



Original Article

Single Administration of a Sustained-Release Formulation of KB-R7785 Inhibits Tympanic Membrane Regeneration in an Animal Model

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OBJECTIVE: A pressure equalization tube placed within the tympanic membrane is the only clinically available method for inhibiting tympanic membrane regeneration. Problems associated with this include associated otorrhea, biofilm formation, medial migration of the tube, tube retention, induction of granulation tissue, and a small but significant rate of cholesteatoma. We aimed to demonstrate that a single administration of a sustained-release polymer formulation of KB-R7785 maintains tympanic membrane perforation for at least 6 months.

MATERIALS and METHODS: Sustained-release KB-R7785 was delivered within a novel polymer hydrogel to 20 mice with bilateral acute tympanic membrane perforations (a total of 40 perforations). The perforations were monitored at 3-month intervals until 9 months.

RESULTS: At 3 months, 90% of perforations were open (n=36/40). At 6 months, 75% of perforations were open (total n=30/40). At 9 months, 22.5% of perforations were open (total n=6/40). The majority of tympanic membrane perforations (75%) were open (not healed) beyond 6 months and close (fully healed) prior to 9 months (77.5%). Once healed, tympanic membranes resembled their normal histological appearance.

CONCLUSION: This study demonstrates that a single administration of a sustained-release polymer formulation of KB-R7785 inhibits tympanic membrane regeneration for 6–9 months.

KEYWORDS: Tympanic membrane, chronic perforation, tympanic perforation, wound healing

INTRODUCTION

Maintaining a chronic perforation in a tympanic membrane (TM) is part of the current standard of care for a number of middle ear diseases, including primary Eustachian tube dysfunction, otitis media with effusion, and recurrent acute otitis media ^[1]. Having a physical structure, a pressure equalization tube (PET), placed within TM is the only clinically available method for inhibiting TM regeneration and maintaining an opening. The presence of PET is associated with a number of problems, including tube associated otorrhea, linked to biofilm formation on the tube, medial migration of the tube, tube retention, induction of granulation tissue, and a small but significant rate of cholesteatoma ^[2-4]. There is a need for the ability to maintain a perforation in the TM (through the inhibition of the normal regenerative mechanisms) that does not require the long-term presence of a physical structure.

KB-R7785 [4-(N-hydroxyamino)-2R-isobutyl-3S-methylsuccinyl]-L-phenylglycine-N-methylamide] is a matrix metalloproteinase (MMP) inhibitor with a particular inhibitory action on MMP-2, MMP-9, disintegrin, and metalloproteinase domain-containing protein 12 (ADAM12) ^[5]. We have previously demonstrated that KB-R7785 treatment of an acute perforation leads to the inhibition of tympanic regeneration that persists for at least 3 months ^[6]. The previously published dosing regimen, for 7 days onto gel foam, is likely to be difficult and inconvenient, and we aimed to develop a solution that was one-time administration. Our previous experiments have shown the potential of perforations to last for at least 3 months, and for our solution to be a true replacement for PET, we estimated that the perforations would need to persist for at least 6 months. Because the hypothesis that KB-R7785 inhibits TM regeneration had already been established, in the present study, we aimed to test the duration for which this inhibition could last using a sustained-release formulation delivered as a biodegradable polymer.

MATERIALS and METHODS

Stanford University's Administrative Panel on Laboratory Animal Care approved all animal work (Protocol 27539, approved June 2014). International standards regarding animal care and handling have been followed during the experiments.

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All mice used in this study were 6–10-week-old male CBA/Cal (15–25 g) mice (Jackson Laboratories, Florida, USA). All recordings by otoscopy and all surgical interventions were performed using inhaled isoflurane at 3%–4% for induction and 1%–2% for maintenance. The study design was a prospective cohort one. The determination of animal numbers in this study was performed using STATA version 1.03 (Statacorp LP, Texas, USA), aiming for an α value of 0.05 and a minimum β value of 0.8. STATA version 13.0 (Statacorp LP, Texas, USA) was used for analysis. In total, 20 mice were used with perforations created bilaterally (a total of 40 perforations). For all statistical analyses, a Pearson's Chi-square test of proportions was used. A significance level of 0.05 was used for the null hypothesis.

KB-R7785

KB-R7785 (4-(N-hydroxyamino)-2R-isobutyl-3S-methylsuccinyl)-L-phenylglycine-N-methylamide) was synthesized and obtained from Stanford University's chemistry department. It was dissolved in the prepolymer solution at 10 mM. The KB-R7785 treatment was initiated for all 40 perforations in 20 mice.

Polymer Construction

Polymer construction has been previously discussed, and the release profiles of various agents have been characterized [7, 8]. The mass ratio of chitosan to lactide was 8:1. 3.3 mM of sodium metabisulfite, the cross-linking agent, was added into the prepolymer solution to form a semi-solid hydrogel within a few minutes. The polymer was designed to dissolve and release KB-R7785 at 10 nM over a 4-week period.

Animal Treatment and Inspection

We used a previously published technique to create TM perforations adapted to mice [6, 9, 10]. In brief, a curved microneedle was used to create a subtotal perforation in the pars tensa of TM [6, 9, 10]. The treatment was performed via the external auditory canal using a syringe and 27-gauge needle through and onto TM to fill the middle ear and into the external ear. The total volume delivered was approximately 0.04 mL in each case. The KB-R7785 polymer treatment was initiated for all 40 perforations in 20 mice. The animals were inspected using otosco-

py under general anesthesia at 3-month intervals. Care was taken to not disturb the TM surface. Otoscopy performed a using the Digital Macroview (Welch Allyn, New York, USA) otoscope. Perforations were measured using ImageJ to determine the perforation size as a percentage of the total pars tensa area [11]. A pinhole perforation was defined as a perforation less than 5% of the surface area. All the animals were sacrificed at 9 months, and TMs were harvested for histology.

Histology

Histology was performed with hematoxylin–eosin staining according to a previously published technique [9].

RESULTS

KB-R7785 delivered via polymer maintains the majority of perforations between 6–9 months

The percentage of healed perforations over time is displayed in Figure 1. At 3 months, 36 perforations were still open ($n=36/40$, 90%). At 6 months, further 6 perforations had healed, leaving a total of 30 perforations open (total $n=30/40$, 75%), 3 of which healed to pinhole size (total $n=27/40$, 67.5% larger than pinhole size). At 9 months, further 21 perforations had healed, leaving a total of 9 perforations open (total $n=9/40$, 22.5%), 3 of which healed to pinhole size (total $n=6/40$, 15% larger than pinhole size). There was no significant difference between the proportion of perforations open at 3 and 6 months ($\chi^2=1.02$, $p=0.31$); however, there were significantly more perforations open at 6 months than at 9 months ($\chi^2=26.8$, $p<0.001$).

Healed TMs after KB-R7785 treatment resemble normal TMs

All healed TMs (Figure 2a) after treatment resembled normal TM histology with an inner mucosal layer, a middle connective tissue layer, and an outer (darker) thin keratinocyte layer. In comparison, all persisting chronic perforations had thickened and disorganized connective tissue layers and thickened keratinocyte layers reaching over the perforation edge to lie adjacent to the mucosal layer (Figure 2b). No inflammation or mucosal thickening was identified in the middle ear mucosa of the specimens at 9 months.

DISCUSSION

Animal Model Limitations

While the mice were alive, it was easy to determine the presence or absence of a perforation via transcanal otoscopy, but it was not possible to determine the true size of the perforations because there was often covering debris or discharge. The angle of TM in the narrow mouse ear canal makes it difficult for a total inspection of the whole pars tensa surface in vivo. This required removal of the debris, which would injure TM and artificially make the perforation seem larger. Therefore, we were unable to comment in vivo on how the perforation was changing or possibly reducing in size accurately. The other limitation was the potential for bias in reporting because only a single observer could report on the perforations. Getting a clearer view of the perforations via the removal of debris for in vivo photography would have disturbed the perforation and introduced another confounding factor in the healing. The disadvantage of KB-R7785 in its current delivery form and this animal model is that it is difficult to maintain a patent perforation in the early days because the polymer itself obstructs the perforation

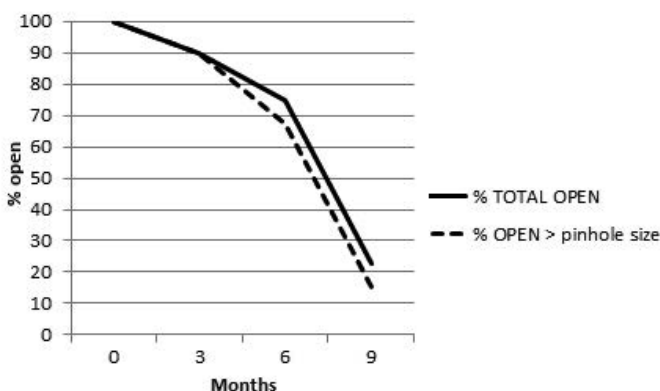


Figure 1. Tympanic membrane perforation open after KB treatment over time. Figure 1 demonstrates the percentage of tympanic membrane perforations ($n=40$) open (not healed) after treatment with KB-R7785 at 3-, 6-, and 9-month inspections, shown in the solid line (% TOTAL OPEN). The majority (75%) were open (not healed) at least for 6 months and close prior to 9 months (77.5%). A small percentage had healed to a small pinhole ($\leq 5\%$ of the total pars tensa surface area) size at each time point, as shown in the dashed line (% OPEN > pinhole size).

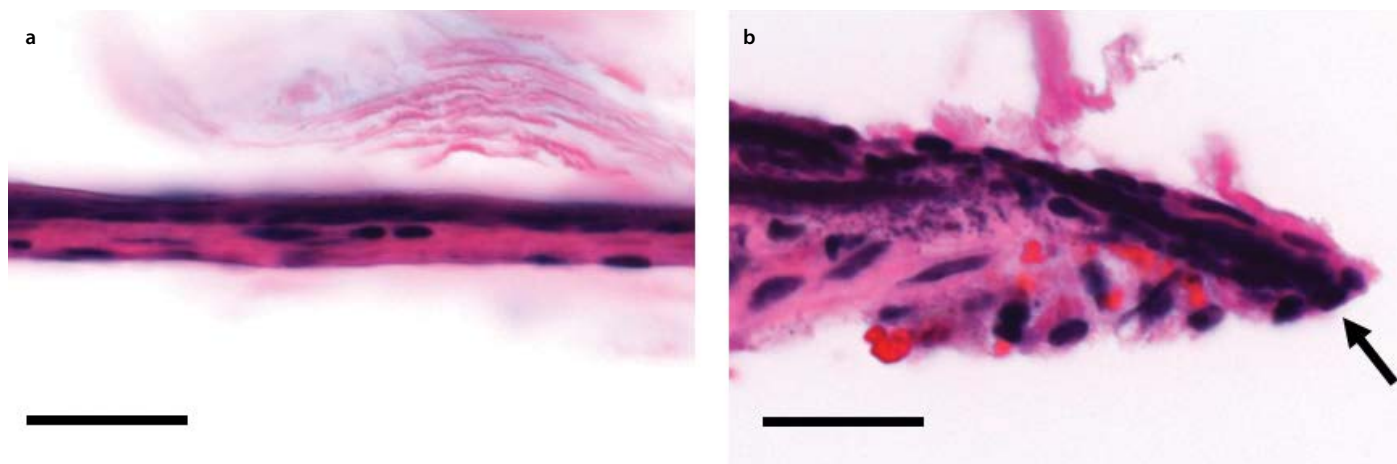


Figure 2. a, b. Tympanic membranes at 9 months following KB-R7785. Figure 2 demonstrates the tympanic membrane after treatment KB-R7785 at 9 months (a) in a healed perforation (b) in a persisting chronic perforation. The arrow identifies the perforation margin showing a thicker keratinocyte and connective tissue layer. Scale bar=5µm (Magnification 40×).

opening. If translated directly, this would prevent the early release of middle ear fluid and ventilation of the middle ear, a benefit of PETs. The polymer also results in temporary hearing loss, which resolves after the polymer dissolves [6]. For this purpose, the delivery vehicle would need to be modified to be delivered adjacent to the perforation opening without obstructing it. This would be difficult to test in a small-sized TM, as observed in the mouse, and would require a larger animal model for proof of concept.

KB-R7785 acts via inhibition of epidermal growth factor receptor ligand shedding

KB-R7785 administration is thought to inhibit TM wound healing via the inhibition of heparin-binding epidermal growth factor-like growth factor (HB-EGF)-mediated epidermal growth factor receptor (EGF-R) ectodomain ligand shedding [6, 12–15]. This migration and proliferation of keratinocytes from progenitor cells located at the attachment of TM to the malleus handle play a crucial role in TM wound healing [9]. The first 7 days appear to be critical in TM wound healing to determine whether a perforation heals or becomes chronic [6, 9]. KB-R7785 also has been identified as having a potential role in modulating proliferation in the gastric mucosa, vascular smooth muscle, tumor cell migration, cerebral ischemia, and apoptosis in the bone marrow [5, 16–20].

KB-R7785 delivered via polymer has potential to replace PETs

Previously, it has been demonstrated that chronic perforations can be created using daily-repeated KB-R7785 (10nM) administration over 1 week onto gel foam into an acute TM perforation. In the present study, a single administration of KB-R7785 via a novel delivery vehicle enabled perforations to be maintained for at least 6 months. This study also showed that this single administration does not produce a persisting perforation, in the majority, beyond 9 months. Given this timing of chronic perforation, this treatment has the potential to replace the physical obstruction method of PETs if further developed into a suitable structure that allows ventilation in the early period. Once TM is healed following KB-R7785 inhibition, the histology takes the appearance of a normal TM. This is important for implications about the potential development of tympanosclerosis and other histological changes observed in TM following subacute or chronic perforation.

KB-R7785 delivered via polymer requires further development before use in human ear

Although the drug release profile of other agents has been investigated in this current delivery system, the current release profile of KB-R7785 needs to be defined. The current proposed polymer has already been shown to be nonototoxic [6]. Other studies delivering KB-R7785 parenterally in rodents have not shown systemic toxicity; however, these were not the primary endpoints measured in these studies [5]. Despite there being no middle ear histological changes detected after tympanic healing, further toxicity studies, specifically for KB-R7785, are needed before it is deemed safe to progress toward human clinical trials.

CONCLUSION

This study demonstrated that a single administration of a sustained-release polymer formulation of KB-R7785 inhibits TM regeneration between 6–9 months.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Stanford University.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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