Innovative dHDPs demonstrated potent antimicrobial activity, possess a limited potential for development of resistance, and reduced the severity of murine Pseudomonas aeruginosa keratitis model. These studies demonstrate that a novel dHDP may have potential to treat patients with sight-threatening bacterial keratitis.

Keywords: antimicrobial peptide, host defense peptide, bacterial keratitis, biofilm

Pathogens cause bacterial keratitis include both Gram-positive and -negative bacteria. Gram-positive causative pathogens include Enterococcus faecium,5 Staphylococcus aureus (including methicillin-resistant Staphylococcus aureus [MRSA]),6 Staphylococcus epidermidis,7 and Streptococcus pneumoniae.8 Gram-negative causative pathogens include Pseudomonas aeruginosa,9 Enterobacter aerogenes,7 and Acinetobacter baumannii.8 The most frequent pathogen in bacterial keratitis is associated with contact lens wear is Gram-negative P aeruginosa while for non-contact lens-related disease the Gram-positive bacterium S. aureus predominates.9,10

Bacterial keratitis is an aggressive corneal infection that, without prompt and effective treatment, can lead to perforation of the cornea and blindness.1 In the United States, there are approximately 50,000 patients annually with these infections, with contact lens wear as the prime risk factor. Bacterial keratitis is presently treated with antibiotics, particularly fluoroquinolones; however, innovation in antimicrobials is needed due to the rapid emergence of bacterial resistance. Designed host defense peptides (dHDPs) are synthetic analogues of naturally occurring HDPs, which provide defense against invading pathogens. This study investigates the use of novel dHDPs for the treatment of bacterial keratitis.

 METHODS. The minimum inhibitory concentrations (MICs) were determined for dHDPs on both Gram-positive and -negative bacteria. The minimum biofilm eradication concentrations (MBEC) and in vitro time-kill assays were determined. The most active dHDP, RP444, was evaluated for propensity to induce drug resistance and therapeutic benefit in a murine Pseudomonas aeruginosa keratitis model.

RESULTS. Designed HDPs were bactericidal with MICs ranging from 2 to >64 μg/mL and MBEC ranging from 6 to 750 μg/mL. In time-kill assays, dHDPs were able to rapidly reduce bacterial counts upon contact with as little as 2 μg/mL. RP444 did not induce resistance after repeated exposure of P aeruginosa to subinhibitory concentrations. RP444 demonstrated significant efficacy in a murine model of bacterial keratitis as evidenced by a significant dose-dependent decrease in ocular clinical scores, a significantly reduced bacterial load, and substantially decreased inflammatory cell infiltrates.

CONCLUSIONS. Innovative dHDPs demonstrated potent antimicrobial activity, possess a limited potential for development of resistance, and reduced the severity of murine P aeruginosa keratitis. These studies demonstrate that a novel dHDP may have potential to treat patients with sight-threatening bacterial keratitis.

Keywords: antimicrobial peptide, host defense peptide, bacterial keratitis, biofilm
Designed Host Defense Peptides for Treating Keratitis

Methods

Designed Host Defense Peptides

Eleven dHDPs, whose amino acid sequences are depicted in Table 1, were synthesized via traditional solid phase synthesis (CSBio, Menlo Park, CA, USA). The discovery and identification of the bactericidal cecropins and magainins and their role in providing “freedom from infection” provided the structural basis and amphipathic properties upon which these dHDPs were designed.16–19 The peptide sequences include use of nonnatural amino acids not encoded by the Universal Genetic Code, and the specific replacement of lysine with ornithine, which has been shown to increase antibacterial activity while also enhancing proteolytic stability.20–21

Bacterial Strains

Bacterial strains were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The Gram-positive bacterial strains included Enterococcus faecium ATCC 700221 (E. faecium 700221), methicillin-resistant Staphylococcus aureus ATCC 33591 (MRSA 33591), Staphylococcus epidermidis ATCC 51625 (S. epidermidis 51625), Streptococcus pneumoniae ATCC 49619 (S. pneumoniae 49619), and Staphylococcus aureus ATCC 49525 (Wright).

The Gram-negative bacterial strains tested were Enterobacter aerogenes ATCC 13048 (E. aerogenes 13048), Acinetobacter baumannii Bouvet and Grimont ATCC 17978-D-5 (A. baumannii 17978-D-5), Pseudomonas aeruginosa ATCC 19660 (P. aeruginosa 19660), and Pseudomonas aeruginosa ATCC 27853 (P. aeruginosa 27853).

The bioluminescence strains, P. aeruginosa 19660 transduced with the Xen5 luciferase gene (here termed P. aeruginosa 19660_Xen5), and Staphylococcus aureus ATCC 49525 (Wright) transduced with the Xen5 luciferase gene (S. aureus 49525_Xen36), were obtained from PerkinElmer (Hopkinton, MA, USA).22,23

In Vitro Bactericidal Activity

The minimum inhibitory concentrations (MICs) of dHDPs were determined by broth microdilution methods approved by the Clinical and Laboratory Standards Institute.24 The minimum biofilm eradication concentrations (MBECs) were determined using the MBEC Assay system (Innovotech, Edmonton, AB, Canada).25 Briefly, the bacteria are grown on 96-well plates with pegs on the lid filled with growth medium enabling biofilm formation on the pegs. The reduction in viable bacteria living in biofilm after exposure to the dHDPs was calculated as the difference between the viable colony forming units (CFU) on the control pegs and those in the treated pegs. The MBEC values for the dHDPs are higher than the corresponding MIC values; thus any bacteria in a biofilm that were shed or dissipated during the biofilm assay have been eradicated by the MBEC level of dHDP.

Antibiotic Resistance Profiling

Subinhibitory concentrations of RP444 and gentamicin were incubated with P. aeruginosa 27853 for 24 hours. The bacteria that showed growth in the highest concentration were repassaged in fresh dilutions containing sub-MIC levels of RP444 or gentamicin. The MIC was reetermined at each time point, and this was repeated for 21 consecutive passages.

Time-Kill Bactericidal Activity

Noninvasive and real-time monitoring of dHDP bactericidal activity was performed using bioluminescent strains of P. aeruginosa and S. aureus.26,27 Cultures of P. aeruginosa 19660_Xen5 and S. aureus 49525_Xen36 were made from a single colony, which was inoculated into 5 mL sterile lysogeny broth (LB). Tubes were incubated at 37°C overnight with agitation. Cultures were diluted 1:10 into fresh LB and incubated at 37°C for 2 hours to achieve mid-logarithmic growth. Optical density measurements (OD, 600 nm) were performed on the bacterial cultures, which were then adjusted to 1×10^6 CFU/mL. Bacterial suspensions (100 μL) were plated in 96-well black-walled plates. The candidate dHDPs were 2-fold serially diluted from 128 μg/mL in LB. Each concentration was done in triplicate, with the final volume being 200 μL. Imaging was performed at select times after the addition of the dHDP, and compared to concurrently run positive control antibiotics, tobramycin and vancomycin, using an IVIS Lumina imaging system (Caliper Life Sciences, Inc., Hopkinton, MA, USA). For imaging, the 96-well plate was positioned on the stage (12.5-cm field of view), with an open emission filter, binning of 4, and Fstop 1. Photons were counted for 30 seconds, and data analysis was performed using the LivingImage software program (version 4.3, Caliper Life Sciences, Inc.).

Animals

All animals received care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 1996) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All studies were approved by the Institutional Animal Care and Use Committee of the University of Houston. C57BL/6 mice, 9 to 13 weeks, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). General anesthesia consisted of an intraperitoneal
injection of ketamine 100 mg/kg and xylazine 10 mg/kg (Vedco, Inc., St. Joseph, MO, USA).

### In Vivo Corneal Epithelial Wound Healing

Rapid clearance occurs following topical ocular application, due in part to test agent drainage, blinking (every 5 minutes in mice), tear film, and tear film turnover. Therefore, in consideration of in vitro to in vivo dosing translation, low, mid, and high doses of 2, 64, and 640 μg/mL were evaluated in a murine model of corneal wound healing. Corneal epithelial scrape wounds (2-mm diameter) were made with an Algerbrush under a dissecting microscope in the right eye. Immediately following wound creation, 5 μL RP444 (2, 64, or 640 μg/mL) or vehicle (PBS) was applied. Topical application of the drops was repeated 5 minutes later, and then again at 6, 12, and 18 hours post wound. Corneal wound areas were assessed by staining with 1.5 μL 1% sodium fluorescein (Sigma-Aldrich Corp., St. Louis, MO, USA) every 6 hours until wound closure (24 hours) and images captured with an Olympus SZX16 stereomicroscope (Center Valley, PA, USA). Wound areas were outlined and measured with ImageJ software (https://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), and expressed as a percentage of the original wound area, at 0 hours. Data were analyzed using 2-way repeated measures ANOVA with Bonferroni’s test for multiple comparisons, with P < 0.05 considered significant.

### Murine P. aeruginosa Keratitis Model

Scarified eyes were infected with 1 × 10⁵ CFU P. aeruginosa ATCC 19660 as outlined by Kolar and colleagues, and dosed with 5 μL PBS or 2, 64, or 640 μg/mL RP444, beginning 8 hours post infection and continuing every 8 hours thereafter for 4 days. The clinical progression of infection was evaluated by capturing digital images using a slit-lamp biomicroscope equipped with a camera module CM 01 (Haag-Streit USA, Mason, OH, USA) on days 1, 3, and 5 post infection. The corneal infection was graded using a clinical scale of 0 to 4: 0, clear or slight opacity, partially covering the pupil; +1, slight opacity, fully covering the cornea; +2, dense opacity, partially or fully covering the pupil; +3, dense opacity fully covering the cornea; and +4, corneal perforation or phthisis.

At days 1, 3, and 5 post infection, corneas from infected eyes of 4 to 10 mice per group were harvested, and two corneas were pooled in 200 μL sterile PBS, generating two to five independent samples. The corneas were homogenized and then briefly sonicated. A 10-μL aliquot of the homogenate was diluted in sterile PBS and 10-fold serial dilutions were plated in duplicate onto Pseudomonas isolation agar plates. The plates were incubated overnight at 37°C and CFU counted. The remainder of the homogenate was processed to quantify the number of infiltrating inflammatory cells by myeloperoxidase (MPO) activity, a standard and well-established method in the study of infectious keratitis.

### RESULTS

#### dHDPs Exhibit Broad-Spectrum Bactericidal Activity

Eleven dHDPs were screened for their bactericidal effectiveness against four strains of Gram-positive and -negative bacteria, representing the most common keratitis pathogens, using the MIC assay (Table 2). RP438, RP442, RP443, and RP444 (shaded columns) were the most active peptides against both Gram-positive and -negative bacterial species and were selected for additional bactericidal screening.

#### dHDPs Are Active Against Gram-Negative and -Positive Biofilms

In addition to inhibiting planktonic growth, the MBEC values demonstrated effective eradication of both Gram-positive and -negative bacteria in their biofilm form (Table 3).
TABLE 3. Minimum Biofilm Eradication Concentration (MBECs, µg/mL) Values Against Gram-Positive and -Negative Biofilm Bacteria

<table>
<thead>
<tr>
<th>dHDPs</th>
<th>RP438</th>
<th>RP442</th>
<th>RP443</th>
<th>RP444</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRSA 33591</td>
<td>94</td>
<td>47</td>
<td>24</td>
<td>47</td>
</tr>
<tr>
<td>S. epidermis 51625</td>
<td>94</td>
<td>47</td>
<td>12</td>
<td>47</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. baumannii 17978D-5</td>
<td>47</td>
<td>6</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>P. aeruginosa 27853</td>
<td>375</td>
<td>750</td>
<td>188</td>
<td>188</td>
</tr>
</tbody>
</table>

Minimum biofilm eradication concentration (MBEC) is the lowest concentration needed to kill biofilm bacteria. Data represent the mean of three replicates from three independent experiments.

dHDPs Rapidly Eradicate *P. aeruginosa* and *S. aureus* in In Vitro Time-Kill Assays

The immediate concentration-dependent bacterial eradication by RP438, RP442, RP443, tobramycin, and vancomycin on *P. aeruginosa* 19660_Xen5 is shown in Figure 1. RP444 killed *P. aeruginosa* immediately in a concentration-dependent manner, suggesting that RP444 exerts its bactericidal activity by disrupting membrane function (Fig. 1). In contrast, all other evaluated dHDPs, including tobramycin, an aminoglycoside that disrupts cell membranes and protein synthesis in Gram-negative bacteria, and vancomycin, a bactericide that acts by inhibiting cell wall synthesis in Gram-positive bacteria, exhibited no trace of antibacterial activity upon direct exposure.

*P. aeruginosa*, as illustrated in Figure 2, was killed by all evaluated dHDPs following 60 minutes of exposure. The order of bactericidal effectiveness was RP444 followed by RP443, with RP442 and RP438 being equipotent. The striking immediate bactericidal activity of the dHDPs relates to direct electrostatic interaction with the bacterial walls. As anticipated, due to their respective modes of action, vancomycin showed no evidence of bactericidal activity during this short incubation time period (2 hours), with tobramycin exhibiting bactericidal effects at 60 minutes. The concentration-dependent time-kill evaluation of RP444 on *P. aeruginosa* 19660_Xen5 is displayed in Figure 3. *P. aeruginosa* was killed by RP444 within 30 minutes with as little as 2 µg/mL peptide.

The bactericidal activities of dHDPs, tobramycin, and vancomycin against *S. aureus* 49525_Xen56 are depicted in Figure 4 following 30 minutes' incubation time. RP442, RP443, and RP444 were effective at killing *S. aureus*, while neither tobramycin nor vancomycin exhibited any bactericidal effect during this exposure time.

**P. aeruginosa** Did Not Develop Resistance Against **RP444**

*P. aeruginosa* did not become resistant to RP444 after 21 rounds of selection whereas gentamicin did, as evidenced by growing at 1024 times the MIC after 21 days (Fig. 5).

**RP444 Did Not Impede In Vivo Corneal Epithelial Wound Healing**

RP444 did not affect corneal epithelial wound healing in an in vivo murine wound healing model, with results obtained from two independent experiments (Fig. 6). No statistically significant difference in closure at 24 hours following topical ocular RP444 administration was observed. Hence, risks associated with delayed healing would not be anticipated following RP444 application.

**RP444 Reduced the Severity of Murine *P. aeruginosa* Keratitis**

RP444 demonstrated significant in vivo activity in reducing clinical scores and corneal opacity in a *P. aeruginosa* model of bacterial keratitis (Fig. 7). Clinical scores were not different among the groups on day 1 post infection. However, scores were lower in treated animals on day 3 post infection, although they were not significantly different (data not shown). A dose-dependent decrease in ocular clinical scores was observed on day 5 post infection with scores of 2.56 ± 0.176, 1.80 ± 0.374, 1.56 ± 0.176, and 1.20 ± 0.200 obtained for the PBS control and 2, 64, and 640 µg/mL groups, respectively. All RP444-treated groups yielded lower clinical scores compared to the control group, with the data for the 64 and 640 µg/mL RP444-treated groups significantly less than for the control group (*P* < 0.001 and 0.0001 for 64 and 640 µg/mL RP444, respectively).

**RP444 Reduced Bacterial Burden in Murine *P. aeruginosa* Keratitis**

RP444 demonstrated statistically significant in vivo efficacy in reducing corneal bacterial burden in the murine *P. aeruginosa* model of bacterial keratitis. The number of viable bacteria recovered from the 64 and 640 µg/mL peptide-treated groups on day 3 (*P* < 0.001 and *P* < 0.001) and day 5 post infection (*P* < 0.01 and *P* < 0.001; Fig. 8A) was significantly lower than from the PBS-treated control. There was no difference in the number of viable bacteria between the control and 2 µg/mL peptide-treated groups at all time points.
RP444 Reduced Inflammatory Cell Infiltration in Murine P. aeruginosa Keratitis

RP444 demonstrated significant in vivo efficacy in reducing inflammatory cell infiltration in the P. aeruginosa model of bacterial keratitis (Fig. 8B). At day 1 post infection there was no significant difference between the vehicle- and RP444-treated corneas at any of the three concentrations. At day 3 post infection, MPO activity in corneas treated with 2, 64, or 640 µg/mL RP444 was dose-dependently lower than for treatment with the PBS vehicle (P < 0.05, P < 0.001, and P < 0.001 for 2, 64, and 640 µg/mL RP444, respectively). At day 5 post infection, MPO activity was lower in corneas treated with RP444, reaching statistical significance in the 64 and 640 µg/mL RP444-treated groups (P < 0.001).

DISCUSSION

Designed host defense peptides are synthetic analogues of naturally occurring HDPs that provide the first line of defense against invading pathogens. Endogenous HDPs were initially recognized for their microbicidal activity but are now recognized as critical immune effector and regulatory molecules that guard against infections and support healing, while
suppressing inflammation.\textsuperscript{13,35,36} To date, the experimental therapeutic benefit of HDPs for bacterial keratitis has been mixed. COL-1, a 20–amino acid peptide, at doses up to 50 \( \mu \)g/mL, was investigated in a rabbit model of \( P. \) aeruginosa keratitis.\textsuperscript{37} COL-1 did not exhibit antimicrobial activity and induced corneal toxicity.\textsuperscript{37} The COL-1 dosing regimen entailed administration beginning 12 to 14 hours post infection and then every 15 minutes for the first hour, followed by dosing every hour for the next 9 hours. Then on days 2 through 4, dosing was every hour for 10 hours. More recently, the topical application of OH-CATH30 was shown to be efficacious in a rabbit de-epithelialization \( P. \) aeruginosa keratitis model when administered as 10 hourly doses of 1 mg/mL, 8 hours post infection.\textsuperscript{38} Additionally, an amphibian skin-derived esculentin, Esc-1a(1-21)NH\textsubscript{2}, was shown to reduce bacterial load and improve ocular clinical scores in a murine \( P. \) aeruginosa-induced keratitis model following topical administration at 88 \( \mu \)g/mL at 5 hours post infection and continuing three times a day for 5 days.\textsuperscript{39}

Here, 11 dHDPs were shown to possess broad-spectrum antimicrobial activity following assessment of bactericidal effectiveness against isolates of both Gram-positive and -negative bacteria, and against drug-resistant pathogens (MRSA and \( P. \) aeruginosa). Empirical antimicrobial management of ocular infections is needed so an antimicrobial treatment possessing broad-spectrum activity with activity against recalcitrant biofilm is critical to limit the potential threat of corneal damage and vision loss.\textsuperscript{40} Four of these dHDPs (RP438, RP442, RP443, and RP444) were evaluated in additional in vitro assays that demonstrated their effectiveness in eradicating bacteria in biofilm. Biofilms are sophisticated colonies of microorganisms encased in a dense extracellular matrix enabling the bacteria to be extremely virulent and resilient, hence the imperative need to find efficacious agents that are able to encroach biofilms and kill bacteria.\textsuperscript{40} Preclinical studies indicate that mature biofilms are a common resilient feature of keratitis and need to be considered when developing therapeutic agents.\textsuperscript{41}

Bacterial eradication should be the primary goal of antibiotic treatment, with bacterial load the main determinant of therapeutic outcome.\textsuperscript{42} Rapid, precise healing at the corneal surface is critical for restoration of the cornea’s important protective and optical functions. Additionally, the rapid elimination of the infective organism should limit the emergence of resistance and the spread of infection. MIC and MBEC values, though useful for bacterial screening, provide no information on the time course of antimicrobial activity or the distinction between bacteriostatic and bactericidal mechanisms of action. Antimicrobial agents that disrupt cell membranes or interfere with essential enzyme function are likely to be bactericidal, whereas agents that inhibit ribosomal
function are most likely bacteriostatic. An innovative time-kill assay using *P. aeruginosa* and *S. aureus* transfected with luciferase reporter genes was utilized to enable real-time evaluation of the effects of DHDPs and the antimicrobial agents tobramycin and vancomycin on bacteria viability. The in vitro time-kill assays demonstrated that RP444 quickly killed bacteria, particularly *P. aeruginosa*, at relatively low doses (2 μg/mL) that were not cytotoxic to ocular cells as evidenced in the corneal epithelial wound study, which utilized repeat dosing of RP444 concentrations up to 640 μg/mL. The rapid destruction of the bacterial cells by potent broad-spectrum topical anti-infectives, thought to be primarily due to peptide–lipid interactions, imply a theoretical reduced likelihood of developing bacterial resistance.6,7 This was supported by RP444’s lack of induction of resistance following repeated incubation with *P. aeruginosa*. Given the favorable therapeutic index with selective disruption of bacterial cells over the in vivo murine corneal epithelial cells, and its rapid bactericidal activity, RP444 was selected for evaluation of its therapeutic potential in a murine model of bacterial keratitis.

The topical application of 64 and 640 μg/mL RP444, at a dosing volume of 5 μL, reduced the severity of murine *P. aeruginosa* keratitis. This is reflected in an improved clinical score, reduced recovery of viable bacteria, and reduced immune cell infiltration.

In summary, a topically applied antimicrobial peptide, RP444, has been identified that exhibits rapid bactericidal activity, broad-spectrum effectiveness against isolates and biofilm, selective targeting of bacterial cell walls, and reduced

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**Figure 7.** RP444 significantly reduced ocular disease in a murine *P. aeruginosa* keratitis model. Data are mean clinical scores ± SE from two independent experiments 5 days post infection with *n* = 9, 5, 9, and 10 mice for 0, 2, 64, and 640 μg/mL groups, respectively. **P < 0.01 and ***P < 0.001; statistically significant difference among control and RP444 treated. The right side of the figure shows representative photographs of the treated infected right eyes compared to the uninfected left eye, illustrating a reduction in pathology (opacity) with RP444 treatment.

**Figure 8.** RP444 topical treatment reduces bacterial load and inflammatory cell infiltration in a murine *P. aeruginosa* keratitis model. (A) Viable bacterial counts in infected corneas treated with RP444 at 64 and 640 μg/mL were significantly lower than in PBS-treated animals at days 3 and 5 post infection. (B) A dose-dependent decrease in inflammatory cell infiltration, as measured by myeloperoxidase (MPO) activity, was observed in RP444-treated animals at days 3 and 5 post infection. Data are from three to six independent samples generated in one (2 and 640 μg/mL) or two experiments (64 μg/mL). Statistical significance was *P < 0.5, **P < 0.01, and ***P < 0.001.
likelihood of developing bacterial resistance. Further preclinical evaluation is under way to evaluate RP444 as a potential therapeutic for the treatment of sight-threatening bacterial keratitis.

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References


34. Schwab U, Gilligan P, Jaynes J, Henke D. In vitro activities of designed antimicrobial peptides against multidrug-resistant bacterial strains.


