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EXPERIMENTAL THERAPEUTICS

Paenipeptin Analogues Potentiate Clarithromycin and Rifampin against *mcr-1*-Mediated Polymyxin-Resistant *Escherichia coli In Vivo*

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ABSTRACT Polymyxin resistance mediated by the *mcr-1* gene threatens the last-resort antibiotics. Linear lipopeptide paenipeptin analogues 1 and 15 disrupted the outer membrane of Gram-negative pathogens and potentiated clarithromycin and rifampin against *mcr-1*-positive *Escherichia coli* from the FDA-CDC Antimicrobial Resistance Isolate Bank. In the presence of paenipeptin, clarithromycin and rifampin resulted in over 3-log reduction of *E. coli in vitro*. Moreover, paenipeptin-antibiotic combinations significantly reduced *E. coli* in a murine thigh infection model.

KEYWORDS lipopeptide, paenipeptin, *mcr-1*, potentiator, combination therapy

Drug-resistant Gram-negative pathogens, including carbapenem-resistant *Enterobacteriaceae* (CRE), have been widely recognized as a serious threat to public health (1, 2). The cyclic cationic lipopeptides, polymyxin and colistin (polymyxin E), are considered the last-resort therapy for the treatment of infections caused by multidrug-resistant Gram-negative pathogens. However, plasmid-mediated, polymyxin-resistant *Escherichia coli* harboring the *mcr-1* gene has been isolated from farm animals and patients (3, 4), which may compromise the therapeutic efficacy of polymyxins. Therefore, rapidly evolving antibiotic resistance requires a matching effort to develop new and effective antimicrobial agents.

Paenipeptin analogues 1 and 15 are novel synthetic linear lipopeptides which were designed through extensive structure-activity relationship (SAR) studies (5). These two compounds share the same peptide structure (Dab-Ile-Dab-dPhe-Leu-Dab-dVal-Leu-Ser, where Dab represents the positively charged 2,4-diaminobutyric acid) but differ in their N-terminal modifications. Analogue 1 possesses an N-terminal C_6 lipid chain, whereas the N terminus of analogue 15 is modified by a hydrophobic carboxybenzyl group. Analogues 1 and 15 are nonhemolytic and showed low cytotoxicity against a human kidney cell line, HEK 293 (6). We previously have shown that these two compounds potentiated clarithromycin and rifampin against carbapenem-resistant *Klebsiella pneumoniae* and *Acinetobacter baumannii in vitro* (6). The objective of this study was to evaluate the efficacy of the combination between paenipeptins and clarithromycin/rifampin against *mcr-1* mediated, polymyxin-resistant *E. coli in vitro* and *in vivo* using a murine thigh infection model.

Paenipeptin analogues 1 and 15 (>95% purity) are synthetic lipopeptides produced by a commercial peptide synthesis company (GenScript Inc., Piscataway, NJ). Clarithromycin, rifampin, and polymyxin B were purchased from Sigma (St. Louis, MO). Polymyxin-resistant *E. coli* strains harboring the *mcr-1* gene were obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank (Table 1). The MIC and minimum bactericidal concentration (MBC) values against *E. coli* isolates were determined as described previously (5). Polymyxin B MIC values for four *mcr-1*-positive-*E. coli* isolates ranged from 4 to 16 μg/ml, which confirmed the polymyxin resistance phenotype.

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TABLE 1 MIC values of polymyxin B, clarithromycin, rifampin, and paenipeptin analogues 1 and 15 against polymyxin-resistant pathogens from the FDA-CDC Antimicrobial Resistance Isolate Bank

	MIC (μg/ml) values for:[a]				
E. coli strain	Polymyxin B	Clarithromycin	Rifampin	Analogue 1	Analogue 15
FDA-CDC AR 0350	4–8	>32	16	16–32	16
FDA-CDC AR 0493	8–16	>32	16	32	16
FDA-CDC AR 0494	8	≥32	16–32	16–32	8–16
FDA–CDC AR 0495	4–16	>32	8–16	16–32	16
ATCC 25922	≤0.5	>32	8–16	8	8–16

[a]The MIC represents the range of individual concentrations measured from at least three independent experiments.

When treated alone by a single antimicrobial agent, these clinical isolates were resistant to clarithromycin and rifampin (MIC, 8 to 32 μg/ml; Table 1), which indicated that clarithromycin and rifampin were excluded by the intact outer membrane permeability barrier in *E. coli* cells. In the presence of paenipeptin analogue 1 or 15 at subinhibitory concentration (4 μg/ml), the MIC of clarithromycin and rifampin was reduced to 0.0019 to 0.125 μg/ml, whereas the MBC ranged from 0.0019 to 0.25 μg/ml (Table 2). Similarly, other researchers reported that a polymyxin derivative, NAB739, potentiated rifampin and other antibiotics against *mcr*-positive, polymyxin-resistant strains (7).

The enhanced antimicrobial activity in the presence of paenipeptin analogues is presumably due to the compromised outer membrane induced by the cationic paenipeptin molecules. To test the mechanism of potentiation, we determined the change of outer membrane permeabilization in the presence of paenipeptin analogue using a fluorescent probe, 1-N-phenylnaphthylamine (NPN) (8, 9). The fluorescence of NPN was measured using a cell imaging multimode reader (Cytation 3; BioTek, Winooski, VT) with an excitation at 350 nm and emission at 420 nm. Polymyxin B, a known outer membrane active compound (10), was used as a positive control. Paenipeptin analogues 1 and 15, at a final concentration of 1.6 to 6.4 μg/ml, significantly increased the uptake of the NPN probe in a concentration-dependent manner, suggesting disruption of the outer membrane in *E. coli* ATCC 25922 (Fig. 1A) and in the *mcr-1*-positive strain, *E. coli* FDA-CDC AR 0494 (Fig. 1B). Paenipeptin treatment may promote the uptake of clarithromycin and rifampin by disrupting the outer membrane of Gram-negative pathogens.

Time-kill kinetics were used to study the bactericidal effect of the combined treatments against a selected polymyxin-resistant strain, *E. coli* FDA-CDC AR 0494, in a microbiological medium, tryptic soy broth. A single treatment by paenipeptin analogue 1 (8 μg/ml), analogue 15 (8 μg/ml), clarithromycin (8 μg/ml), or rifampin (4 μg/ml) alone reduced the growth rate of *E. coli* by 6 h but failed to inhibit pathogen growth for 24 h. At the same tested concentrations, paenipeptin analogue 1, in combination with clarithromycin or rifampin, resulted in 3.7- and 4.4-log reduction within 24 h, respectively (Fig. 2). Analogue 15 in the presence of clarithromycin or rifampin exhib-

TABLE 2 MIC (μg/ml) and MBC (μg/ml) values for clarithromycin and rifampin in the presence of paenipeptin analogue 1 or analogue 15 at 4 μg/ml against polymyxin-resistant isolates from the FDA-CDC Antimicrobial Resistance Isolate Bank[a]

	Data (μg/ml) for the indicated strain in the presence of:							
	Analogue 1				Analogue 15			
	Clarithromycin		Rifampin		Clarithromycin		Rifampin	
E. coli strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
FDA-CDC AR 0350	0.0313–0.0625	0.125	≤0.0019	0.0078	0.0313–0.0625	0.0313	≤0.0019	0.0156
FDA-CDC AR 0493	0.0625	0.125	≤0.0019	0.0019	0.0625	0.0625	≤0.0019	0.0019
FDA-CDC AR 0494	0.0313	0.0625	≤0.0019	0.0039	0.0156–0.0313	0.0313	≤0.0019	0.0019
FDA-CDC AR 0495	0.0625–0.125	0.125	≤0.0019	0.0019	0.125	0.25	≤0.0019	0.0019
ATCC 25922	0.0313	0.0625	≤0.0019	0.0019	0.0078	0.0625	≤0.0019	0.0019

[a]The MIC represents the range of individual concentrations measured from at least three independent experiments.

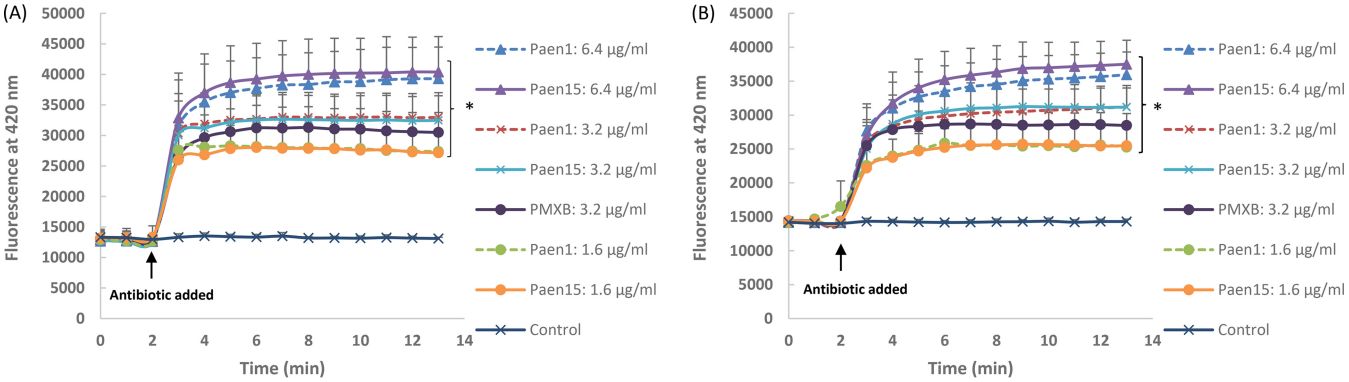

FIG 1 Time course of increase in 1-N-phenylnaphthylamine (NPN) fluorescence intensity in the presence of intact *Escherichia coli* cells at different concentrations of paenipeptin analogues 1 and 15. (A) *E. coli* ATCC 25922; (B) *E. coli* FDA-CDC AR 0494. Paen1, analogue 1; Paen15, analogue 15; PMXB, polymyxin B. The final antibiotic concentrations after mixing with NPN and cells were reported. Values are expressed as means (three independent repeats), and error bars represent standard deviations. An asterisk indicates statistical significance ($P < 0.05$) at the final data points between the control and antimicrobial treatment groups.

ited very similar inactivation kinetics. These results are consistent with our previous report in which paenipeptin analogues potentiated antibiotics against carbapenem-resistant *K. pneumoniae* and *A. baumannii in vitro* (6). Because of the high similarity in time-kill kinetics for both analogues, we selected analogue 1 for further acute toxicity and therapeutic efficacy studies in mice.

The animal experiments have been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Arkansas for Medical Sciences (approval numbers 3760 and 3922). An acute toxicity study was conducted to evaluate the tolerance of paenipeptin analogue 1 in female CD-1 mice ($n = 4$; 6 weeks old). Briefly, mice were treated by subcutaneous injection of a single dose of paenipeptin analogue 1 at 40, 60, 80, 100, or 120 mg/kg. The treated mice were monitored for 10 days, and the survival rate was recorded. All mice in the 40- to 80-mg/kg groups showed normal eating and drinking activity and survived the whole 10-day period. However, analogue 1 at 100 and 120 mg/kg resulted in one and two deaths out of four animals, respectively. Therefore, analogue 1 was tested at 40 and 60 mg/kg for its therapeutic activity in mice.

A neutropenic murine thigh infection model was used to evaluate the therapeutic

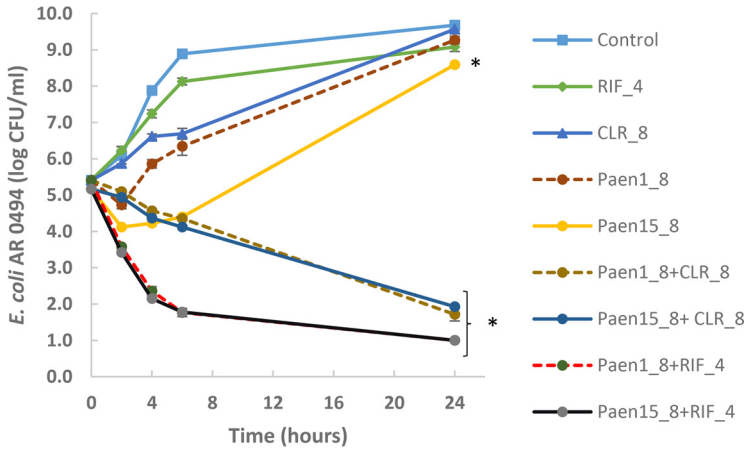

FIG 2 Time-kill curves of *Escherichia coli* FDA-CDC AR 0494 with exposure to paenipeptin analogues alone or in combination with clarithromycin/rifampin in tryptic soy broth. RIF_4, 4 μg/ml rifampin; CLR_8, 8 μg/ml clarithromycin; Paen1_8, 8 μg/ml analogue 1; Paen15_8, 8 μg/ml analogue 15; Paen1_8+CLR_8, 8 μg/ml analogue 1 plus 8 μg/ml clarithromycin; Paen15_8+CLR_8, 8 μg/ml analogue 15 plus 8 μg/ml clarithromycin; Paen1_8+RIF_4, 8 μg/ml analogue 1 plus 4 μg/ml rifampin; Paen15_8+RIF_4, 8 μg/ml analogue 15 plus 4 μg/ml rifampin. Values are expressed as means (four independent repeats), and error bars represent standard deviations. An asterisk indicates statistical significance ($P < 0.05$) at the final data points between the control and antimicrobial treatment groups.

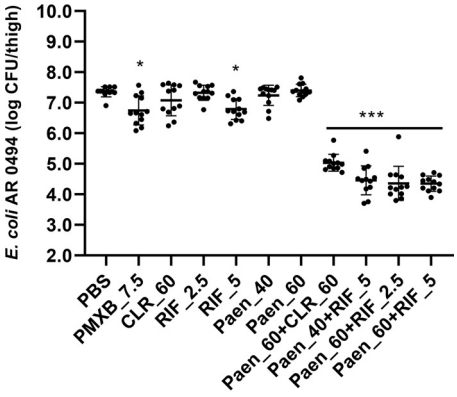

FIG 3 Paenipeptin analogue 1 in combination with clarithromycin or rifampin was efficacious against *Escherichia coli* FDA-CDC AR 0494 in a murine thigh infection model. Treatment groups (antimicrobial injection time, hours postinfection): PBS, phosphate-buffered saline; PMXB_7.5, 7.5 mg/kg polymyxin B (2 h); CLR_60, 60 mg/kg clarithromycin (2 and 5 h); RIF_2.5, 2.5 mg/kg rifampin (2 and 5 h); RIF_5, 5 mg/kg rifampin (2 and 5 h); Paen_40: 40 mg/kg analogue 1 (2 h); Paen_60, 60 mg/kg analogue 1 (2 h); Paen_60+CLR_60, 60 mg/kg analogue 1 (2 h) plus 60 mg/kg clarithromycin (2 h and 5 h); Paen_40+RIF_5, 40 mg/kg analogue 1 (2 h) plus 5 mg/kg rifampin (2 h and 5 h); Paen_60+RIF_2.5, 60 mg/kg analogue 1 (2 h) plus 2.5 mg/kg rifampin (2 h and 5 h); Paen_60+RIF_5, 60 mg/kg analogue 1 (2 h) plus 5 mg/kg rifampin (2 h and 5 h). Statistical significance between the antimicrobial treatment groups and the PBS control group is indicated by an asterisk(s) (*, $P < 0.05$; ***, $P < 0.001$).

efficacy of the combined treatments against *E. coli* FDA-CDC AR 0494 *in vivo*. Female CD-1 mice (6 weeks old) were rendered neutropenic by intraperitoneal injection of cyclophosphamide on 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to bacterial inoculation. On the day of inoculation, *E. coli* cells (3.4×10^5 CFU in 100 μl saline) were intramuscularly injected into each posterior thigh. At 2 and 5 h postinfection, mice were treated with single or combined antibiotics in phosphate-buffered saline (PBS) by subcutaneous injection. At 8 h postinfection, mice were euthanized by CO_2, and tissues from each thigh were collected and homogenized for bacterial count determination. Mice showed normal eating and drinking activity in all treatment groups.

As showed in Fig. 3, polymyxin B at 7.5 mg/kg and rifampin at 5 mg/kg reduced 0.6 log of the polymyxin-resistant *E. coli* strain. All other single treatments, including clarithromycin (60 mg/kg), rifampin (2.5 mg/kg), and paenipeptin analogue 1 (40 and 60 mg/kg), didn't significantly inhibit the growth of the bacterium in thigh tissues. In contrast, all combined treatments significantly reduced bacterial counts in thigh tissues. For example, paenipeptin analogue 1 at 60 mg/kg, in combination with clarithromycin at 60 mg/kg, showed 2.3-log reduction compared with the PBS control. Moreover, paenipeptin-rifampin combinations displayed 2.9- to 3.0-log reductions in comparison with the PBS control group (Fig. 3). Therefore, paenipeptin analogue 1 potentiated clarithromycin and rifampin *in vivo* against polymyxin-resistant *E. coli*. Combination therapy has been proved as a promising strategy to overcome antibiotic resistance. *Coli*stin and clarithromycin combination therapy resulted in 2.9-log reduction of *mcr-1*-positive *K. pneumoniae* in a mouse thigh infection model (11). Similarly, combination therapy of colistin plus rifampin or minocycline was efficacious against NDM- and MCR-1 coproducing *E. coli* in a murine thigh infection model (12).

In conclusion, cationic paenipeptin analogues 1 and 15 disrupted the outer membrane of Gram-negative bacteria and thus may promote the entry of hydrophobic antibiotics into bacterial cells. Paenipeptin analogue 1 potentiated clarithromycin and rifampin against *mcr-1*-positive *E. coli in vitro* and *in vivo*. This is the first report on the combination effect between potentiator paenipeptin and other antibiotics *in vivo*. Pharmacokinetics and pharmacodynamics studies of paenipeptin analogues will be needed in the future to develop optimal treatment strategies and to prevent the emergence of resistance (13).

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