates than TMT-3, or at least that such variants were balanced by the selection of variants in which amino acid extension resulted in significantly lower transit than that of TMT-3. We were therefore surprised when not only did the post-selection TMT-3(18) library sequence sample indicate collapse to only three 18-mer variants, but that two of these variants exhibited significantly higher trans-TM transit than was observed for the TMT-3 12-mer, while one transported similarly to TMT-3. Moreover, the variant represented by only one of thirty copies in the sequencing sample exhibited the highest transport rate. These paradoxical results could be explained by differences in the efficiency of *E. coli* transfection and/or replication induced by the 18-mer peptides relative to the 12-mer and each other.

The amino acid composition of the 18-mers as presented in Figure 4 identifies a common feature in the added amino acids of those 18-mers with the highest trans-TM transport rates. TMT3(18)-1, TMT3(18)-2 and TMT2(18)-2 are all characterized by the addition of an aliphatic residue at position 4 and an aromatic residue at position 5 of the auxiliary 6-mer.

Peptides lacking these features showed distinctly lower transport. No other common amino acid features emerge from this analysis, with the number and distribution of basic and acidic, as well as hydrophilic versus hydrophobic, showing no clear relationship to transport rate.

Interestingly, the optimal peptides appear to be characterized by prominent structural presentation of candidate motifs first identified in the 12-mers. We hypothesize that the extended structures (6 added as residues) allow these motifs to be arranged at the end of the peptide structure, permitting optimal access to their targets on the TM. **Though the peptides' mechanism of action is not fully clear, a major question arises as to whether the two motifs might utilize a common or different binding sites, and perhaps alternate transporting mechanisms. This is currently under investigation.**

The predicted tertiary structural analysis of the peptides (Figure 6) suggests that TMT-2 and its derived 18-mers have quite different surface topologies when compared to TMT-3 and its 18-mers derivatives. This difference, between a globular versus a linear structure, may contribute to the lower transport rate of TMT-2 and its 18-mers compared to those based on TMT-3. The greater potential flexibility of linear peptides could facilitate interaction with molecular targets on the TM. However, regardless of the structure, presentation of one of two conserved motifs at the end of the peptides was associated with higher transport rates. This would presumably facilitate binding of the motifs to sites on the TM that are related to the active transport process. This finding underscores the potential importance of these motifs to trans-TM transport.

The identity of binding partners on the TM that might be involved in trans-TM transport is unknown. Sequence and structural analysis did not provide any obvious clues, and BLASTP [38] analysis of the 12-mer and 18-mer peptides did not reveal any relationship to potential ligands for cell surface molecules that might initiate or mediate transport. However, the sequence of the trans-TM peptides appears to be highly specific to their function. Previously we established that an excess of free 12-mer peptide was able to block the transport of phage bearing the same peptide [31], indicating that it is the peptide sequence alone that determines the recognition of targets on the TM that are presumably involved in transport, not a combination of peptide and the phage pIII protein. While we have not as yet identified any targeted receptors or transporters that might contribute to trans-TM transport, experiments based on the affinity of the peptides for TM protein targets are currently under way to identify such molecular substrates.

Since our observation in this study that increasing the length of two trans-TM peptides from 12 to 18 amino acids increased transport rate, one can speculate whether further increases in peptide length might yield additional enhancement. Indeed, the greater degree of conformational variability of longer peptides could have such an effect. Since we found evidence that presentation of key amino acid motifs was related to transport rate, longer peptides selected for transport efficiency could produce even more effective presentation. Moreover, given the size of phage particles and the pIII protein relative to the peptides studied, it does not seem likely that the greater size of longer peptides would exert any negative effects.

In any event, the optimization of active trans-TM transport increases the potential utility of this family of peptides for the noninvasive delivery of therapeutic cargo into the ME. Peptide transport appears to have no deleterious effects on the TM, ME or inner ear [39]. Moreover, the ability to carry large particles (M13 phage is approximately 1 μm in length) suggests that large drug packages such as liposomes [40] or micronized drug particles could be effectively delivered by these peptides.

In summary, we were able to optimize the trans-TM transport of two 12-mer peptides using amino acid extension and phage display. This resulted in three peptides with increased transport compared to their parent 12-mers scaffold, and one 18-mer with about a 9-fold increase in uptake. This lead peptide could be employed to support the active delivery of drugs, gene therapy vectors or lytic phage species that could target OM pathogens, as alternatives to existing therapies for ME disease. The 6-mer extensions do not share sequence homology that would explain enhanced transport. Rather, structural predictions of the peptides strongly implicate the positioning of conserved motifs in peptide structures that would facilitate interaction with binding partners.

Acknowledgements

This work was financially supported by the TRIH initiative of Action on Hearing Loss, and by the National Institute of Health / National Institute on Deafness and Other Communication Disorders (NIH/NIDCD) under grant DC012595; and grant DC000129, and by the Veterans Administration under grant BX001205.

Biography

Arwa Kurabi, PhD, is a protein biologist and faculty member at the UCSD School of Medicine. She studies middle and inner ear disease, drug delivery and leukocyte function.

Daniel Schaerer, MD, is an ENT resident at UCSD who recently completed an NIH research fellowship.

Lisa Chang, BS, was a UCSD undergraduate student majoring in Human Biology.

Kwang Pak, BS, is a Research Associate at UCSD who studies middle and inner ear disease.

Allen F Ryan, PhD, is an auditory biologist and faculty member at UCSD School of Medicine and the San Diego VA Medical Center. He studies middle and inner ear disorders.

References

1. Coco AS. Cost-effectiveness analysis of treatment Options for acute otitis media. *Ann Fam Med*. 2007;5(1):29–38. [PubMed: 17261862]
2. Casselbrant M, Mandel E, Rockette H, et al. Recent Advances in Otitis Media: Incidence of OM and bacteriology of acute OM during the first two years of life. Toronto, Decker, 1993.
3. Pichichero ME. Ten-year study of acute otitis media in Rochester NY. *Pediatr Infect Dis J*. 2016;35(9):1027–1032. [PubMed: 27182898]
4. Teele DW, Klein JO, Rosner B. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. *J Infect Dis*. 1989;160(1):83–94. [PubMed: 2732519]

5. Wallis S, Atkinson H, Coatesworth AP. Chronic otitis media. *Postgrad Med.* 2015;127(4):391–395. [PubMed: 25913599]
6. Rovers MM. The burden of otitis media. *Vaccine.* 2008;26 Suppl 7:G2–4. [PubMed: 19094933]
7. Klein JO. The burden of otitis media. *Vaccine.* 2000;19 Suppl 1:S2–8. [PubMed: 11163456]
8. Ahmed S, Shapiro NL, Bhattacharyya N. Incremental health care utilization and costs for acute otitis media in children. *Laryngoscope.* 2014;124(1):301–305. [PubMed: 23649905]
9. Cullen KA, Hall MJ, Golosinskiy A. Ambulatory surgery in the United States, 2006. *Natl Health Stat Report.* 1, 2009;(11):1–25.
10. Stangerup SE, Tos M, Arnesen R, et al. A cohort study of point prevalence of eardrum pathology in children and teenagers from age 5 to age 16. *Eur Arch Otorhinolaryngol.* 1994;251(7):399–403. [PubMed: 7857627]
11. Joglekar S, Morita N, Cureoglu S, et al. Cochlear pathology in human temporal bones with otitis media. *Acta Otolaryngol.* 2010;130(4):472–476. [PubMed: 19895333]
12. Friel-Patti S, Finitzo T. Recent Advances in Otitis Media: Speech-language learning in the first 3 years in children with tympanostomy tubes for recurrent or persistent OM. Toronto, Decker, 523–552, 1993.
13. Klausen O, Møller P, Holmeffjord A, et al. Lasting effects of otitis media with effusion on language skills and listening performance. *Acta Otolaryngol* 2000;543:73–76.
14. Wendler-Shaw P, Menyuk P, Teele D. Recent Advances in Otitis Media: Effects of otitis media in first year of life on language production in the second year of life. Toronto, Decker, 1993.
15. Friel-Patti S, Finitzo-Hieber T, Conti G, et al. Language delay in infants associated with middle ear disease and mild, fluctuating hearing impairment. *Pediatr Infect Dis J.* 1982;1(2):104–109
16. Klein J, Chase C, Teele D, et al. Recent Advances in Otitis Media: OM and the development of speech, language and cognitive abilities at 7 years of age. Decker, Toronto, 1988.
17. Arguedas A, Kvaerner K, Liese J, et al. Otitis media across nine countries: disease burden and management. *Int J Pediatr Otorhinolaryngol.* 2010;74(12):1419–1424. [PubMed: 20965578]
18. American Academies of Family Physicians, ENT-HNS and Pediatrics. OM with effusion. *Pediatrics.* 2004;113:1412–1429.
19. Coco A, Vernacchio L, Horst M, Anderson A. Management of acute otitis media after publication of the 2004 AAP and AAFP clinical practice guideline. *Pediatrics.* 2010;125(2):214–220. [PubMed: 20100746]
20. Grossman Z, Branski D. Changing the therapeutic approach to acute OM in children. *Harefuah.* 2004;143(4):277–282.
21. Heidemann CH, Lous J, Berg J, et al. Danish guidelines on management of otitis media in preschool children. *Int J Pediatr Otorhinolaryngol.* 2016;87:154–163. [PubMed: 27368465]
22. Vergison A. Microbiology of otitis media: a moving target. *Vaccine.* 2008;26 Suppl 7:G5–10. [PubMed: 19094935]
23. Casey J, Adlowitz D, Pichichero M. New patterns in the otopathogens causing acute OM 6–8 years after introduction of pneumococcal conjugate vaccine. *Pediatr Infect Dis J.* 2010;29(4):304–309. [PubMed: 19935445]
24. Schraff SA. Contemporary indications for ventilation tube placement. *Curr Opin Otolaryngol Head Neck Surg.* 2008;16(5):406–411. [PubMed: 18797280]
25. Daly KA, Hunter LL, Lindgren BR, et al. Chronic otitis media with effusion sequelae in children treated with tubes. *Arch Otolaryngol Head Neck Surg.* 2003;129(5):517–522. [PubMed: 12759263]
26. de Beer BA, Schilder AG, Ingels K, et al. Hearing loss in young adults who had ventilation tube insertion in childhood. *Ann Otol Rhinol Laryngol.* 2004;113(6):438–444. [PubMed: 15224825]
27. Johnston LC, Feldman HM, Paradise JL, et al. Tympanic membrane abnormalities and hearing levels at the ages of 5 and 6 years in relation to persistent otitis media and tympanostomy tube insertion in the first 3 years of life: a prospective study incorporating a randomized clinical trial. *Pediatrics.* 2004;114(1):e58–67. [PubMed: 15231974]

28. Pichichero ME, Reed MD. Variations in amoxicillin pharmacokinetic/pharmacodynamic parameters may explain treatment failures (4) acute otitis media. *Pediatric Drugs*. 2009;11:243–249. [PubMed: 19566108]
29. McFarland LV. Epidemiology, risk factors and treatments for antibiotic-associated diarrhea. *Dis. 1998;16(5):292–307*. [PubMed: 9892789]
30. Zielnik-Jurkiewicz B, Bielicka A. Antibiotic resistance of *Streptococcus pneumoniae* in children with acute otitis media treatment failure. *Int J Pediatr Otorhinolaryngol*. 2015;79(12):2129–2133. [PubMed: 26454530]
31. Kurabi A, Pak K, Bernhardt M, et al. Discovery of a biological mechanism of active transport through the tympanic membrane to the middle ear. *Sci Rep*. 2016;6:22663. [PubMed: 26946957]
32. Grau-Campistany A, Strandberg E, Wadhvani P, et al. Hydrophobic mismatch demonstrated for membranolytic peptides, and their use as molecular rulers to measure bilayer thickness in native cells. *Sci Rep*. 2015;5:9388. [PubMed: 25807192]
33. Wu CH, Liu IJ, Lu RM, et al. Advancement and applications of peptide phage display technology in biomedical science. *J Biomed Sci*. 2016;23:8. [PubMed: 26786672]
34. Hernandez M, Leichtle A, Pak K, et al. Myeloid differentiation primary response gene 88 is required for the resolution of otitis media. *J Infect Dis*. 2008;198(12):1862–1869. [PubMed: 18986247]
35. Leichtle A, Hernandez M, Ebmeyer J, et al. CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF^{-/-} mice. *J Immunol*. 2010;184(6):3087–3097. [PubMed: 20164426]
36. Lear S, Cobb SL. Pep-Calculator: a set of web utilities for the calculation of peptide and peptoid properties and automatic mass spectral peak assignment. *J Comput Aided Mol Des*. 2016;30(3):271–277. [PubMed: 26909892]
37. Maupetit J, Derreumaux P, Tuffery P. PEP-FOLD: an online resource for de novo peptide structure prediction [Web Server issue]. *Nucleic Acids Res*. 2009;37:W498–503. [PubMed: 19433514]
38. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol*. 1990;215(3):403–410. [PubMed: 2231712]
39. Kurabi A, Beasley KA, Chang L, et al. Peptides actively transported across the tympanic membrane: Functional and structural properties. *PLoS One*. 2017;12(2):e0172158. [PubMed: 28234923]
40. Bulbake U, Doppalapudi S, Kommineni N, et al. Liposomal formulations in clinical use: an updated review. *Pharmaceutics*. 2017;9(2):e12. [PubMed: 28346375]

Figure 1.

Transport rate for the two 12-mer peptides chosen as scaffolds for extension, TMT-2 and TMT-3, versus the transport rate observed for the two 18-mer phage libraries after three rounds of selection, after 15 minutes or 1 hour of incubation on the TM.

Figure 2.

2A. Sequences of 30 phage plaques selected randomly after the third round of selection for the TMT-2 (18) library. The sample contained a highly diverse population of sequences, indicating failure of the library to show significant collapse. Two sequences were isolated three times each, suggesting superior transport to the remaining sequences.

2B. Sequences of 30 phage plaques selected randomly after the third round of selection for the TMT-3 (18) library. The library exhibited almost complete collapse into one extension sequence, with only two others represented in the sample.

Figure 3.

Comparison of trans-TM transport for phage bearing the 12-mer backbone peptides TMT-2 or TMT-3, versus candidates from the 18-mer screen. Of those peptide phage represented more than once in the TMT-2(18) sequenced sample, TMT-2(18)-2 exhibited TM transit 2.1 fold higher than that of the parent 12-mer. Of the three peptides in the TMT-3(18) sequenced sample, TMT-3(18)-1 was transported at 8.9 times the rate of TMT-3 itself, while TMT-3(18)-2 was 3 times higher.

Figure 4.

PepCalc analysis of the 6-mer extensions of the 18-mers evaluated in this study. The extensions show no strong relationships based on sequence homology. The six N-terminal amino acids added to each 12-mer backbone are on the left. Amino acid characteristics and hydrophilicity/-phobicity are indicated as is the peptide net charge at physiological pH.

Figure 5.

The amino acid sequence of the 6-mer extensions of the six 18-mer peptides for which trans-TM transport was measured in this study. The amino acids are colored by side chain characteristics to show any potential homology. The phylogenetic tree sequence analysis is illustrated at the bottom. No robust sequence features are shared between the different 6-mer peptide groups.

Figure 6.

(A) 3-D structural predictions for the 12-mer and 18-mer peptides evaluated for transport, generated with PEPFOLD software. The peptides are arranged in order of the amount recovered from the ME after a 1-hour incubation on the TM (Figure 3). The final 7 amino acids of the M13 pIII protein are included in the predictions, and are colored gray. The conserved ST(K/R) and PxxP motifs are indicated in blue and green, respectively. The remaining amino acids of the expressed peptide are in white. Both side and head-on (inset box) views are presented. In the side view, the terminal amino acids of M-13 pIII are positioned at the lower left. In the head-on view, they are directly behind the expressed peptide.