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EXPERIMENTAL THERAPEUTICS

Short Proline-Rich Lipopeptide Potentiates Minocycline and Rifampin against Multidrug- and Extensively Drug-Resistant *Pseudomonas aeruginosa*

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ABSTRACT A series of 16 short proline-rich lipopeptides (SPRLPs) were constructed to mimic longer naturally existing proline-rich antimicrobial peptides. Antibacterial assessment revealed that lipopeptides containing hexadecanoic acid (C_{16}) possess optimal antibacterial activity relative to others with shorter lipid components. SPRLPs were further evaluated for their potential to serve as adjuvants in combination with existing antibiotics to enhance antibacterial activity against drug-resistant *Pseudomonas aeruginosa*. Out of 16 prepared SPRLPs, C_{12}-PRP was found to significantly potentiate the antibiotics minocycline and rifampin against multidrug- and extensively drug-resistant (MDR/XDR) *P. aeruginosa* clinical isolates. This nonhemolytic C_{12}-PRP is comprised of the heptapeptide sequence PRPRPRP-NH_2 acylated to dodecanoic acid (C_{12}) at the N terminus. The adjuvant potency of C_{12}-PRP was apparent by its ability to reduce the MIC of minocycline and rifampin below their interpretative susceptibility breakpoints against MDR/XDR *P. aeruginosa*. An attempt to optimize C_{12}-PRP through peptidomimetic modification was performed by replacing all L- to D-amino acids. C_{12}-PRP demonstrated that it was amenable to optimization, since synergism with minocycline and rifampin were retained. Moreover, C_{12}-PRP displayed no cytotoxicity against human liver carcinoma HepG2 and human embryonic kidney HEK-293 cell lines. Thus, the SPRLP C_{12}-PRP is a lead adjuvant candidate that warrants further optimization. The discovery of agents that are able to resuscitate the activity of existing antibiotics against drug-resistant Gram-negative pathogens, especially *P. aeruginosa*, is of great clinical interest.

KEYWORDS proline-rich antimicrobial peptides, lipopeptides, adjuvant, *Pseudomonas aeruginosa*, minocycline, rifampin

The increasing incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* infection imposes a significant burden in our current health care system (1, 2). Infections caused by *P. aeruginosa* are difficult to treat, since this pathogen often harbors multiple resistance mechanisms against most currently used antibiotics (3, 4). Intrinsic resistance in *P. aeruginosa* is a major hurdle to overcome. The protective outer membrane (OM) of *P. aeruginosa* is comprised of selective porins and a polar lipopolysaccharide (LPS) barrier that is 12 to 100 times less permeable than that of *Escherichia coli* (5). Compounds which are able to cross the OM and enter the periplasm are prone to efflux by up to 12 overexpressed multidrug efflux

Received 20 November 2017 **Returned for modification** 18 December 2017 **Accepted** 27 January 2018

Accepted manuscript posted online 5 February 2018

Citation Domalaon R, Sanchak Y, Koskei LC, Lyu Y, Zhanel GG, Arthur G, Schweizer F. 2018. Short proline-rich lipopeptide potentiates minocycline and rifampin against multidrug- and extensively drug-resistant *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 62:e02374-17. https://doi.org/10.1128/AAC.02374-17.

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systems that prevent most antibiotics from reaching the required intracellular concentration for their antibacterial action (6, 7). As a result, there is currently a strong interest to identify novel agents that are able to enhance membrane permeability and compromise active efflux in Gram-negative bacillary pathogens such as *P. aeruginosa* (8).

Proline-rich antimicrobial peptides (PRAMPs) are amphiphilic cationic peptides typically possessing potent Gram-negative but poor Gram-positive antibacterial activity (9, 10). They are characterized by an unusual large amount of L-proline residues (typically 25 to 50% of sequence composition) and frequently contain a repeating PXP or PXXP motif, where X may be any amino acid but is typically L-arginine (9, 10). Well-known examples include mammal-derived Bac7(1-35) (11) (sequence, RRIRPRPRLPRPRPRPLPF PRPGPRPIPRPLPFP) and PR-39 (12) (sequence, RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPP RFPPRFP-NH_2) and insect-derived apidaecins (13). PRAMPs eradicate bacteria in a dose-dependent bimodular fashion. At low concentrations, they are believed to target the 70S ribosome and the DnaK chaperone (14, 15). Conversely, they eradicate pathogens via lysis at high concentrations (15). PRAMPs enter the OM via a poorly understood mechanism (presumably through a "self-promoted" uptake mechanism similar to that of most cationic peptides) (16). Inner membrane transporters SbmA and MdtM proteins facilitate their promiscuous cytosolic uptake (17, 18). However, Gram-negative *P. aeruginosa* does not express both SbmA and MdtM; therefore, the mode of action of PRAMPs is restricted to membrane rupture and lysis (19). With the urgent need for new therapeutic agents/strategies to treat drug-resistant Gram-negative bacterial infections, PRAMPs are considered an emerging source of potential new antibiotics.

In addition to antimicrobials that directly kill bacteria, adjuvants that sensitize resistant pathogens to existing antibiotics are widely studied (20, 21). In fact, several combinations of a β-lactamase inhibitor (adjuvant) and a β-lactam (antibiotic) are already used to treat drug-resistant Gram-negative bacillary infections (22, 23). Adjuvants act on bacterial processes that may elicit direct or indirect advantageous effects toward its partner antibiotic. For instance, adjuvants that inhibit β-lactamase enzymes prevent the degradation of β-lactam antibiotics. Adjuvants that disrupt the bacterial membrane may enhance cellular permeation of otherwise membrane-impermeable antibiotics.

In this study, we evaluated the antibacterial activity of synthetic short proline-rich lipopeptides (SPRLPs) against clinically relevant Gram-positive and Gram-negative pathogens. The short peptide sequence of SPRLPs was inspired by the repeating PXP motif apparent in longer PRAMPs. Moreover, we assessed the potential of these SPRLPs to serve as adjuvants in combination with clinically used antibiotics against *P. aeruginosa*. Our results revealed an amphiphilic nonhemolytic noncytotoxic L-lipopeptide lead sequence that strongly potentiates minocycline and rifampin against MDR/XDR *P. aeruginosa*. Furthermore, the adjuvant potency is retained in its enantiomeric D-SPRLP counterpart.

RESULTS AND DISCUSSION

SPRLP design is inspired by repeating PXP motif in PRAMPs. Inspired by peptide sequences of longer and naturally occurring PRAMPs such as Bac7(1-35) and PR-39, we prepared shorter synthetic versions possessing a lipoheptapeptide sequence of PRPZ PRP, where Z represents either R, G, L or W (Table 1). The observed PXP repeats in naturally occurring PRAMPs were retained in the heptapeptide sequence. Position Z was incorporated to introduce amino acid variability, resulting in four sequence subsets, namely PRP, PGP, PLP, and PWP sequences (Table 1). Amino acid variability was integrated in the design to "fine-tune" the overall physicochemical property of SPRLP at the heptapeptide portion. For instance, incorporation of L-arginine imparts an additional protonizable guanidine side chain, whereas L-leucine imparts additional hydrophobicity. L-Tryptophan was added for its aromatic ring side chain, while L-glycine was selected to see the effect of replacing the carbon-based side chain groups with hydrogen. Aliphatic lipids such as octanoic acid (C_8), dodecanoic acid (C_{12}), and hexadecanoic acid (C_{16}) were ligated to the N terminus of the cationic heptapeptide to

TABLE 1 SPRLP sequences under consideration

Compound	Sequence	Molecular mass (g/mol of TFA salt)[a]
C_8-PRP	$CH_3(CH_2)_6CO$-PRPRPRP-NH_2	1,342.33
C_{12}-PRP	$CH_3(CH_2)_{10}CO$-PRPRPRP-NH_2	1,398.44
C_{16}-PRP	$CH_3(CH_2)_{14}CO$-PRPRPRP-NH_2	1,454.55
Ad-PRP	Adamantyl-CH_2CO-PRPRPRP-NH_2	1,392.39
C_8-PGP	$CH_3(CH_2)_6CO$-PRPGPRP-NH_2	1,129.17
C_{12}-PGP	$CH_3(CH_2)_{10}CO$-PRPGPRP-NH_2	1,185.28
C_{16}-PGP	$CH_3(CH_2)_{14}CO$-PRPGPRP-NH_2	1,241.39
Ad-PGP	Adamantyl-CH_2CO-PRPGPRP-NH_2	1,179.23
C_8-PLP	$CH_3(CH_2)_6CO$-PRPLPRP-NH_2	1,185.28
C_{12}-PLP	$CH_3(CH_2)_{10}CO$-PRPLPRP-NH_2	1,241.39
C_{16}-PLP	$CH_3(CH_2)_{14}CO$-PRPLPRP-NH_2	1,297.50
Ad-PLP	Adamantyl-CH_2CO-PRPLPRP-NH_2	1,235.34
C_8-PWP	$CH_3(CH_2)_6CO$-PRPWPRP-NH_2	1,258.34
C_{12}-PWP	$CH_3(CH_2)_{10}CO$-PRPWPRP-NH_2	1,314.44
C_{16}-PWP	$CH_3(CH_2)_{14}CO$-PRPWPRP-NH_2	1,370.55
Ad-PWP	Adamantyl-CH_2CO-PRPWPRP-NH_2	1,308.40
C_{12}-prp	$CH_3(CH_2)_{10}CO$-prprprp-NH_2 (all D-peptide)	1,398.44

[a]TFA, trifluoroacetic acid.

vary the hydrophobic or amphiphilic moment in the SPRLPs (24–26). We also included the more rigid and conformationally constrained lipid 1-adamantaneacetic acid (Ad) in our study. The bulky hydrophobic adamantane moiety is perceived to be less prone to toxicity issues than longer aliphatic hydrocarbons. The C terminus of each peptide was also amidated. Sixteen acylated SPRLPs were synthesized to explore the effect of peptide sequence and amphiphilicity on their biological activity.

SPRLPs composed of longer hydrocarbons demonstrate antibacterial activity. The synthesized SPRLPs were evaluated for their antibacterial potency against a panel of Gram-positive and Gram-negative bacteria (Tables 2 and 3). Some of the included

TABLE 2 Biological activity of SPRLPs belonging to PRP and PGP sequence subsets

Organism	MIC (μg/ml)							
	C_8-PRP	C_{12}-PRP	C_{16}-PRP	Ad-PRP	C_8-PGP	C_{12}-PGP	C_{16}-PGP	Ad-PGP
Staphylococcus aureus ATCC 29213	>128	128	8	>128	>128	>128	32	>128
MRSA[a] ATCC 33592	>128	>128	16	>128	>128	>128	32	>128
MSSE[b] CANWARD-2008 81388	>128	32	4	>128	>128	128	8	>128
MRSE[c] CAN-ICU 61589	>128	128	8	>128	>128	>128	16	>128
Enterococcus faecalis ATCC 29212	>128	>128	16	>128	>128	>128	16	>128
Enterococcus faecium ATCC 27270	>128	128	8	>128	>128	>128	16	>128
Escherichia coli ATCC 25922	>128	>128	16	>128	>128	>128	32	>128
E. coli CAN-ICU 61714[d]	>128	>128	8	>128	>128	>128	32	>128
E. coli CAN-ICU 63074[e]	>128	>128	8	>128	>128	>128	32	>128
E. coli CANWARD-2011 97615[f]	>128	>128	8	>128	>128	>128	32	>128
Pseudomonas aeruginosa ATCC 27853	>128	>128	32	>128	>128	>128	128	>128
P. aeruginosa CAN-ICU 62308[g]	>128	>128	32	>128	>128	>128	128	>128
P. aeruginosa CANWARD-2011 96846[h]	>128	>128	64	>128	>128	>128	128	>128
P. aeruginosa PAO1	>512	128	32	>512	>512	>512	64	>512
Stenotrophomonas maltophilia CAN-ICU 62584	>128	>128	64	>128	>128	>128	128	>128
Acinetobacter baumannii CAN-ICU 63169	>128	>128	16	>128	>128	>128	32	>128
Klebsiella pneumoniae ATCC 13883	>128	>128	64	>128	>128	>128	64	>128
MHC[i]	>512	>512	16	>512	>512	>512	16	>512

[a]MRSA, methicillin-resistant *S. aureus*.
[b]MSSE, methicillin-susceptible *Staphylococcus epidermidis*.
[c]MRSE, methicillin-resistant *S. epidermidis*. Ceftazidime resistant.
[d]Gentamicin resistant.
[e]Amikacin intermediate resistant.
[f]Gentamicin, tobramycin, and ciprofloxacin resistant; *aac(3′)iia*.
[g]Gentamicin resistant.
[h]Gentamicin and tobramycin resistant.
[i]Minimum concentration (μg/ml) that resulted in 5% red blood cell hemolysis.

TABLE 3 Biological activity of SPRLPs belonging to PLP and PWP sequence subsets

Organism	MIC (μg/ml)							
	C_8-PLP	C_{12}-PLP	C_{16}-PLP	Ad-PLP	C_8-PWP	C_{12}-PWP	C_{16}-PWP	Ad-PWP
Staphylococcus aureus ATCC 29213	>128	128	64	>128	>128	32	8	>128
MRSA[a] ATCC 33592	>128	128	64	>128	>128	32	8	>128
MSSE[b] CANWARD-2008 81388	>128	64	32	>128	>128	16	4	>128
MRSE[c] CAN-ICU 61589	>128	64	64	>128	>128	16	8	>128
Enterococcus faecalis ATCC 29212	>128	128	64	>128	>128	32	8	>128
Enterococcus faecium ATCC 27270	>128	128	64	>128	>128	32	8	>128
Escherichia coli ATCC 25922	>128	>128	128	>128	>128	128	32	>128
E. coli CAN-ICU 61714[d]	>128	>128	64	>128	>128	128	32	>128
E. coli CAN-ICU 63074[e]	>128	>128	64	>128	>128	128	16	>128
E. coli CANWARD-2011 97615[f]	>128	>128	128	>128	>128	128	64	>128
Pseudomonas aeruginosa ATCC 27853	>128	>128	>128	>128	>128	>128	64	>128
P. aeruginosa CAN-ICU 62308[g]	>128	>128	>128	>128	>128	>128	64	>128
P. aeruginosa CANWARD-2011 96846[h]	>128	>128	>128	>128	>128	>128	64	>128
P. aeruginosa PAO1	>512	512	32	>512	>512	64	32	>512
Stenotrophomonas maltophilia CAN-ICU 62584	>128	>128	>128	>128	>128	>128	64	>128
Acinetobacter baumannii CAN-ICU 63169	>128	>128	128	>128	>128	128	64	>128
Klebsiella pneumoniae ATCC 13883	>128	>128	128	>128	>128	>128	64	>128
MHC[i]	>512	>512	16	>512	>512	64	16	>512

[a]MRSA, methicillin-resistant *S. aureus*.
[b]MSSE, methicillin-susceptible *Staphylococcus epidermidis*.
[c]MRSE, methicillin-resistant *S. epidermidis*. Ceftazidime resistant.
[d]Gentamicin resistant.
[e]Amikacin intermediate resistant.
[f]Gentamicin, tobramycin, and ciprofloxacin resistant; *aac(3′)iia*.
[g]Gentamicin resistant.
[h]Gentamicin and tobramycin resistant.
[i]Minimum concentration (μg/ml) that resulted in 5% red blood cell hemolysis.

pathogens were collected from patients visiting or admitted to participating Canadian hospitals through the CAN-ICU (27) and CANWARD (28) national surveillance studies. Antibacterial activity was assessed using MICs against various clinical pathogens.

The Gram-negative-specific antibacterial activity of naturally existing PRAMPs was not observed for the synthesized SPRLPs. Among the four sequence subsets, peptides acylated with C_{16} displayed better antibacterial activity than those acylated with C_8, C_{12}, or Ad. Three of the four peptides comprising C_{16} showed promising activity. C_{16}-PRP displayed broad-spectrum activity (Table 2), with an MIC range of 4 to 16 μg/ml against Gram-positive bacteria and an MIC range of 8 to 16 μg/ml against the Gram-negative bacterium *E. coli*. Moderate activity against Gram-positive bacteria (MIC range of 8 to 32 μg/ml) was demonstrated by C_{16}-PGP (Table 2). The lipopeptide C_{16}-PWP also exhibited good activity (MIC range of 4 to 8 μg/ml) against Gram-positive bacteria (Table 3). Overall, the SPRLPs reported herein appeared to be active mostly against Gram-positive organisms.

Nonspecific membrane lysis limits therapeutic potential. Since PRAMPs are able to kill bacteria through membrane lysis, it is imperative to evaluate whether these synthesized SPRLPs also lyse eukaryotic membranes. The ability to lyse porcine red blood cells was assessed, and the minimum concentration resulting in 5% erythrocyte hemolysis (MHC) was reported (Tables 2 and 3). SPRLPs acylated with the long hydrocarbon C_{16} showed high hemolytic activity. C_{16}-PRP, C_{16}-PGP, C_{16}-PLP, and C_{16}-PWP resulted in 5% red blood cell hemolysis at 16 μg/ml. These data corroborate that the observed antibacterial activity of these four SPRLPs is through nonspecific membrane lysis and therefore greatly limits their therapeutic potential. The lipopeptide C_{12}-PWP demonstrated marginal hemolytic activity, with an MHC of 64 μg/ml. However, all other SPRLPs were nonhemolytic (MHC > 512 μg/ml).

Potentiation of minocycline and rifampin by an SPRLP against MDR/XDR *P. aeruginosa*. Adjuvants typically do not kill the pathogens directly but are able to help their antibiotic partner broaden their antibacterial spectrum or maximize their antibac-

terial activity. A literature search revealed only one report of a PRAMP that can synergize a clinically used antibiotic against Gram-negative bacilli. The long proline-rich peptide dimer A3-APO, consisting of 41 amino acids, was found to potentiate chloramphenicol against *Klebsiella pneumoniae* in a checkerboard assay (29). However, an amphiphilic lysine-based peptide-like agent was reported to potentiate rifampin in *E. coli* (30). We therefore were interested in studying whether our short proline-rich heptapeptide-based SPRLPs possessed adjuvant properties. Certainly, it is advantageous to have a lead molecule of shorter peptide sequence, as it is more cost-effective and more amenable to peptidomimetic modifications for further optimization.

We evaluated the activity of SPRLPs in combination with 15 clinically used antibiotics (see Tables S1 to S3 in the supplemental material) against *P. aeruginosa*. The antibiotics tested included fluoroquinolones (moxifloxacin, ciprofloxacin, and levofloxacin), aminoglycosides (gentamicin, tobramycin, and amikacin), cephalosporins (ceftazidime and cefotaxime), carbapenems (meropenem and doripenem), aztreonam, rifampin, minocycline, colistin, and fosfomycin. The SPRLPs were screened initially at a fixed concentration of 8 μg/ml (5 μM) in combination with antibiotics against wild-type *P. aeruginosa* PAO1. We assessed potentiation based on at least a 4-fold absolute reduction in MIC of the antibiotic, after which synergism was further validated by a conventional checkerboard assay. Fractional inhibitory concentration (FIC) indexes of $\leq$0.5, 0.5 $< x \leq 4$, and $>$4 were interpreted as indicating synergistic, indifferent, and antagonistic interactions, respectively (31). The FIC index was obtained by adding the FIC values of the antibiotic and the SPRLP adjuvant. The FIC of antibiotics was calculated by dividing the MIC of the antibiotic in the presence of the adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of the adjuvant was calculated by dividing the MIC of the adjuvant in the presence of the antibiotic by the MIC of the adjuvant alone.

Of the 15 clinically used antibiotics and 16 short synthetic SPRLPs, an initial screening revealed potentiation of minocycline and rifampin with the amphiphilic lipopeptide C_{12}-PRP (Table S1). Further validation by a checkerboard assay confirmed the synergistic combinations against wild-type *P. aeruginosa* strain PAO1. C_{12}-PRP with either minocycline or rifampin yielded an FIC index of 0.19 or 0.14, respectively. These findings warranted further studies, since C_{12}-PRP is nonhemolytic even at a high concentration of 512 μg/ml (Table 2). We evaluated whether the observed synergism was retained against MDR/XDR *P. aeruginosa* clinical isolates. But, we also investigated the capability of C_{12}-PRP to reduce the absolute MICs of minocycline and rifampin below their susceptibility breakpoints. No established minocycline and rifampin susceptibility breakpoints currently exist for *Pseudomonas aeruginosa* from either the CLSI or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Therefore, we cautiously used established breakpoints for other organisms that are as similar as possible to *Pseudomonas aeruginosa* for our comparison. According to CLSI (32), the susceptibility breakpoint of minocycline for *Acinetobacter* spp. is $\leq$4 μg/ml, while the susceptibility breakpoint of rifampin for *Staphylococcus* spp. is $\leq$1 μg/ml.

The combination of minocycline and C_{12}-PRP was found to be strongly synergistic against all eight tested MDR/XDR *P. aeruginosa* isolates (Table 4). Moreover, the MIC of minocycline in the presence of 8 μg/ml (5 μM) C_{12}-PRP was reduced below the susceptibility breakpoint in seven out of nine strains. Significant potentiation was also observed for the combination of rifampin and C_{12}-PRP against MDR/XDR *P. aeruginosa* isolates (Table 5). At 8 μg/ml (5 μM) C12-PRP, the MIC of rifampin reached the susceptibility breakpoint in five out of nine strains. Indeed, the SPRLP C_{12}-PRP was able to enhance the antibacterial potency of minocycline and rifampin against wild-type and clinical isolates of *P. aeruginosa*. The potential of this SPRLP as a lead adjuvant candidate is apparent. The mechanism of antibiotic potentiation certainly warrants further study in the future. However, membrane perturbation that results in enhanced antibiotic uptake is a likely possibility. PRAMPs are known to disrupt bacterial membranes, which is more pronounced in *P. aeruginosa* than in other Gram-negative bacilli (19). This suggests that the SPRLP C12-PRP may potentiate minocycline and rifampin through OM permeabilization of *P. aeruginosa*. Membrane perturbation may also

TABLE 4 Adjuvant potency of amphiphilic C_{12}-PRP in combination with minocycline against wild-type and MDR/XDR *P. aeruginosa*[a]

P. aeruginosa strain	MIC_{MIN} (μg/ml)	$MIC_{C12\text{-}PRP}$ (μg/ml)	FIC index	Absolute MIC_{MIN}[b] (μg/ml)	Potentiation (fold)[c]
PAO1	8	128	0.19	1	8
259-96918	16	>128	$0.12 < x < 0.19$	2	8
260-97103	16	128	0.12	1	16
262-101856	64	>128	$0.12 < x < 0.25$	16	4
264-104354	32	>128	$0.06 < x < 0.12$	2	16
91433[d]	32	>128	$0.12 < x < 0.25$	8	4
100036	16	>128	$0.12 < x < 0.25$	4	4
101243[d]	2	128	0.31	0.5	4
101885	16	64	0.37	4	4

[a]MIN, minocycline; MDR, multidrug resistant; XDR, extensively drug resistant.
[b]MIC of minocycline in the presence of 8 μg/ml (5 μM) C_{12}-PRP.
[c]Degree of antibiotic potentiation in the presence of 8 μg/ml (5 μM) C_{12}-PRP.
[d]Colistin resistant.

compromise the activity of integral membrane proteins such as multidrug efflux pumps, essentially halting antibiotic resistance through active efflux.

The type of fatty acyl ligated to the peptide sequence PRPRPRP-NH_2 is important for adjuvant activity. Since the lead adjuvant C_{12}-PRP was discovered from a synergy scan having a fixed 8-μg/ml (5 μM) SPRLP concentration, combinations of minocycline or rifampin with other PRP subset lipopeptides warranted further investigation. Therefore, we assessed the interaction of either C_8-PRP, C_{16}-PRP, or Ad-PRP with minocycline or rifampin by a checkerboard assay. The three synthetic SPRLPs displayed indifferent interactions with minocycline and rifampin (Table S4). Interestingly, C_{16}-PRP did not display synergism with either antibiotic, even though our initial data suggested that it can disrupt and lyse membranes. These data suggest that the aliphatic lipid C_{12} is optimal for amphiphilic SPRLPs to potentiate minocycline and rifampin against *P. aeruginosa*.

The D-lipopeptide counterpart of C_{12}-PRP retains adjuvant potency. The amphiphilic C_{12}-PRP is considered susceptible to nonspecific proteolytic degradation, since host enzymes (e.g., human proteases) easily recognize L-amino acid peptide bonds. Therefore, lead peptide agents typically undergo peptidomimetic modifications to increase serum stability (10, 33). We explored one approach to optimize C_{12}-PRP by synthesizing the same sequence but with D- instead of L-amino acids, yielding the D-lipopeptide analog C_{12}-prp. Peptide bonds formed by D-amino acids are less prone to mammalian proteases (34). Like C_{12}-PRP, C_{12}-prp was found to be inactive (MIC > 128 μg/ml) against wild-type and clinical isolates of *P. aeruginosa*. The adjuvant properties of C_{12}-PRP were retained, but the potency was slightly reduced in the D-lipopeptide analog. C_{12}-prp potentiated minocycline and rifampin against wild-type and MDR/XDR

TABLE 5 Adjuvant potency of amphiphilic C_{12}-PRP in combination with rifampin against wild-type and MDR/XDR *P. aeruginosa*[a]

P. aeruginosa strain	MIC_{RMP} (μg/ml)	$MIC_{C12\text{-}PRP}$ (μg/ml)	FIC index	Absolute MIC_{RMP}[b] (μg/ml)	Potentiation (fold)[c]
PAO1	8	128	0.14	1	8
259-96918	16	>128	$0.01 < x < 0.14$	2	8
260-97103	16	128	0.16	2	8
262-101856	512	>128	$0.25 < x < 0.37$	512	None
264-104354	8	>128	$0.06 < x < 0.12$	0.5	16
91433[d]	16	>128	$0.06 < x < 0.09$	1	16
100036	16	>128	$0.01 < x < 0.14$	1	16
101243[d]	4	128	0.09	0.125	32
101885	16	64	0.25	2	8

[a]RMP, rifampin; MDR, multidrug resistant; XDR, extensively drug resistant.
[b]MIC of rifampin in the presence of 8 μg/ml (5 μM) C_{12}-PRP.
[c]Degree of antibiotic potentiation in the presence of 8 μg/ml (5 μM) C_{12}-PRP.
[d]Colistin resistant.

TABLE 6 Adjuvant potency of amphiphilic C_{12}-prp in combination with minocycline against wild-type and MDR/XDR *P. aeruginosa*[a]

P. aeruginosa strain	MIC_{MIN} (μg/ml)	$MIC_{C12\text{-}prp}$ (μg/ml)	FIC index	Absolute MIC_{MIN}[b] (μg/ml)	Potentiation (fold)[c]
PAO1	8	>128	$0.25 < x < 0.31$	2	4
259-96918	16	>128	$0.12 < x < 0.25$	4	4
260-97103	16	>128	$0.12 < x < 0.19$	2	8
262-101856	64	>128	$0.12 < x < 0.25$	16	4
264-104354	32	>128	$0.12 < x < 0.19$	4	8
91433[d]	32	>128	$0.12 < x < 0.19$	4	8
100036	16	>128	$0.25 < x < 0.37$	8	2
101243[d]	4	>128	$0.12 < x < 0.37$	2	2
101885	16	>128	$0.25 < x < 0.37$	8	2

[a]MIN, minocycline; MDR, multidrug resistant; XDR, extensively drug resistant.
[b]MIC of minocycline in the presence of 8 μg/ml (5 μM) C_{12}-prp.
[c]Degree of antibiotic potentiation in the presence of 8 μg/ml (5 μM) of all D-lipopeptide C_{12}-prp.
[d]Colistin resistant.

P. aeruginosa isolates (Tables 6 and 7). Furthermore, 8 μg/ml (5 μM) of C_{12}-prp reduced the MICs of minocycline (Table 6) and rifampin (Table 7) below susceptibility breakpoints in some MDR/XDR *P. aeruginosa* clinical isolates. These results suggest that C_{12}-PRP is amenable to peptidomimetic alterations and that further lead optimizations are possible.

Amphiphilic C_{12}-PRP is not cytotoxic to eukaryotic cells. Our initial assessment of the effect of SPRLPs on eukaryotic membranes revealed that C_{12}-PRP is nonhemolytic. In fact, the concentration resulting in 5% red blood cell hemolysis for C_{12}-PRP was >512 μg/ml. At 512 μg/ml (366 μM), C_{12}-PRP resulted in only 4.6% $\pm$ 0.2% hemolysis (Fig. 1A). We then evaluated the potential toxicity of C_{12}-PRP against two eukaryotic cell lines, which included human liver carcinoma HepG2 and human embryonic kidney HEK-293, by its ability to inhibit cellular proliferation and cellular viability (Table S5). We used colistin (also known as polymyxin E) and adriamycin as internal controls to represent clinically used peptide antibiotics and anticancer drugs, respectively. Amphiphilic C_{12}-PRP did not inhibit cellular proliferation of either cell line (Fig. 1B and C) up to the highest concentration tested (50 μM), notably 10-fold higher than the C_{12}-PRP's adjuvant working concentration (5 μM). Interestingly, a 1.5 μM concentration of the antibiotic colistin inhibited the proliferation of HepG2 cells to 50% (50% inhibitory concentration [IC_{50}]). The anticancer drug adriamycin inhibited the growth of both cell lines at very low concentrations. We further evaluated cytotoxicity by assessing the effect of the agents on the global oxidoreductive metabolism of cells through the MTS assay (Fig. 1D and E). Neither C_{12}-PRP nor colistin killed either cell line up to the highest concentration tested (50 μM). Congruent with results from the proliferation assay, adriamycin drastically reduced the viability of both cell lines at a low concentration. Our

TABLE 7 Adjuvant potency of amphiphilic C_{12}-prp in combination with rifampin against wild-type and MDR/XDR *P. aeruginosa*[a]

P. aeruginosa strain	MIC_{RMP} (μg/ml)	$MIC_{C12\text{-}prp}$ (μg/ml)	FIC index	Absolute MIC_{RMP}[b] (μg/ml)	Potentiation (fold)[c]
PAO1	8	>128	$0.12 < x < 0.19$	1	8
259-96918	16	>128	$0.03 < x < 0.16$	2	8
260-97103	16	>128	$0.06 < x < 0.19$	4	4
262-101856	512	>128	$0.12 < x < 0.25$	256	2
264-104354	8	>128	$0.12 < x < 0.25$	2	4
91433[d]	16	>128	$0.12 < x < 0.14$	2	8
100036	16	>128	$0.06 < x < 0.19$	4	4
101243[d]	4	>128	$0.06 < x < 0.19$	0.5	8
101885	16	>128	$0.12 < x < 0.25$	4	4

[a]RMP, rifampin; MDR, multidrug resistant; XDR, extensively drug resistant.
[b]MIC of rifampin in the presence of 8 μg/ml (5 μM) C_{12}-prp.
[c]Degree of antibiotic potentiation in the presence of 8 μg/ml (5 μM) of all D-lipopeptide C_{12}-prp.
[d]Colistin resistant.

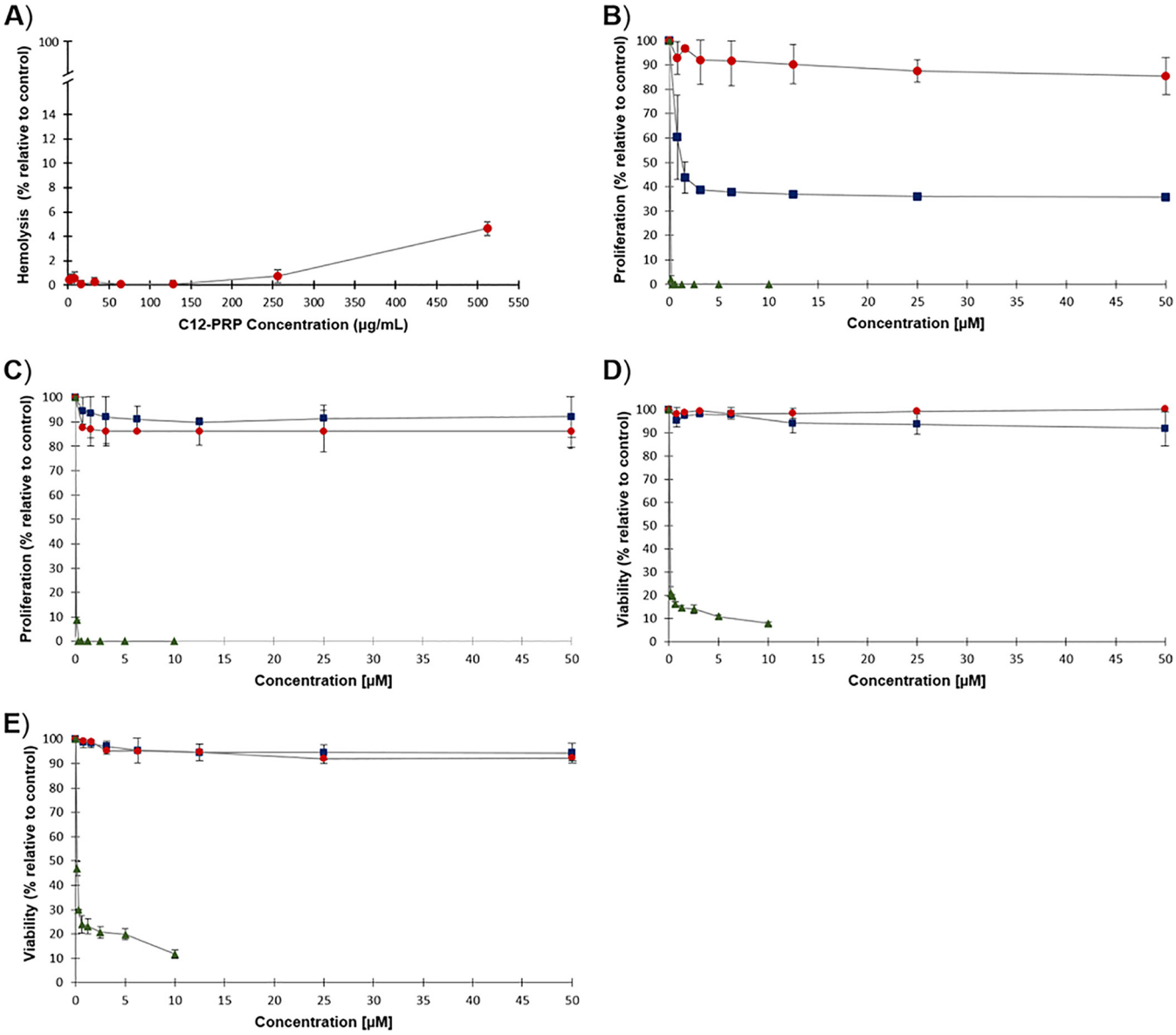

FIG 1 Evaluation of cytotoxicity of amphiphilic C_{12}-PRP (red circles) via hemolytic activity against erythrocytes (A), inhibition of cellular proliferation against human liver carcinoma HepG2 cells (B), inhibition of cellular proliferation against human embryonic kidney HEK-293 cells (C), cytotoxic effects against human liver carcinoma HepG2 cells (D), and cytotoxic effects against human embryonic kidney HEK-293 cells (E). All experiments were performed in three or more replicates. Colistin (blue squares) and adriamycin (green triangles) were used to represent clinically used peptide antibiotics and anticancer drugs, respectively. Error bars indicate standard deviations of results from three independent experiments ($n = 3$).

data presented herein strongly suggest that the amphiphilic C_{12}-PRP is not cytotoxic to eukaryotic cells.

Conclusion. An amphiphilic short proline-rich lipopeptide that synergizes with two clinically used antibiotics was identified for the first time. The nonhemolytic lipopeptide C_{12}-PRP, with a short sequence of C_{12}-PRPRPRP-NH_2, potentiates minocycline and rifampin against wild-type and MDR/XDR *P. aeruginosa* isolates. More importantly, C_{12}-PRP significantly reduced the MICs of minocycline and rifampin against *P. aeruginosa* below their interpretative susceptibility breakpoints. Furthermore, our data strongly suggest that C_{12}-PRP is noncytotoxic. However, instability to proteases remains a drawback. Our initial attempt of optimization by incorporating D-amino acids retained the desired adjuvant property of the lipopeptide, and therefore, C_{12}-PRP appeared to be amenable to peptidomimetic modification. Envisioned future work includes further

optimization to bestow protease stability and to enhance the adjuvant profile of C_{12}-PRP, by incorporating peptoids and unnatural amino acids to the C_{12}-PRP structure. Indeed, peptide-based antibacterial drug candidates such as murepavadin (also known as POL7080) (35) and brilacidin (36), both in phase-2 clinical trials, were optimized to remove their "peptide-like" nature prior to clinical validation. Furthermore, the *in vivo* efficacy of the lipopeptide-antibiotic combination will be assessed in insect models of infection. Mode-of-action studies to explore the effects of the lead lipopeptide on the OM, inner membrane, and proton motive force that can result in increased intracellular concentrations of minocycline and rifampin are planned (37, 38).

MATERIALS AND METHODS

Peptide preparation. All lipopeptides were synthesized on solid-phase methylbenzhydrylamine (MBHA) Rink amide resin by following a standard fluorenylmethyloxycarbonyl (Fmoc) chemistry protocol (39, 40). Amino acids with reactive side chain functional groups were masked with protecting groups inert to solid-phase peptide synthesis conditions yet labile upon peptide cleavage from the solid support. Therefore, Fmoc-Arg(Pbf)-OH and Fmoc-Trp(Boc)-OH were purchased to prevent the guanidine and indole side chain, respectively, to cause unwanted reactions. The coupling reagent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and *N*-methylmorpholine were used to induce peptide bond formation between amino acids. All reagents and solvents were purchased from commercially available sources and used without further purification.

SPRLPs were purified via reverse-phase flash chromatography using C_{18} (40- to 63-μm) silica gel purchased from Silicycle (USA). Purity was assessed by high-performance liquid chromatography (HPLC) and determined to be >95%. Each peptide was characterized using nuclear magnetic resonance (NMR) and mass spectrometry (MS). One (^{1}H and ^{13}C)- and two-dimensional NMR experiments were performed on either a Bruker AMX-500 or Bruker AMX-300 instrument (Germany). Electrospray ionization mass spectrometry (ESI-MS) experiments were performed on a Varian 500-MS ion trap mass spectrometer (USA), and high-resolution matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) experiments were done on a Bruker Ultraflextreme mass spectrometer (Germany) coupled to a time-of-flight mass analyzer.

Bacterial strains. Isolates used in this study were obtained from either the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study (27), or the Canadian Ward Surveillance (CANWARD) study (28). Clinical isolates belonging to the CAN-ICU and CANWARD studies were recovered from patients suffering presumed infectious diseases who were admitted to a participating medical center across Canada during the time of study. MDR *P. aeruginosa* strains in this study refer to those that are resistant to aminoglycosides, fluoroquinolones, cephalosporins, and carbapenems, while XDR strains are those that are resistant to aminoglycosides, fluoroquinolones, cephalosporins, carbapenems, aztreonam, and penicillin–β-lactamase inhibitor combinations (37, 38).

Antimicrobial susceptibility assay. A broth microdilution susceptibility test following the Clinical and Laboratory Standards Institute (CLSI) guidelines (32) was performed to assess the *in vitro* antibacterial activity of SPRLPs. Bacterial cultures grown overnight were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of 5×10^5 CFU/ml. The assay was done on a 96-well plate in which the agents of interest were 2-fold serially diluted in MHB and incubated with equal volumes of inoculum at 37°C for 18 h. MIC was determined as the lowest concentration to inhibit visible bacterial growth in the form of turbidity, which was confirmed using an EMax Plus microplate reader (Molecular Devices, USA) at a wavelength of 590 nm. The wells containing MHB broth with or without bacterial cells were used as positive or negative controls, respectively.

Hemolytic assay. The ability of SPRLPs to lyse eukaryotic red blood cells was quantified based on the amount of hemoglobin released upon incubation with pig erythrocytes, in accordance with published protocols (37, 39). Fresh pig blood drawn from the pig antecubital vein was centrifuged at $1,000 \times g$ for 5 min at 4°C, washed with phosphate-buffered saline (PBS) three times, and resuspended in the same buffer, consecutively. Agents of interest were then 2-fold serially diluted in PBS on a 96-well plate and mixed with equal volumes of the erythrocyte solution. After 1 h of incubation at 37°C, intact cells were pelleted by centrifugation at $1,000 \times g$ for 5 min at 4°C. The supernatant was then transferred to a new 96-well plate. The hemoglobin released was measured with an EMax Plus microplate reader (Molecular Devices, USA) at a 570-nm wavelength. Erythrocytes in PBS with or without 0.1% Triton X-100 were used as negative or positive controls, respectively.

Synergy scan testing. The synergy assay was performed on a 96-well plate in which the agents of interest were 2-fold serially diluted in working MHB. Prior to serial dilution, SPRLP was added to the working MHB medium so that a fixed final concentration of 8 μg/ml (5 μM) SPRLP per well was achieved. To ensure that the assay was working, the MIC determination test of the studied antibiotic (without SPRLP) was included on the same plate. Similar MIC results between the assay comparator and an independent MIC determination test (on a different plate) ensured the validity of the synergy test. Bacterial cultures grown overnight were diluted in saline to 0.5 McFarland turbidity, followed by 1:50 dilution in MHB (without SPRLP) and inoculation into each well to a final concentration of approximately 5×10^5 CFU/ml. Wells containing only MHB (without SPRLP) with or without bacterial cells were used as positive or negative controls, respectively. The plate was then incubated at 37°C for 18 h and examined

for visible turbidity, which was confirmed using an EMax Plus microplate reader (Molecular Devices, USA) at a wavelength of 590 nm. An antibiotic MIC reduction of ≥4-fold in the presence of 8 μg/ml (5 μM) SPRLP denoted a positive synergy result and was further validated by a checkerboard assay.

Checkerboard assay. The checkerboard assay was done on a 96-well plate as previously described (38, 41). The agent of interest was 2-fold serially diluted along the *x* axis, while the adjuvant was 2-fold serially diluted along the *y* axis to create a matrix in which each well consisted of a combination of both agent and adjuvant at different concentrations. Bacterial cultures grown overnight were diluted in saline to 0.5 McFarland turbidity, followed by 1:50 dilution in MHB and inoculation on each well to a final concentration of approximately 5×10^5 CFU/ml. Wells containing only MHB with or without bacterial cells were used as positive or negative controls, respectively. The plate was incubated at 37°C for 18 h and examined for visible turbidity, which was confirmed using an EMax Plus microplate reader (Molecular Devices, USA) at a wavelength of 590 nm. The fractional inhibitory concentration (FIC) of antibiotic was calculated by dividing the MIC of antibiotic in the presence of adjuvant by the MIC of antibiotic alone. Similarly, the FIC of adjuvant was calculated by dividing the MIC of adjuvant in the presence of antibiotic by the MIC of adjuvant alone. The FIC index was obtained by the summation of both FIC values. The FIC index was interpreted as synergistic, indifferent, or antagonistic for values of ≤ 0.5, $0.5 < x \leq 4$, or >4, respectively (31).

Proliferation assay. The CyQuant Direct cell proliferation assay kit (ThermoFisher, Canada) was used to assess the effect of C_{12}-PRP on cell proliferation according to the manufacturer's protocol. Briefly, human embryonic kidney cells (HEK-293) and HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were dispersed into 96-well plates (8,000 cells/well in 100 μl). Wells with medium but no cells were used as blanks. After 24 h, various concentrations of C_{12}-PRP, colistin, and adriamycin were added to the wells containing cells but also the blanks. After incubation of the cells with the compounds for 48 h, the CyQuant Direct detection reagent was added to the wells. The plates were incubated at 37°C for 1 h, and the fluorescence (excitation, 480 nm; emission, 535 nm) was read using a SpectraMax M2e (Molecular Devices, USA). As a positive control, CyQuant Direct detection reagent was added to a plate with untreated cells and incubated for 1 h, followed by fluorescence reading. The number of cells in each well was determined by detaching the cells by trypsin followed by counting on a CoulterZM counter to ensure approximately equal numbers of cells per well.

Cytotoxicity assay. The cytotoxic effects of C_{12}-PRP was assessed by measuring its effect on the viability of HEK-293 or HepG2 cells. The cells were dispersed into 96-well plates, and after 24 h, C_{12}-PRP, colistin, or adriamycin was added as described in the proliferation assay. After incubation for 48 h, the viability of the cells was determined with the MTS reagent (Promega, Canada) as previously described (42).

Statistical analysis. Data herein represent the means ± standard deviations (error bars) of the results of at least three independent experiments. The null hypothesis was evaluated via one-way analysis of variance (ANOVA), where the confidence interval was set to be 95% ($P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02374-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Council of Canada (NSERC) (NSERC-DG 261311-2013) and a Manitoba Health Research Council (MHRC) graduate studentship scholarship.

We thank Charles M. Nyachoti (University of Manitoba) for generously supplying fresh porcine erythrocytes.

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