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EXPERIMENTAL THERAPEUTICS

Development of a Broad-Spectrum Antimicrobial Combination for the Treatment of *Staphylococcus aureus* and *Pseudomonas aeruginosa* Corneal Infections

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ABSTRACT *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most common causes of bacterial keratitis and corresponding corneal blindness. Accordingly, such infections are predominantly treated with broad-spectrum fluoroquinolones, such as moxifloxacin. Yet, the rising fluoroquinolone resistance has necessitated the development of alternative therapeutic options. Herein, we describe the development of a polymyxin B-trimethoprim (PT) ophthalmic formulation containing the antibiotic rifampin, which exhibits synergistic antimicrobial activity toward a panel of contemporary ocular clinical *S. aureus* and *P. aeruginosa* isolates, low spontaneous resistance frequency, and *in vitro* bactericidal kinetics and antibiofilm activities equaling or exceeding the antimicrobial properties of moxifloxacin. The PT plus rifampin combination also demonstrated increased efficacy in comparison to those of either commercial PT or moxifloxacin in a murine keratitis model of infection, resulting in bacterial clearance of 70% in the animals treated. These results suggest that the combination of PT and rifampin may represent a novel antimicrobial agent in the treatment of bacterial keratitis.

KEYWORDS *Pseudomonas aeruginosa*, *Staphylococcus aureus*, keratitis, polymyxin, rifampin, trimethoprim

Bacterial keratitis (corneal infection) is a potentially devastating, vision-threatening ocular disease that requires emergent antimicrobial treatment to prevent scarring, corneal perforation, and/or endophthalmitis (1, 2). Globally, bacterial keratitis is a leading cause of blindness, with 2 million new cases reported annually (3, 4). The most common etiologies of keratitis include *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which both induce rapid destructive disease courses that are clinically indistinguishable, thus requiring immediate empirical antimicrobial therapy (5–8).

Fluoroquinolone-based ophthalmic solutions have been widely favored for the treatment of ocular infections because of their broad-spectrum activity, excellent tissue penetrance, and patient tolerability (9). However, emerging resistance among both *S. aureus* and *P. aeruginosa* has rendered many of these drugs ineffective, resulting in increased disease severity and worse visual outcomes (10, 11). Indeed, resistance rates as high as 71% have been reported among *S. aureus* ocular isolates to levofloxacin, a broad-spectrum fluoroquinolone and the only Food and Drug Administration (FDA)-approved antibiotic for the treatment of bacterial keratitis (9, 12). With limited commercially available alternatives, clinicians have resorted to the off-label use of broad-spectrum fluoroquinolones such as moxifloxacin as the predominant bacterial keratitis treatment choice (13).

Citation Chojnacki M, Philbrick A, Wucher B, Reed JN, Tomaras A, Dunman PM, Wozniak RAF. 2019. Development of a broad-spectrum antimicrobial combination for the treatment of *Staphylococcus aureus* and *Pseudomonas aeruginosa* corneal infections. Antimicrob Agents Chemother 63:e01929-18. https://doi.org/10.1128/AAC.01929-18.

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Received 10 September 2018
Returned for modification 6 October 2018
Accepted 25 October 2018
Accepted manuscript posted online 12 November 2018
Published 21 December 2018

Predictably, the emergence of antibiotic resistance has now also significantly limited the effectiveness the broad-spectrum fluoroquinolones. Indeed, antibiotic resistance surveillance programs have revealed moxifloxacin resistance routinely occurs in 40% to 60% of methicillin-resistant *S. aureus* (MRSA) ocular isolates within the United States (5, 6, 12, 14). While resistance among *P. aeruginosa* ocular isolates has remained low nationally, broad-spectrum fluoroquinolone resistance in India has increased significantly, from 19% in 2007% to 52% in 2009, necessitating the development of alternative therapeutic options (15).

Polymyxin B-trimethoprim (PT) is a broad-spectrum antibiotic combination commonly used for the treatment of mild bacterial conjunctivitis (16). The polymyxin B component is active against Gram-negative organisms, including *P. aeruginosa*, whereas trimethoprim primarily targets Gram-positive organisms such as *S. aureus* (17–19). Resistance rates among ocular isolates to either polymyxin B or trimethoprim in isolation are relatively low in comparison to those for the fluoroquinolones, with 3% of *P. aeruginosa* isolates demonstrating polymyxin B resistance and trimethoprim resistance rates of 3% and 7% for methicillin-susceptible *S. aureus* (MSSA) and MRSA, respectively (12). Despite the limited bacterial resistance, PT is rarely considered a bacterial keratitis treatment option because of its low antimicrobial potency, slow killing kinetics, weak antibiofilm activity, and limited tissue penetration relative to those of fluoroquinolones (20–23).

Herein, we sought to develop an improved PT-based ophthalmic combination that overcomes these inherent liabilities. Screens of an FDA-approved drug library and the subsequent hit characterization arrived at a PT plus rifampin triple antibiotic formulation that displays potent, synergistic *in vitro* antimicrobial activities and *in vivo* efficacy that equals or exceeds the performance of broad-spectrum fluoroquinolones and may represent a promising new bacterial keratitis treatment option.

RESULTS

Identification of compounds that improve the antimicrobial activity of polymyxin B-trimethoprim. Combination therapies offer the potential for synergistic activity that surpasses each component in isolation. Thus, as a first step toward improving the antimicrobial performance of polymyxin B-trimethoprim (PT), a synergy screen was performed to identify drug combinations that may enhance the antimicrobial potency of PT toward *S. aureus* and/or *P. aeruginosa*.

Accordingly, an 853-member FDA-approved drug library was screened for agents that confer antibacterial activity toward *S. aureus* strain UAMS-1 in the presence of an otherwise subinhibitory concentration of PT. To do so, a total of 10^5 CFU were inoculated in individual wells of a microtiter plate supplemented with 0.25× MIC of PT (1.2 $\mu g \cdot ml^{-1}$). Library members were added at a low (5 μM) or high (50 μM) concentration and then scored to identify agents that inhibited *S. aureus* growth following an overnight incubation. Such agents were expected to represent compounds that display antimicrobial activity on their own and/or potentiate the antimicrobial activity of polymyxin B-trimethoprim. In parallel, the library was also screened with low and high concentrations of agents that inhibited the growth of *P. aeruginosa* PAO1 cells in medium supplemented with 0.5× MIC of PT (0.2 $\mu g \cdot ml^{-1}$).

The *S. aureus* screening results revealed that 153 library compounds inhibited bacterial growth at 50 μM in the presence of 0.25× MIC PT, and of those, 75 also displayed antibacterial activity at 5 μM, suggesting that they represent the most potent antistaphylococcal agents and/or potentiators of the antimicrobial performance of polymyxin B-trimethoprim (see Table S1 in the supplemental material). For *P. aeruginosa*, a total of 63 compounds inhibited growth at 50 μM in the presence of an otherwise subinhibitory concentration of PT, 32 of which also inhibited growth at 5 μM (Table S1).

Given the fundamental need for broad-spectrum ophthalmic antimicrobials, we considered that the most therapeutically relevant PT combination(s) would include agents that display potent activities toward both organisms. In that regard, a compar-

TABLE 1 Selected agents with activity against *S. aureus* and *P. aeruginosa* as well as performance in relevant assays

Drug	*S. aureus*[a]		*P. aeruginosa*[a]		*P. aeruginosa* biofilm AK signal[b]	*A. baumannii*[c]			
	5 μM	50 μM	5 μM	50 μM		MH	Serum	Surfactant	AK signal
Rifabutin	−	−	−	−	+	−	+	−	+
Rifapentine	−	−	−	−	+	−	+	−	+
Rifampin	−	−	−	−	+	−	−	−	+
Rifaximin	−	−	−	−	+	−	−	−	+
Pefloxacin	−	−	−	−	+	−	+	−	+
Sparfloxacin	−	−	−	−	+	−	+	−	+
Levofloxacin	+	−	−	−	+	−	−	+	+
Sarafloxacin	−	−	−	−	+	−	+	−	+
Sitafloxacin	−	−	−	−	+	−	+	−	+
Tobramycin	+	−	−	−	+	−	+	−	+/−
Lomefloxacin	−	−	−	−	+	−	+	+	+
Besifloxacin	−	−	−	−	+	−	+	−	+
Danofloxacin	−	−	−	−	+	−	+	+	+
Enrofloxacin	−	−	−	−	+	−	+	−	+
Nadifloxacin	−	−	−	−	+	−	+	−	+
Clinafloxacin	−	−	−	−	+	−	+	−	+

[a]Growth (+) or no growth (−) of *S. aureus* UAMS-1 in the presence of 1.2 $\mu g \cdot ml^{-1}$ polymyxin B-trimethoprim or *P. aeruginosa* PAO1 in the presence of 0.2 $\mu g \cdot ml^{-1}$ polymyxin B-trimethoprim at the indicated drug concentrations.
[b]Killing (+) of established biofilms as measured by the adenylate kinase reporter assay for bacterial cell death (24).
[c]Antimicrobial effects of each drug toward *A. baumannii* in Mueller Hinton (MH) broth, human serum, lung surfactant, or established biofilm (25).

ison of the screening results from each organism revealed that 18 test agents exhibited activities against both *S. aureus* and *P. aeruginosa* at 5 μM and 50 μM, 14 of which were also previously reported to exhibit activities toward established *P. aeruginosa* biofilms (24) and *Acinetobacter baumannii*, a rare but significant cause of keratitis (25). Thus, these 14 compounds (Table 1) were considered to be of greatest interest and included 4 rifamycins (rifabutin, rifapentine, rifampin, and rifaximin), 2 quinolones (sarafloxacin and pefloxacin), and 8 fluoroquinolones (sparfloxacin, sitafloxacin, lomefloxacin, besifloxacin, danofloxacin, enrofloxacin, nadifloxacin, and clinafloxacin). A representative rifamycin (rifampin) and fluoroquinolone (besifloxacin) were selected for further study. Additionally, the aminoglycoside tobramycin was also selected for further characterization because of current use in clinical practice, despite demonstrating activity against *P. aeruginosa* at 5 μM and 50 μM but only at a high concentration (50 μM) toward *S. aureus*.

Rifampin and tobramycin improve the antimicrobial activity of polymyxin B-trimethoprim. To distinguish the agents with independent antibacterial activity from those that exhibit true synergistic antimicrobial activity when combined with polymyxin B-trimethoprim, fractional inhibitory concentrations (FICs) were measured for rifampin, tobramycin, and besifloxacin. To do so, the MIC of each drug in isolation was compared to the MIC of the drug when in combination with PT; an FIC index of <0.5 was considered synergistic, with 0.5 to 1 being additive, 1 to 4 considered indifferent/neutral, and >4 indicating antagonism (26).

FIC testing revealed that rifampin plus PT exhibited the greatest synergistic/additive effect toward both *S. aureus* and *P. aeruginosa* (Table 2). More specifically, for *S. aureus* UAMS-1 cells, the MICs of polymyxin B-trimethoprim and rifampin in isolation were 2.1 $\mu g \cdot ml^{-1}$ and 0.01 $\mu g \cdot ml^{-1}$, respectively. However, the combination of rifampin plus PT resulted in $\geq$6-fold and 3-fold improvements in the antimicrobial activities of rifampin (0.0015 $\mu g \cdot ml^{-1}$) and PT (0.67 $\mu g \cdot ml^{-1}$), respectively, resulting in a synergistic effect (FIC, 0.469). Against *P. aeruginosa* strain PAO1, the MICs of PT and rifampin in isolation were 0.41 $\mu g \cdot ml^{-1}$ and 15.6 $\mu g \cdot ml^{-1}$, respectively, whereas the combination of rifampin with polymyxin B-trimethoprim resulted in $>$8-fold and $>$2-fold improvements in the antimicrobial activities of rifampin (1.9 $\mu g \cdot ml^{-1}$) and PT (0.21 $\mu g \cdot ml^{-1}$), respectively, resulting in an additive effect (FIC, 0.637).

Conversely, neither the combination of tobramycin or besifloxacin with PT exhibited improved activity toward both *S. aureus* and *P. aeruginosa*. While tobramycin plus PT

TABLE 2 MIC and FIC testing of PT, rifampin, tobramycin, and besifloxacin alone and in combination toward *P. aeruginosa* PAO1 and *S. aureus* UAMS-1

Organism	MIC ($\mu g \cdot ml^{-1}$)								FIC index[a]
	Alone				Combination				
	PT[b]	Rifampin	Tobramycin	Besifloxacin	PT	Rifampin	Tobramycin	Besifloxacin	
P. aeruginosa	0.41	15.6			0.21	1.9			0.637
S. aureus	2.1	0.01			0.67	0.0015			0.469
P. aeruginosa	0.41		0.48		0.32		0.12		1.03
S. aureus	2.1		1.9		1.3		0.48		0.872
P. aeruginosa	0.41			1.56	0.205			0.78	1
S. aureus	2.1			0.12	1.05			0.06	1

[a]FIC, fractional inhibitory concentration.
[b]PT, polymyxin B-trimethoprim.

exhibited an additive antimicrobial effect toward *S. aureus* (FIC, 0.872) the combination exhibited a neutral effect (FIC, 1.03) toward *P. aeruginosa* (Table 2). Besifloxacin showed modest improvements in antimicrobial activity when in combination with PT, yet the resulting FIC indexes against *S. aureus* and *P. aeruginosa* were both 1, indicating a neutral effect. Given the fact that besifloxacin did not demonstrate the ability to potentiate the antimicrobial effect of PT, it was not considered for additional study. However, tobramycin and rifampin did display the ability to improve the potency of PT, albeit to differing magnitudes, and were thus advanced for further characterization.

Efficacy of polymyxin B-trimethoprim in combination with rifampin or tobramycin toward clinical ocular isolates. To assess whether the improved antimicrobial performance of PT in combination with tobramycin or rifampin is maintained across clinically relevant strains, FIC testing was expanded to include a panel of contemporary ocular isolates. Seven *P. aeruginosa* clinical keratitis isolates were collected from the University of Rochester and genetically characterized according to common virulence factors and antibiotic resistance profiles to ensure diversity (see Table S2), whereas 20 *S. aureus* ocular clinical isolates collected from diverse geographical regions of the United States were acquired from International Health Management Associates.

FIC testing revealed that the antimicrobial effects of PT plus rifampin were conserved across both sets of clinical isolates, whereas the properties of PT plus tobramycin were less conserved. More specifically, for all strains tested, polymyxin B-trimethoprim plus rifampin displayed a severalfold improvement over the activity of each drug in isolation, resulting in average FICs of 0.463 ($\pm$ 0.04) and 0.509 ($\pm$ 0.04) toward the *S. aureus* and *P. aeruginosa* clinical isolate sets, respectively (Table 3). Conversely, the addition of tobramycin to polymyxin B-trimethoprim exhibited more variability, resulting in FIC values of 1.14 ($\pm$ 0.17) for *P. aeruginosa* and 0.936 ($\pm$ 0.37) for *S. aureus* isolates (see Table S3). Thus, in contrast to PT plus tobramycin, the antimicrobial potency of PT plus rifampin was well preserved across a diverse set of clinical isolates, supporting the therapeutic promise of this combination.

Antibiofilm activity of polymyxin B-trimethoprim combinations. As a second means to compare the therapeutic potential of PT plus rifampin or tobramycin, we assessed the antimicrobial properties of each toward established biofilms, given the likely role of biofilms in potentiating corneal disease (27, 28).

As shown in Fig. 1A, the combination of PT plus rifampin showed synergistic antibiofilm activity toward 48-h established *S. aureus* UAMS-1 biofilms that exceeded the antimicrobial activity of moxifloxacin (MIC, 0.5 $\mu g \cdot ml^{-1}$). More specifically, while PT (alone) did not exhibit significant antibiofilm activity at any concentration tested, both rifampin (alone) and moxifloxacin displayed dose-dependent reductions in biofilm-associated cells, resulting in a maximum 4-log reduction in biofilm-associated bacteria at 32$\times$ the planktonic MIC of the strain. Strikingly, the combination of polymyxin B-trimethoprim plus rifampin demonstrated the most potent *S. aureus* antibiofilm activity, with significant antimicrobial activity observed at 2$\times$ MIC and a maximal 6-log reduction in biofilm-associated *S. aureus* at 32$\times$ MIC. Similarly, the treatment of 48-h

TABLE 3 FIC testing of PT plus rifampin against clinical isolates

Strain	MIC ($\mu g \cdot ml^{-1}$)				FIC index[a]
	Alone		Combination		
	PT[b]	Rifampin	PT	Rifampin	
P. aeruginosa					
PAO1	0.41	15.6	0.21	1.9	0.634
RW01	0.41	15.6	0.13	1.9	0.439
RW02	0.41	15.6	0.16	1.9	0.512
RW03	0.51	15.6	0.16	3.9	0.564
RW04	0.32	15.6	0.13	1.9	0.528
RW05	0.32	31.25	0.13	1.9	0.467
RW06	0.32	15.6	0.13	1.9	0.528
RW07	0.32	15.6	0.13	1.9	0.528
S. aureus					
UAMS-1	2.1	0.01	0.67	0.0015	0.469
1110936	1.6	0.05	0.67	0.0035	0.489
1111063	2.1	0.1	0.67	0.0076	0.395
1071788	1.6	0.01	0.67	0.0015	0.569
1103430[c]	2.1	0.01	0.67	0.0015	0.469
1094094[c]	2.1	0.01	0.67	0.0015	0.469
1094140[c]	2.1	0.01	0.67	0.0015	0.469
1094147	2.1	0.01	0.67	0.0015	0.469
1094166	2.1	0.01	0.67	0.0015	0.469
1094178	2.1	0.1	0.67	0.0076	0.395
1144105[c]	1.6	0.05	0.67	0.0035	0.489
1097612	2.1	0.05	0.67	0.0035	0.389
1097630	2.1	0.01	0.67	0.0015	0.469
1122181[c]	2.1	0.01	0.67	0.0015	0.469
1122187[c]	2.1	0.05	0.67	0.0035	0.389
1122190[c]	1.6	0.05	0.67	0.0035	0.489
1122197[c]	2.1	0.01	0.67	0.0015	0.469
1132822[c]	2.1	0.01	0.67	0.0015	0.469
1101442[c]	1.6	0.05	0.67	0.0035	0.489
1142776[c]	2.1	0.01	0.67	0.0015	0.469
1125120[c]	2.1	0.05	0.67	0.0076	0.471
Vig R	0.65	0.03	0.31	0.0078	0.75

[a]FIC, fractional inhibitory concentration.
[b]PT, polymyxin B-trimethoprim.
[c]Methicillin-resistant *Staphylococcus aureus* (MRSA).

established *P. aeruginosa* strain PAO1 biofilms with PT, rifampin, and PT plus rifampin showed that the combination displayed increased antimicrobial activity in comparison to that of each agent in isolation and approached the antibiofilm activity of moxifloxacin (Fig. 1B). Indeed, PT (alone) exhibited mild antimicrobial activity against biofilms, resulting in a 2-log reduction in CFU $\cdot ml^{-1}$ at 32× the planktonic PT MIC for the strain (0.41 $\mu g \cdot ml^{-1}$). Treatment with rifampin (alone) produced a dose-dependent antibiofilm effect, with a maximum 5-log reduction in CFU at a concentration 32× MIC. The combination of PT plus rifampin outperformed each drug alone in a dose-dependent manner starting at 2× MIC, resulting in a maximum 6-log reduction in CFU at 32× MIC, which equaled the performance of moxifloxacin (MIC, 1 $\mu g \cdot ml^{-1}$).

By comparison, PT plus tobramycin displayed less antimicrobial activity and did not exhibit a strong additive/synergistic antimicrobial effect toward biofilms formed by either organism (Fig. 1C and D). For *S. aureus*, neither PT, tobramycin, nor their combination (tobramycin plus PT) displayed >1-log reduction in biofilm-associated cells at any concentration tested. Toward *P. aeruginosa* biofilms, the antimicrobial effects of tobramycin alone and in combination with PT mimicked one other, exhibiting dose-dependent decreases in biofilm-associated cells and maximum 5-log reductions at 32× MIC that were inferior to the activity of moxifloxacin.

Altogether, these results indicate that polymyxin B-trimethoprim plus rifampin may represent the more promising PT combination for the treatment of ocular biofilm-associated infections. Furthermore, on the basis if the aforementioned conserved

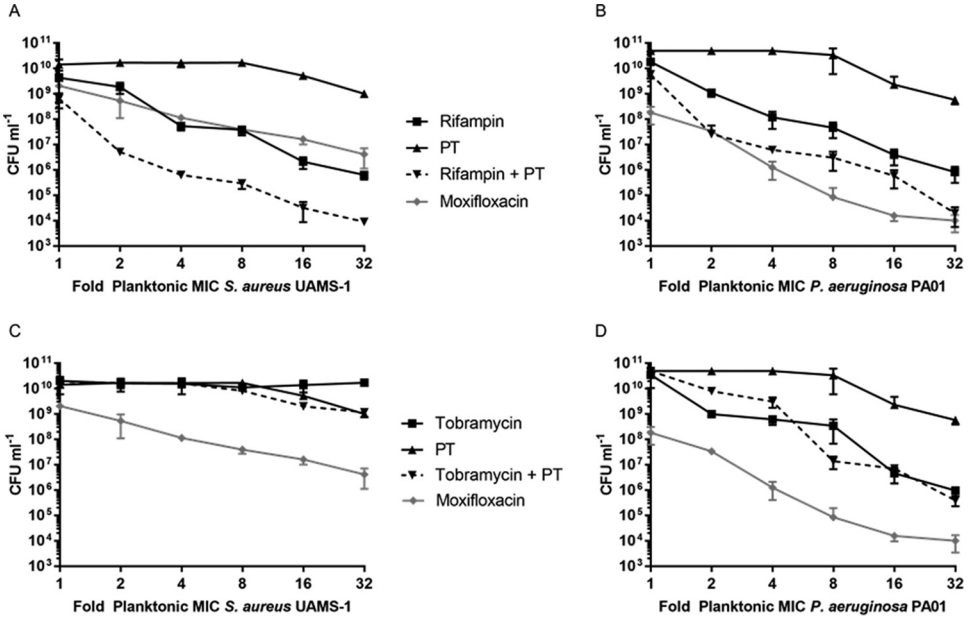

FIG 1 Antibiofilm activity of polymyxin B-trimethoprim with/without rifampin and polymyxin B-trimethoprim with/without tobramycin. Biofilms established for 48 to 72 h were treated with increasing concentrations of indicated drug concentration as derived by fold-increase over MIC planktonic cells. (A) Activity of rifampin with/without PT against *S. aureus* UAMS-1. (B) Activity of rifampin with/without PT against *P. aeruginosa* PAO1. (C) Activity of tobramycin with/without PT against *S. aureus* UAMS-1. (D) Activity of tobramycin with/without PT against *P. aeruginosa* PAO1. Performance of moxifloxacin and standard deviations shown ($n = 3$).

synergistic activity of the combination toward clinical isolates, we chose to continue the characterization of PT plus rifampin.

Bactericidal activity of polymyxin B-trimethoprim in combination with rifampin. As noted above, the limited inherent weaker bactericidal activity of PT has limited its utility as a bacterial keratitis therapeutic. Thus, we performed standard kill-curve assays to assess whether PT plus rifampin was capable of overcoming this liability. To do so, *S. aureus* UAMS-1 or *P. aeruginosa* PAO1 was grown to exponential phase and then treated with 2× MIC of rifampin, PT, or PT plus rifampin; the number of viable CFU remaining was then measured over the course of 24 h.

As shown in Fig. 2A, while the treatment of *S. aureus* cells with PT or rifampin in isolation resulted in increased cell death over time, the combination displayed more rapid bactericidal activity and equaled the performance of moxifloxacin. More specifically, both PT (alone) and rifampin (alone) resulted in 3-log to 4-log reductions in *S. aureus* cells at 4 h posttreatment. The combination of PT plus rifampin displayed more rapid bactericidal activity, with a 6-log reduction in viable cells at 4 h posttreatment,

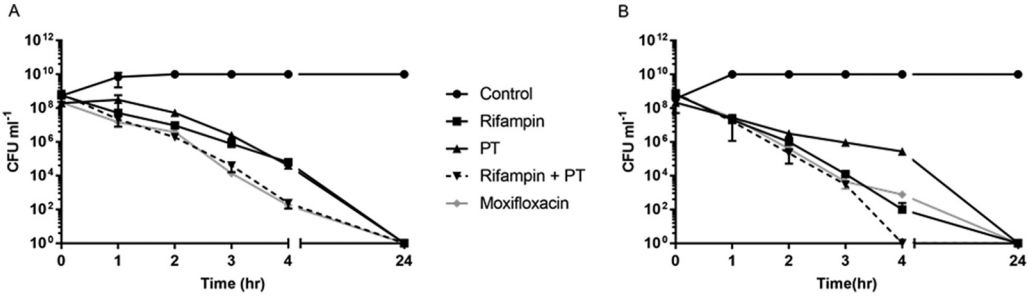

FIG 2 Bactericidal kinetics of polymyxin B-trimethoprim with/without rifampin. Bacterial cultures were treated with 2× MIC of the indicated drug and resulting CFU were plotted over time. (A) Activity of rifampin with/without PT against *S. aureus* UAMS-1. (B) Activity of rifampin with/without PT against *P. aeruginosa* PAO1. Performance of moxifloxacin and standard deviations shown ($n = 3$).

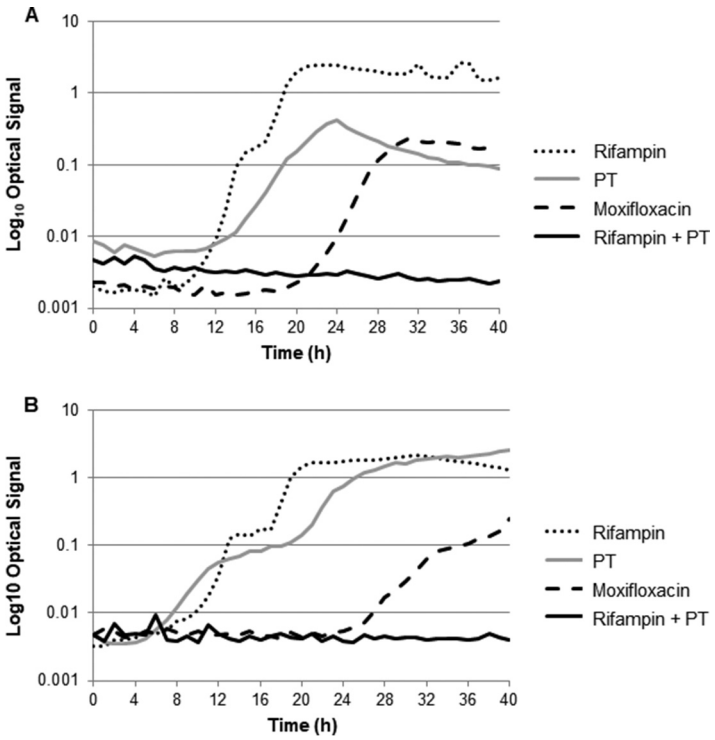

FIG 3 Antimicrobial resistance. Shown are optical signal measures of *P. aeruginosa* PAO1 cells (A) or *S. aureus* UAMS-1 cells (B) in medium supplemented with 1× MIC of polymyxin B-trimethoprim with/without rifampin or moxifloxacin. Plots are representative data from one of six independent assays.

which was nearly identical to the antimicrobial effects of moxifloxacin at all time points. Against *P. aeruginosa*, the combination displayed more rapid bactericidal activity than PT (alone), rifampin (alone), or moxifloxacin (Fig. 2B). Indeed, both rifampin and moxifloxacin treatment resulted in 5-log and 6-log CFU reductions at 4 h, respectively, and no viable cells at 24 h. Similarly, PT alone resulted in a 3-log CFU reduction at 4 h and eradication of viable cells by 24 h. However, the combination of PT plus rifampin resulted in notable improvement in bactericidal activity, resulting in the complete eradication of viable cells by 4 h.

Improved antimicrobial resistance profile of polymyxin B-trimethoprim plus rifampin. While the emergence of bacterial antibiotic resistance is inevitable, the propensity to develop resistance is, in part, contingent upon the mutability of an agent's intracellular target and may arise more slowly with combination therapies that affect multiple cellular targets. Accordingly, we hypothesized that the resistance to the PT plus rifampin combination may develop slower than that to either PT or rifampin in isolation.

To test that prediction, the spontaneous rates of *S. aureus* and *P. aeruginosa* resistance to rifampin (alone), PT (alone), and PT plus rifampin were measured and compared to that for moxifloxacin. To do so, the growth of each organism was monitored continuously in the presence of 1× MIC of each agent over the course of 40 h by using a BacterioScan automated laser microbial growth monitor. The results revealed that *P. aeruginosa* cultures treated with rifampin (alone) or PT (alone) were either at or below the level for growth detection for the first 10 to 12 h of incubation but increased dramatically ≥12 h, suggesting the emergence of resistance (representative data shown in Fig. 3A). Indeed, plating and subsequent susceptibility testing confirmed that cells recovered from rifampin (alone) or PT (alone) and exhibited a 2× to 8× increase in MIC (data not shown). Moxifloxacin-treated cells did not exhibit a growth phenotype until approximately 22 h posttreatment, suggesting that *P. aeruginosa* fluoroquinolone resistance was slower to develop; testing of the resulting cells

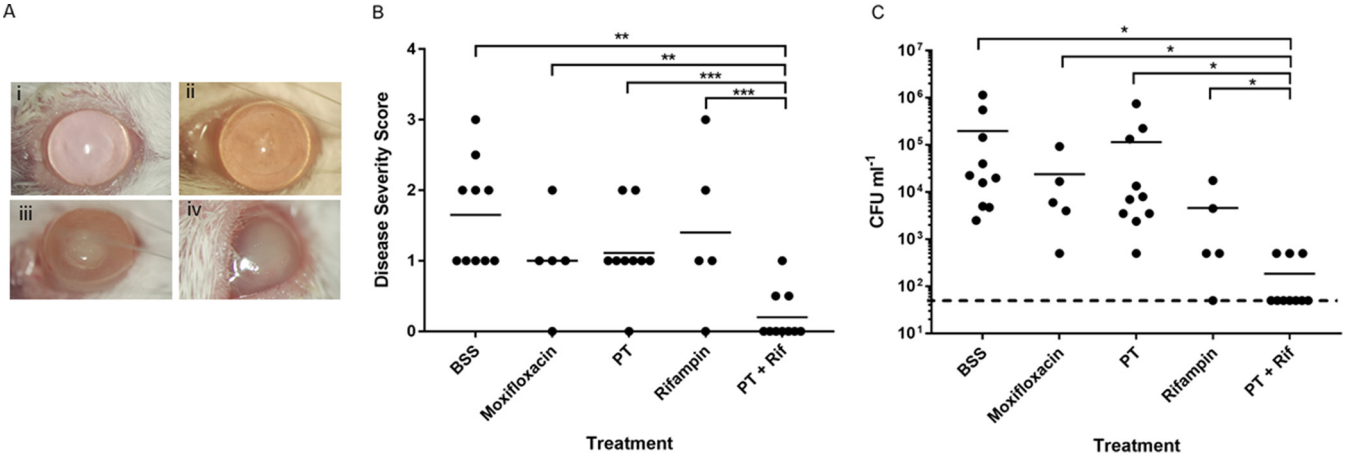

FIG 4 Antimicrobial efficacy of polymyxin B-trimethoprim plus rifampin in a murine model of *S. aureus* keratitis. (A) Representative grade 0 (i), grade 1 (ii), grade 2 (iii), and grade 3 (iv) *S. aureus* corneal infection. (B) Disease severity scores after 72-h treatments with BSS (negative control), moxifloxacin, PT (alone), rifampin (alone), and PT plus rifampin. (C) CFU recovered from inoculated animals after 3-day treatment with either BSS, moxifloxacin, polymyxin B-trimethoprim (PT), rifampin, or polymyxin B-trimethoprim plus rifampin. Each symbol represents an individual animal. The lower limit of detection is indicated with the horizontal dashed line, and each group average is indicated by a horizontal line. ***, $P < 0.05$; **, $P < 0.1$; *, $P < 0.15$ by Student's *t* test.

confirmed a ≥2-fold MIC increase. In six independent experiments, cells treated with the combination of rifampin plus PT did not show any growth over the course of 40 h, and there were no recoverable viable cells at the study conclusion.

Similar resistance profiles were observed for *S. aureus* strain UAMS-1 cells (representative data shown in Fig. 3B). The treatment with either rifampin (alone) or PT (alone) enabled outgrowth starting at 8 h, while moxifloxacin-treated cells demonstrated growth starting at 26 h; the emergence of resistance was confirmed by MIC testing. In contrast, *S. aureus* cells treated with the combination of rifampin and PT did not demonstrate any growth during the 40-h time period in six independent studies. Collectively, these results suggest that in comparison to that with moxifloxacin, PT (alone), or rifampin (alone), both *S. aureus* and *P. aeruginosa* have lower propensities to develop resistance to the combination of PT plus rifampin.

Antimicrobial efficacy of polymyxin B-trimethoprim plus rifampin in a murine keratitis model of *S. aureus* infection. The aforementioned studies indicate that the combination of PT plus rifampin displays promising *in vitro* antimicrobial activity against both *P. aeruginosa* and *S. aureus* that is comparable, and in some cases, superior, to the broad-spectrum fluoroquinolone moxifloxacin. To determine if this activity would extend to a relevant *in vivo* setting, a murine model of *S. aureus* keratitis was used to measure the antimicrobial efficacy of the PT plus rifampin combination.

Corneal infections in BALB/c mice were established using a *S. aureus* ocular clinical isolate with moderate moxifloxacin resistance (27 $\mu g \cdot ml^{-1}$ MIC). Each animal was then treated 4 times daily for 72 h with either balanced salt solution (BSS), commercially available moxifloxacin, commercially available PT, rifampin, or the combination of PT plus rifampin. Daily, the severity of corneal disease was assessed by slit lamp photography (Fig. 4A) and scored on the basis of previously established metrics of disease progression (29). After 72 h of treatment, the eyes received a final disease severity score and were harvested to determine the bacterial burden.

The slit lamp evaluation suggested that corneal infections were achieved in 100% of animals; however, differences among the treatment groups with respect to disease severity were apparent starting at 24 h (data not shown). By 72 h, there were statistically significant reductions in disease severity in the moxifloxacin, PT (alone), and PT plus rifampin groups compared to that in the BSS-treated group. However, the PT-plus-rifampin-treated group demonstrated the greatest reduction in disease severity, with 7 of 10 mice receiving a score of 0, indicating no evidence of clinical infection (Fig. 4B). There was no significant improvement noted for the rifampin (alone) treatment group.

As shown in Fig. 4C, in comparison to those treated with BSS, commercial moxifloxacin, commercial PT, or rifampin, animals treated with PT plus rifampin displayed an impressive reduction in bacterial burden. Specifically, 3 days of PT plus rifampin treatment resulted in a >3-log reduction in bacterial burden in comparison to that in the vehicle-treated animals (2.5×10^5 CFU per eye), with no organisms recovered from 7 of 10 animals and a single colony recovered from each of the remaining 3 animals. By comparison, moxifloxacin, PT, and rifampin treatments displayed reduced antimicrobial efficacies, resulting in 1.2-log, 0.4-log, and 1.75-log reductions in recoverable organisms, despite treatment with a commercial formulation of polymyxin B-trimethoprim or moxifloxacin that exceeded the MIC for the strain by 1,500-fold or 200-fold, respectively. In summary, these results suggest that the PT plus rifampin combination may represent a new and promising treatment option for bacterial keratitis, including infections caused by fluoroquinolone-resistant strains.

DISCUSSION

S. aureus and *P. aeruginosa* are recognized as members of the ESKAPE bacterial pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.) that have a high propensity to "escape" the activity of antibiotics due to resistance (30, 31). Both organisms are well recognized to represent predominant causes of severe and debilitating human disease, including infections of the cornea.

Broad-spectrum fluoroquinolones have been successfully used off label for broad-spectrum empirical infectious keratitis treatment, yet the emergence of the corresponding resistance has led to clinical failure and highlighted the need for new therapeutic alternatives (11, 32). While specialty compounded antibiotics such as vancomycin or tobramycin are available in some academic centers to circumvent the emerging resistance, there are limited alternative commercial options for treatment. To this end, we considered that the rational development of existing drug combinations may identify novel synergistic antimicrobial combinations that could simultaneously overcome resistance issues and provide a more-rapid-to-market bacterial keratitis therapeutic option in comparison to the development of a new chemical entity.

Polymyxin B-trimethoprim, a currently available topical ophthalmic antibiotic combination for the treatment of bacterial conjunctivitis, was selected as the platform for our combination synergy screen on the basis of the inherent low-level resistance among *S. aureus* and *P. aeruginosa* strains associated with ocular infection (22). Recognizing that limitations in potency, antibiofilm activity, and slow bactericidal activity have limited the use of PT in treating bacterial keratitis, our immediate goal was to arrive at a PT-based antimicrobial combination that overcomes these liabilities.

The results presented here demonstrate that PT plus rifampin exhibits increased potency, more rapid bactericidal activity, and improved antibiofilm activity in comparison to those of PT, approaching or improving upon the activity of moxifloxacin, a current keratitis treatment. Moreover, the PT plus rifampin combination also demonstrated a lower propensity to develop resistance than either agent in isolation or moxifloxacin. The improved activities of the combination may be due, in part, to their independent mechanisms of antimicrobial action; rifampin inhibits bacterial DNA transcription by binding to RNA polymerase, trimethoprim inhibits bacterial DNA synthesis by inhibiting dihydrofolate reductase, and polymyxin B acts as a detergent by disrupting the outer and inner membranes primarily of Gram-negative bacteria, although it may also have some activity toward Gram-positive organisms (18, 33–35). Thus, while further characterization is required, it may be reasonable to speculate that the observed synergistic/improved bactericidal effects of the combination may be, in part, attributable to the improved antibiotic penetration due to compromised polymyxin B-associated membrane integrity. Alternatively (or in addition), the improved effects may be manifested as a consequence of simultaneously crippling multiple cellular processes. Regardless, given the disparate modes of action of the antimicrobial components of the combination, it is reasonable to assume that its correspondingly low spontaneous resistance frequency is

a function of the multiple mutations that would be required to overcome the function of each antibiotic. Indeed, the simultaneous targeting of disparate pathways is a common strategy to overcome existing resistance in a variety of infections, including HIV, malaria, and tuberculosis (36, 37).

As an early assessment of the antimicrobial activity of PT plus rifampin within a host, the combination's performance was evaluated in a murine keratitis model of *S. aureus* infection. Utilizing a clinically relevant ocular isolate of *S. aureus* with moderate moxifloxacin resistance, we demonstrated the superiority of the PT plus rifampin combination, leading to the nearly complete eradication of bacteria from the eye following a 3-day treatment. This is in sharp contrast to treatment with moxifloxacin, rifampin, or PT (alone), in which there was no statistical difference between these treatment groups and the control vehicle-treated group. Interestingly, while the strain used for those studies was a moxifloxacin-resistant keratitis isolate, both moxifloxacin and PT were used in vast excess to the *in vitro* MIC values (200-fold and 1,500-fold, respectively) and yet were unable to eradicate the infection. Moreover, the combination of PT plus rifampin displayed synergistic activity in an *in vivo* setting, outperforming either agent in isolation. These results suggest that PT plus rifampin may be effective for treating contemporary strains of *S. aureus* that have already demonstrated resistance and would likely fail treatment with other commercially available ophthalmic antibiotics, including moxifloxacin.

While there may be unforeseen effects of combination drugs with respect to toxicity, both rifampin and PT are currently FDA-approved drugs with favorable safety profiles; thus, their combination may provide a significant advantage for further drug development. Rifampin, which does not have a current commercial ophthalmic formulation, has historically been used as an ophthalmic solution (38–41) with demonstrated efficacy against *P. aeruginosa*, *Proteus* sp., and trachoma. Additionally, early animal studies have revealed excellent corneal penetration of rifampin (42). However, it is important to recognize that combinations of drugs may exhibit different pharmacokinetic properties compared to those of each component in isolation, necessitating formulation testing and refinement to optimize the compatibility, stability, and effective delivery properties of the antimicrobials in mixture.

In summary, rifampin plus PT may represent a novel antibiotic combination with broad-spectrum antimicrobial activity, rapid bactericidal action, antibiofilm activity, reduced resistance frequency, and potent *in vivo* activity for the treatment of bacterial keratitis. Moreover, these results provide further credence to the overarching concept that developing combinations of previously FDA-approved drugs may provide a path forward for novel antimicrobial therapies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in these studies include the conventional laboratory strains *P. aeruginosa* PAO1 and *S. aureus* UAMS-1, both of which are antibiotic susceptible and capable of forming robust biofilms (43). Seven *P. aeruginosa* clinical keratitis isolates were collected from the Flaum Eye Institute (Rochester, NY), and 20 *S. aureus* clinical isolates from geographically distinct regions of the country were purchased from International Health Management Associates (IHMA; Schaumburg, IL). A moxifloxacin-resistant clinical isolate of *S. aureus* was obtained from the University of Rochester Medical Center (Rochester, NY). For the experiments requiring planktonic cultures, the indicated organism was grown at 37°C overnight in Luria-Bertani (LB) broth (BD, Franklin Lakes, NJ), diluted 1:100 in fresh medium, grown to early exponential phase (optical density at 600 nm [OD_{600}] of 0.18 or 0.4 for *S. aureus* and *P. aeruginosa*, respectively), and processed. *P. aeruginosa* biofilms were established by inoculating individual wells of flat-bottom polystyrene plates (Falcon; Corning Life Sciences, Durham, NC) with overnight cultures in 200 μg of fresh LB broth to a final concentration of 1×10^7 CFU $\cdot$ ml^{-1}. *S. aureus* biofilms were established similarly with the exception that polystyrene plates were pretreated with 20% human serum diluted in carbonate buffer overnight at 4°C. The plates were then placed in a 37°C humidified incubator for 72 h to allow static biofilm formation. Nonadherent cells were aspirated, and the biofilms were washed twice with phosphate-buffered saline (PBS) prior to each experiment.

Chemicals and compound libraries. Tobramycin, rifampin, neomycin, ciprofloxacin, ampicillin, gentamicin, besifloxacin, and ceftriaxone were purchased from Sigma-Aldrich (St. Louis, MO). The compounds already in ophthalmologic solution (moxifloxacin and polymyxin B-trimethoprim) were

obtained from the Flaum Eye Institute (Rochester, NY). A library of 853 FDA-approved drugs was obtained from Selleck Chemical (Houston, TX).

***Pseudomonas aeruginosa* clinical isolate characterization.** A series of genotyping and biological assays were used as a preliminary means to evaluate the clonality of the clinical *P. aeruginosa* isolates collected. Genomic DNA was purified from each isolate with Qiagen DNeasy kits (Hilden, Germany) and used as a template for PCRs to detect the presence of the virulence factors *exoS*, *exoY*, *exoU*, *exoT*, and *pcrV* via previously published primers (see Table S4 in the supplemental material) (44–47). The antibiotic susceptibility profiles of each isolate toward moxifloxacin, tobramycin, and PT, as well as four other antibiotics, were evaluated according to MIC guidelines (48). The ability of isolate to form a biofilm was quantified. To do so, the wells of a polystyrene plate containing 72-h established biofilms were washed with 0.8% sodium chloride (NaCl) to remove nonadherent cells and stained with crystal violet. To quantify the biofilm staining, the plates were washed 3 times with water, the stain was suspended in 30% glacial acetic acid, and the optical density (OD_{600}) of each well was determined using an automated plate reader, as previously described (49).

Selleck library screening. As a prerequisite to screening, the highest concentration of polymyxin B-trimethoprim that was tolerated by—and enabled the growth of—*P. aeruginosa* strain PAO1 and *S. aureus* strain UAMS-1 was determined. To do so, standard MIC plate assays were performed, in which approximately 1×10^5 CFU was added to each well of a microtiter plate in the presence of 2-fold increasing concentrations of PT and incubated for 16 h at 37°C. The MIC was identified as the drug concentration that did not enable growth, as measured by the unaided eye. To appreciate the more subtle effects of lower concentrations of drugs, aliquots (20 μl) were plated from wells representing the 1×, 0.5×, 0.25×, and 0.125× MIC for each organism to determine the number of viable CFU. The concentration of PT that enabled approximately a 1-log increase in CFU during a 16-h incubation was selected for use in the screen, as this represented a concentration with moderate antibacterial activity but that did not lead to an inhibition of growth after an overnight incubation.

For Selleck library screening, individual wells of 96-well microtiter plates containing 88 μl of fresh medium were inoculated with approximately 10^5 CFU of *P. aeruginosa* strain PAO1 or *S. aureus* UAMS-1. Next, 2 μl of PT was added to a final concentration of 0.2 $\mu g \cdot ml^{-1}$ or 1.25 $\mu g \cdot ml^{-1}$ for PAO1 and UAMS-1, respectively. Library members were then added at either 5 μM or 50 μM final concentration. Plates were incubated at 37°C for 16 h, and hits were identified as wells with no visible growth.

Fractional inhibitory concentration testing. In a checkerboard format, *S. aureus* strain UAMS-1 or *P. aeruginosa* strain PAO1 was added to individual wells of a microtiter plate. The rows of each plate were treated with increasing concentrations of PT (0 to 12.5 $\mu g \cdot ml^{-1}$), whereas each column was treated with increasing concentrations of rifampin, tobramycin, or besifloxacin. The plates were incubated for 16 h, at which time the concentration of each test agent that inhibited bacterial growth (alone) and at various combination concentrations was determined. The fractional inhibitory concentration (FIC) index was calculated using the following formula: FIC = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). A synergistic interaction was defined as an FIC value of <0.5, an additive interaction as an FIC of 0.5 to 1.0, neutral interaction as an FIC of 1 to 4, and an antagonistic interaction as an FIC of >4 (26).

Biofilm susceptibility testing. Biofilms were established as described above in "Bacterial strains and growth conditions" and then treated with 100 μl of fresh medium supplemented with 1×, 2×, 4×, 8× or 16× MIC of the test agent toward planktonic PAO1 or UAMS-1 cells. After 24 h, the remaining medium and nonadherent cells were removed, and the biofilms were suspended in 0.8% NaCl and plated to quantify surviving bacteria. The susceptibilities of biofilms treated with a combination of compounds were determined in the same manner as above, utilizing a ratio of 1:1 of the planktonic MIC of each compound (e.g., 0.41 $\mu g \cdot ml^{-1}$ PT and 15.6 $\mu g \cdot ml^{-1}$ rifampin for PAO1). The biofilms were treated with the agents in concentrations increasing from 0 to 32× MIC.

Antimicrobial kill curves. Overnight cultures of the indicated organisms were used to inoculate (1:100 dilution) 100 ml of fresh medium and were grown to mid-exponential phase. The test compound(s) was added at 2× MIC, and the culture was allowed to incubate. Aliquots were taken at 1, 2, 3, 4, and 24 h, serially diluted, and plated for the enumeration of CFU.

Resistance measures. Spontaneous resistance to the indicated test agent(s) was measured for *P. aeruginosa* strain PAO1 and *S. aureus* strain UAMS-1. A 50-μl suspension from each treatment condition was transferred to a BacterioScan (St. Louis, MO) collection cuvette containing 1.95 ml of Mueller-Hinton broth. Growth was measured using the BacterioScan 216Dx laser microbial growth monitor with optical measurements taken every 3 min over 40 h. After 40 h, the samples were plated to measure viable CFU and were retested for drug susceptibility; each experiment was performed in triplicates.

Murine corneal infection model and treatment. Female BALB/c mice four to six weeks of age were obtained from Charles River (Washington, MA) and housed according to the approved University of Rochester Medical Center Council on Animal Research (UCAR) protocol. A clinical *S. aureus* strain with moderate moxifloxacin resistance (MIC, 26 $\mu g \cdot ml^{-1}$) was grown overnight at 37°C on both Mueller-Hinton agar (Fisher Scientific, Hampton, NH) and in Mueller-Hinton broth. Broth cultures were centrifuged at 2,000 × *g* for 10 min at 4°C, and bacterial cell pellets were resuspended in PBS in a volume equivalent to the starting volume. The mice were anesthetized with 100 $mg \cdot kg^{-1}$ ketamine (Par Pharmaceutical, Chestnut Ridge, NY) and 10 $mg \cdot kg^{-1}$ xylazine (Akorn, Inc., Lake Forest, IL), and 0.5% proparacaine (Akorn, Inc, Lake Forest, IL) was applied to the right eye of each mouse. Excess proparacaine was blotted from the ocular surface and, using a 27-guage needle inoculated with a single colony, three 2-mm scratches were made across the right cornea. The scratches were then inoculated with a 5-μl volume of bacterial culture containing 10^7 CFU. After the infection, the eyes were assigned a disease

severity score every 24 h, using a previously validated standard: 0, no evidence of clinical infection; 1, opacity <4 mm; 2, opacity >4 mm; 3, dense opacity covering the entire cornea; 4, perforation of the cornea (29). In the event of a perforation, the mouse was euthanized immediately via carbon dioxide. Treatments were administered in 5-μl aliquots to the infected eyes every 6 h beginning 6 h postinoculation and ending 72 h postinoculation. The treatments included balanced salt solution (BSS) ophthalmologic solution (Alcon Laboratories, Inc, Fort Worth, TX), moxifloxacin (5.4 mg · ml^{-1}), PT (1,000 units polymyxin B and 1 mg · ml^{-1} trimethoprim), rifampin (0.5 mg · ml^{-1}), and PT plus rifampin (1,000 units polymyxin B, 1 mg · ml^{-1} trimethoprim, and 0.5 mg · ml^{-1} rifampin). After the animals were euthanized, the whole eyes were removed and homogenized to determine the number of viable *S. aureus* colonies present. To do so, samples were suspended in tubes containing 1.4-mm ceramic beads (Fisher Scientific, Hampton, NH) and 0.5 ml PBS and homogenized using the Fisherbrand Bead Mill homogenizer. Aliquots (0.1 ml) were then removed, serially diluted in 0.8% NaCl, and plated on mannitol salt agar plates (Fisher Scientific, Hampton, NH) for enumeration after incubating at 37°C for 16 h.

Statistics. To compare disease severity scores and CFU between treatment groups in the *in vivo* murine keratitis model, standard *t* tests were applied. *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01929-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

R.A.F.W. was supported in part by an unrestricted grant from the Research to Prevent Blindness Foundation to the Flaum Eye Institute, an American Society of Cataract and Refractive Surgery Foundation research grant (AWD0000831), and an NIH K08 award (EY029012-01). *In vivo* studies were also supported in part by a University of Rochester URVentures Technology Development Award to P.M.D. and R.A.F.W. and by Arcum Therapeutics. M.C. was supported in part by the Training Program in Oral Sciences (T90DE021985).

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