Designed Host Defense Peptides for the Treatment of Bacterial Keratitis

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PURPOSE. To limit corneal damage and potential loss of vision, bacterial keratitis must be treated aggressively. Innovation in antimicrobials is required due to the need for empirical treatment and 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
against Gram-positive and -negative bacteria.\textsuperscript{13,14} The direct killing of bacteria via perturbation of the cell membrane infers a reduced likelihood of inducing bacterial resistance, a critical key in fighting antibiotic-resistant pathogens.\textsuperscript{15}

There is an urgent and profound need for the design and clinical trial of dHDPs to treat sight-threatening infectious keratitis. Here, 11 novel dHDPs were evaluated for their bactericidal effectiveness against isolates and biofilm cultures of both Gram-positive and -negative bacteria, and against drug-resistant MRSA and \textit{P. aeruginosa}. The most promising of these novel peptides, RP444, was evaluated for potential to induce resistance and efficacy in a murine model of \textit{P. aeruginosa} 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.\textsuperscript{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.\textsuperscript{20–21}

**Bacterial Strains**

Bacterial strains were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The Gram-positive bacterial strains included \textit{Enterococcus faecium} ATCC 700221 (\textit{E. faecium} 700221), methicillin-resistant \textit{Staphylococcus aureus} ATCC 35951 (MRSA 35951), \textit{Staphylococcus epidermidis} ATCC 51625 (\textit{S. epidermidis} 51625), \textit{Streptococcus pneumoniae} clinical development of \textit{RP436} (\textit{S. pneumoniae} 49619), and \textit{Staphylococcus aureus} ATCC 49525 (Wright).

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

The bioluminescence strains, \textit{P. aeruginosa} 19660 transfected with the Xen5 luciferase gene (here termed \textit{P. aeruginosa} 19660_Xen5), and \textit{Staphylococcus aureus} ATCC 49525 (Wright) transfected with the Xen5 luciferase gene (\textit{S. aureus} 49525_Xen36), were obtained from PerkinElmer (Hopkinton, MA, USA).\textsuperscript{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.\textsuperscript{24} The minimum bactericidal concentrations (MBECs) were determined using the MBEC Assay system (Innovotech, Edmonton, AB, Canada).\textsuperscript{25} Briefly, the bacteria were 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 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 \textit{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 redetermined 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 \textit{P. aeruginosa} and \textit{S. aureus}.\textsuperscript{26–27} Cultures of \textit{P. aeruginosa} 19660_Xen5 and \textit{S. aureus} 49525_Xen36 were made from a single colony, which was inoculated into 5 mL sterile L-hysogeny 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 \(1 \times 10^7\) CFU/mL. Bacterial suspensions (100 mL) were plated in 96-well black-walled plates. The candidate dHDPs were 2-fold serially diluted from 128 mg/mL in LB. Each concentration was done in triplicate, with the final volume being 200 mL. 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 fps 1. Photons were counted for 30 seconds, and data analysis was performed using the Living Image 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%(vol/vol) H2O2 in PBS. The absorbance was measured for up to 3 hours at 450 nm. A standard curve was generated using purified MPO (Calbiochem, San Diego, CA, USA) on the same plate. Results were expressed as relative units of MPO activity per cornea (one MPO unit is proportional to 2 x 10⁵ infiltrating neutrophils).

Data are expressed as mean ± standard error (SE). Comparisons were performed using a 1-way analysis of variance (ANOVA) followed by a post hoc Dunnett’s test. A P value < 0.05 was considered statistically significant.

Table 2. Minimum Inhibitory Concentrations (MICs, µg/mL) Against Gram-Positive and -Negative Bacteria

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<td>&gt;64</td>
<td>16</td>
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</tbody>
</table>

Minimum inhibitory concentration (MIC) is the lowest concentration of dHDP that shows no growth after a 24-hour incubation. Data represent the mean of three replicates from three independent experiments.

* RP438, RP442, RP443, and RP444 columns represent the most potent broad-spectrum dHDPs.

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 demonstrate 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>Gram-positive bacteria</th>
<th>dHDPs</th>
<th>RP438</th>
<th>RP442</th>
<th>RP443</th>
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<td>94</td>
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<td>12</td>
<td>47</td>
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<tr>
<td>Gram-negative bacteria</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>P. aeruginosa 27853</td>
<td></td>
<td>375</td>
<td>750</td>
<td>188</td>
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</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* immediate 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.

**FIGURE 1.** RP444 kills *P. aeruginosa* upon direct application. RP438, RP442, RP443, RP444, tobramycin, and vancomycin were applied to *P. aeruginosa* 19660_Xen5, and bactericidal effectiveness was assessed immediately after direct contact at time zero. The bioluminescence of viable bacteria was evaluated noninvasively with an IVIS Lumina bioimaging system. Data represent the mean ± SE of triplicate replicates from two independent experiments. For some points, the error bars are shorter than the height of the symbols.
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.24 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. \cite{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 µg/mL, was investigated in a rabbit model of \textit{P. aeruginosa} keratitis. \cite{37} COL-1 did not exhibit antimicrobial activity and induced corneal toxicity. \cite{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 \textit{P. aeruginosa} keratitis model when administered as 10 hourly doses of 1 mg/mL, 8 hours post infection. \cite{38} Additionally, an amphibian skin-derived esculentin, Esc-1a(1-21)NH2, was shown to reduce bacterial load and improve ocular clinical scores in a murine \textit{P. aeruginosa}-induced keratitis model following topical administration at 88 µg/mL at 5 hours post infection and continuing three times a day for 5 days. \cite{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 \textit{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. \cite{40} Four of these dHDPs (RP438, RI41-2, 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. \cite{41} Preclinical studies indicate that mature biofilms are a common resilient feature of keratitis and need to be considered when developing therapeutic agents. \cite{42}

Bacterial eradication should be the primary goal of antibiotic treatment, with bacterial load the main determinant of therapeutic outcome. \cite{43} 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

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{RP442, RP443, and RP444 exhibit antibacterial activity against \textit{S. aureus}. The concentration-dependent profile of dHDPs, tobramycin, and vancomycin against \textit{S. aureus} 49525.Xen36 following 30 minutes. The bioluminescence of viable cells was quantitated with an IVIS Lumina imaging system. Data represent the mean ± SE of triplicate replicates from two independent experiments. For some points, the error bars are shorter than the height of the symbols.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{\textit{P. aeruginosa} did not develop resistance against RP444. After 21 days of culturing \textit{P. aeruginosa} ATCC 27853 with gentamicin, resistance was observed as 1024 times the MIC was needed for growth to occur whereas no resistance was observed with RP444. Assay was performed in duplicate; means are shown.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure6.png}
\caption{RP444 treatment does not affect in vivo wound closure. Mice were wounded and treated with vehicle (PBS) or RP444 (2, 64, or 640 µg/mL) every 6 hours for 24 hours. Corneal wound areas were assessed by fluorescein staining every 6 hours. The percentage of wound remaining open was determined by comparison to the original wound area. Data represent mean ± SE and were analyzed with 2-way repeated measures ANOVA and Bonferroni’s correction for multiple comparisons. *$P < 0.05$; statistically significant difference between vehicle and RP444 640 µg/mL treatment; n = 7–10 mice/group.}
\end{figure}
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
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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