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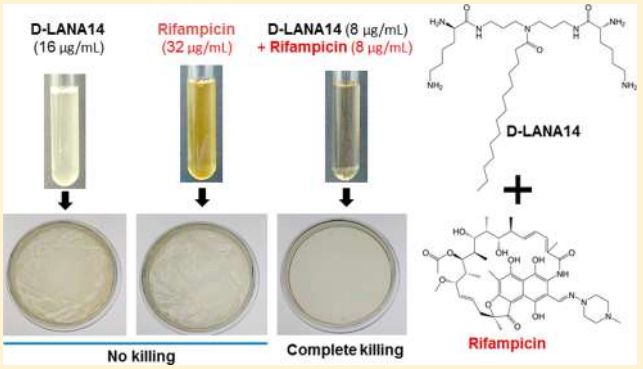
Lysine-Based Small Molecule Sensitizes Rifampicin and Tetracycline against Multidrug-Resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

Mohini Mohan Konai and Jayanta Haldar*

Antimicrobial Research Laboratory, New Chemistry Unit and School of Advanced Materials, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bengaluru 560064, Karnataka, India

Supporting Information

ABSTRACT: The priority pathogen list published by the World Health Organization (WHO) has categorized carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as the top two critical pathogens, and hence, the development of novel antibacterial strategies to tackle such bacteria is highly necessary. Toward this aim, herein we report the efficacy of the combination of a lysine-based membrane-active small molecule, D-LANA-14 (D-lysine conjugated aliphatic norspermidine analogue bearing tetradecanoyl chain) and the obsolete/inactive antibiotics (such as tetracycline and rifampicin) to combat these superbugs. The combination of D-LANA-14 and the antibiotics tetracycline or rifampicin showed not only synergistic activity against growing planktonic cells of meropenem-resistant *A. baumannii* and *P. aeruginosa* clinical isolates but was also able to disrupt their established biofilms. More importantly, this synergistic effect was retained under the *in vivo* scenario, wherein the combination showed excellent efficacy in mice model of burn-wound infection with a drastic reduction of bacterial burden. A combined treatment of D-LANA-14 (40 mg/kg) and rifampicin (40 mg/kg) showed 4.9 log and 4.0 log reduction in *A. baumannii* and *P. aeruginosa* viability, respectively. On the contrary, individual treatment of D-LANA-14 decreased bacterial burden by 2.3 log (*A. baumannii*) and 1.3 log (*P. aeruginosa*) and rifampicin reduced about 3.0 log (*A. baumannii*) and 1.6 log (*P. aeruginosa*). Owing to the membrane-active nature imparted by D-LANA-14, bacteria could not develop resistance against the combined treatment, whereas a high-level of resistance development was observed against the last resort Gram-negative antibiotic, colistin. Taken together, the results therefore indicate a great potential of this novel combination to be developed as therapeutic regimen to combat infections caused by critical Gram-negative pathogens.



KEYWORDS: *Gram-negative superbugs, antibiotic-resistance, biofilms, burn-wound infections, combination therapy, membrane-targeting compound*

The Gram-negative bacterial infections are becoming increasingly difficult to treat because of the dearth of effective antibiotics in the clinical pipeline.[1−3] A recent report published by the World Health Organization (WHO) identified *A. baumannii* and *P. aeruginosa* as the top two critical pathogens.[3] An epidemic outbreak of these dangerous pathogens, in both nosocomial and community associated infections, has raised a serious challenge in the face of public health around the world. A rapid propensity of resistance development against conventional antibiotics and the biofilm-forming capability of these pathogens has further deteriorated the situation.[4−6] As a result, the infections caused by these pathogens are becoming increasingly impossible to treat with existing antibacterial therapy. Among the numerous strategies employed by the research community worldwide to combat the threats created by these pathogens, the combination strategy has emerged as a potential approach, which may provide an immediate solution to this growing problem.[7,8] The combined treatment with multiple antibacterial agents or rationally developed sensitizers of obsolete antibiotics has garnered a lot of attention in the recent past.[9−20] The presence of outer and inner membranes in Gram-negative bacteria plays a significant role in the development of antibiotic resistance (Figure 1). The lipopolysaccharide (LPS)-containing polar outer membrane prevents the passive diffusion of hydrophobic antibiotics (such as rifampicin), and hence, this class of bacteria are intrinsically resistant to this antibiotic.[21] They have also acquired resistance to multiple antibiotics through either impaired influx or increased efflux of antibiotics from the cells or through both in many cases (Figure 1). The development of

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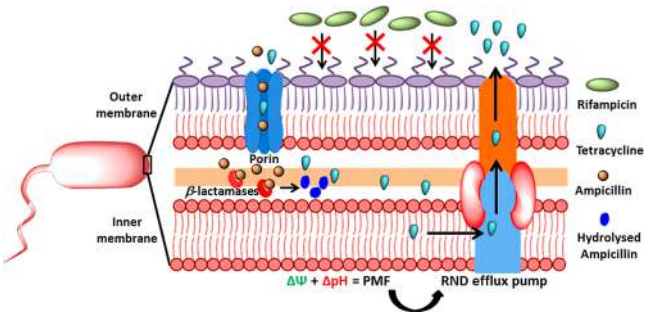

Figure 1. Two membrane cell envelope structure of Gram-negative bacteria and the important role they play in the development of antibiotic resistance. Gram-negative bacteria are intrinsically resistant to hydrophobic antibiotics (e.g., rifampicin) owing to the presence of polar lipopolysaccharide (LPS) present in the outer membrane. They have acquired resistance against multiple antibiotics (e.g., tetracycline family) because of impaired influx, increased outflow of antibiotics, or both. Conversely, the penicillin class of antibiotics (e.g., ampicillin) is primarily ineffective because of the enzymatic degradation of the drug. PMF stands for proton motif force, which is the summation of transmembrane electrical potential, ΔΨ and ΔpH (which originates because of the difference of proton distribution across the membrane).

resistance against the tetracycline class of antibiotics is one such example. In the case of acquired-resistance bacteria, the impaired influx of antibiotics is primarily due to the changes that happen in the porins, which is also escalated by lowering the passive diffusion of the drug.[21,22] The increased efflux of antibiotics out of the bacterial cell is accomplished by the efflux pumps, which are overexpressed in the case of drug-resistant bacteria. An additional special class of efflux pump is also expressed in drug-resistant bacteria, called the resistance-nodulation-division (RND), dedicated only to the efflux of antibiotics.[21−24] This class of efflux pumps mainly functions by using the energy originating from the proton motive force (PMF), which is generated as the result of the difference in distribution of various ions and protons across the inner membrane.[25] Hence, by destabilizing the membranes of Gram-negative bacteria, all such membrane-associated functions could be hampered, making it a potential strategy to sensitize many obsolete/inactive antibiotics against these critical superbugs.

On the basis of this hypothesis, the membrane-active property of our previously reported compound, D-LANA-14 (D-lysine conjugated aliphatic norspermidine analogue bearing tetradecanoyl chain) was first investigated by performing the studies targeting both outer as well as inner membrane. Next, the sensitizing efficacy of D-LANA-14 was evaluated for antibiotics such as tetracycline and rifampicin. The synergistic effect of the combinations was investigated by performing a checkerboard assay against various *A. baumannii* and *P. aeruginosa* clinical isolates, which are highly resistant to meropenem. Furthermore, the efficacy was assessed by performing time-kill kinetics, and the ability to disrupt preformed biofilms was also investigated. More importantly, *in vivo* efficacy was examined in a mouse model of burn-wound infection caused by such critical pathogens. Finally, the propensity of resistance development was studied for the combination.

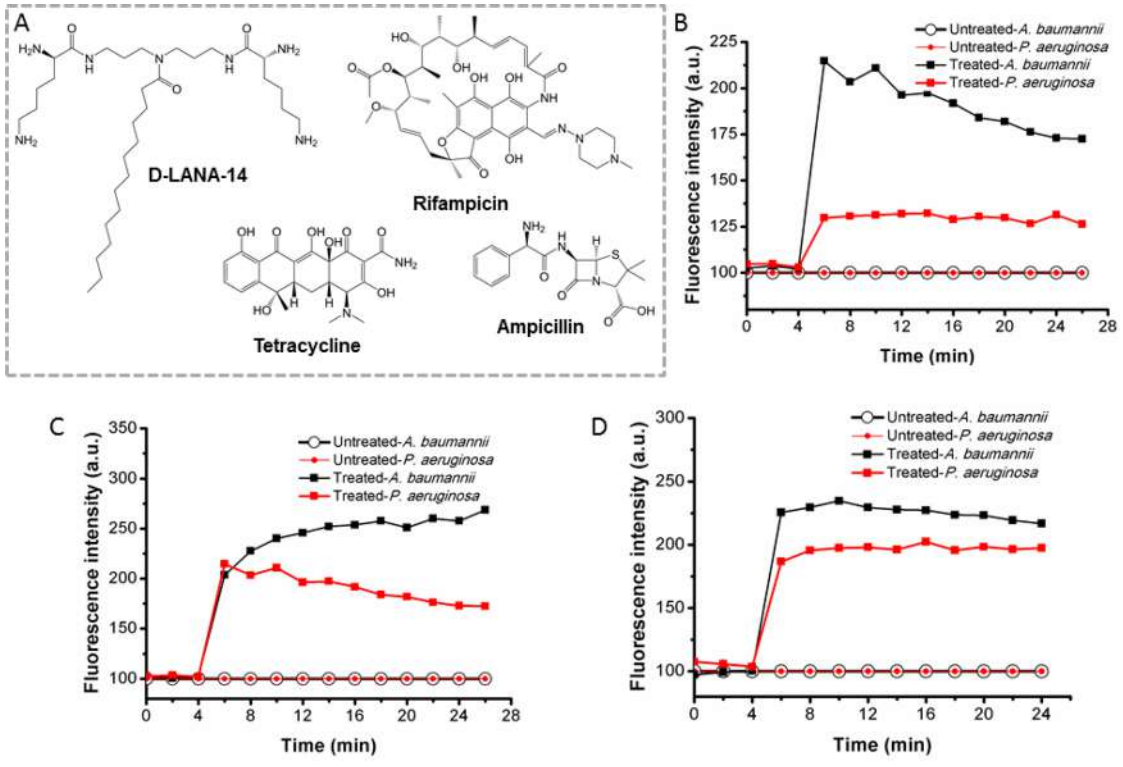

Figure 2. (A) Chemical structure of D-LANA-14 and antibiotics used in the study; (B) Outer membrane permeabilization by D-LANA-14 (8 μg/mL); the study was performed by using NPN (*N*-phenyl-1-naphthylamine) as fluorescence probe (the emission at 420 nm was recorded by exciting at a wavelength of 350 nm); (C) Cytoplasmic membrane depolarization by D-LANA-14 (8 μg/mL); the experiment was performed by using the dye $DisC_3$ (5) as florescence probe (the emission at 670 nm was recorded by exciting at a wavelength of 622 nm); (D) Uptake of tetracycline in the presence of D-LANA-14 (8 μg/mL), the tetracycline fluorescence was recorded at 535 nm by exciting at a wavelength of 405 nm.

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Table 1. Antibacterial Activity of of D-LANA-14, Antibiotics, and the Combinations

Bacterial Strains	D-LANA-14 only MIC (μg/mL)	Antibiotic only MIC (μg/mL)	MIC of antibiotics (μg/mL) in presence of D-LANA-14			FICI[a]
			MIC/4	MIC/8	MIC/16	
Tetracycline						
A. baumannii-R674	32	128	4	8	16	0.19-0.28
A. baumannii-R676	32	128	8	16	32	0.25-0.31
A. baumannii-MTCC1425	32	0.5	0.25	0.25	0.25	0.56-0.75
P. aeruginosa-R590	32	128	4	8	16	0.19-0.28
P. aeruginosa-R596	64	>128	32	64	64	<0.50-<0.63
P. aeruginosa-R2477	64	>128	32	64	64	<0.50-<0.63
P. aeruginosa- R2536	64	>128	32	64	64	<0.50-<0.63
P. aeruginosa-MTCC424	8	8	2	2	4	0.38-<0.56
Rifampicin						
A. baumannii-R674	32	128	2	4	8	0.13-0.27
A. baumannii-R676	32	64	2	8	16	0.19-0.31
A. baumannii-MTCC1425	32	4	<0.25	<0.25	0.5	<0.19-<0.31
P. aeruginosa-R590	32	128	2	4	8	0.13-0.27
P. aeruginosa-R596	64	32	0.5	1-2	2	0.13-0.27
P. aeruginosa-R2477	64	32	0.25-1	0.5-2	1-4	0.09-0.28
P. aeruginosa-R2536	64	32	0.5-1	1-2	2-4	0.13-0.28
P. aeruginosa-MTCC424	8	32	4	8	8	0.31-0.38
Ampicillin						
A. baumannii-R674	32	>128	>64	>64	>64	ND[b]
A. baumannii-R676	32	>128	>64	>64	>64	ND
A. baumannii-MTCC1425	32	>128	>64	>64	>64	ND
P. aeruginosa-R590	32	>128	>64	>64	>64	ND
P. aeruginosa-R596	64	>128	>64	>64	>64	ND
P. aeruginosa-R2477	64	>128	>64	>64	>64	ND
P. aeruginosa- R2536	64	>128	>64	>64	>64	ND
P. aeruginosa-MTCC424	8	64	≥64	≥64	≥64	ND

[a]FICI stands for fractional inhibitory concentration index. [b]ND stands for not determined.

RESULTS AND DISCUSSION

D-LANA-14 and Its Membrane Activity against Gram-Negative Superbugs. The molecule selected for the study is an optimized lead compound, D-LANA-14, identified through a structure−activity-relationship (SAR) study earlier.[26] The name D-LANA-14 stands for D-lysine conjugated aliphatic norspermidine analogue bearing tetradecanoyl chain (Figure 2A). This molecule was reported to display potent anti-MRSA efficacy, which killed both growing planktonic as well as stationary-phase MRSA by targeting their membrane.[27] More importantly, it showed minimal toxicity toward different mammalian cells (such as human red blood cells and the RAW-cell line), and no adverse effect was observed toward mice skin even at 200 mg/kg.[26,27] It also displayed potent antibiofilm efficacy toward MRSA and subsequently also revealed anti-Ebola activity.[28] In the case of Gram-negative bacteria, however, D-LANA-14 displayed a lower efficacy with slower killing kinetics, which was possibly due to the complex cell envelope structure of this class of bacteria. Nevertheless, a significant activity seen for D-LANA-14 prompted us to interrogate its efficacy to potentiate the obsolete antibiotics toward top two critical bacteria, *A. baumannii* and *P. aeruginosa*. Toward this aim, the membrane-active nature of D-LANA-14 was first examined by performing studies such as outer membrane and inner membrane permeabilization for such pathogens. The results suggested that D-LANA-14 significantly permeabilized the outer membrane of both *A. baumannii* and *P. aeruginosa* clinical isolates, as indicated by the increased fluorescence intensity upon D-LANA-14 treatment (Figure 2B). D-LANA-14 was also found to cause depolarization of cytoplasmic membrane potential as demonstrated in the Figure 2C. In order to test whether this membrane activity of D-LANA-14 could increase the accumulation of antibiotic in the bacterial cell or not, the uptake of antibiotic was investigated in the presence of D-LANA-14. Tetracycline was used as the model antibiotic in the study, whose uptake can be monitored easily by fluorescence measurement. The results suggested that D-LANA-14 indeed increased the concentration of tetracycline inside the bacterial cells, which was demonstrated by the increased fluorescence intensity of tetracycline after treatment with D-LANA-14 (Figure 2D). Taken together, the results therefore laid the foundation for the rational development of combination therapy by using D-LANA-14 and obsolete/inactive antibiotics to combat critical Gram-negative pathogens in a synergistic fashion.

***In Vitro* Antibacterial Efficacy.** The antibacterial efficacy was tested against various multidrug-resistant *A. baumannii* and *P. aeruginosa* clinical isolates, which have developed a high level of resistant against meropenem (Table S1). To compare the results, pathogenic MTCC strains (meropenem-sensitive) were also included in the study. Individual MIC values of D-LANA-14 and the antibiotics (such as tetracycline and rifampicin) were first determined, followed by evaluating the efficacy of drug combinations by the checkerboard assay. However, D-LANA-14 was found to be moderately active against all the tested bacteria (Table 1), displaying MIC in the concentration range of 32−64 μg/mL with a notable exception of 8 μg/mL against *P. aeruginosa*-MTCC424. By contrast, the antibiotic tetracycline displayed very high MIC of ≥128 μg/mL against these clinical isolates but was potent against the MTCC strains

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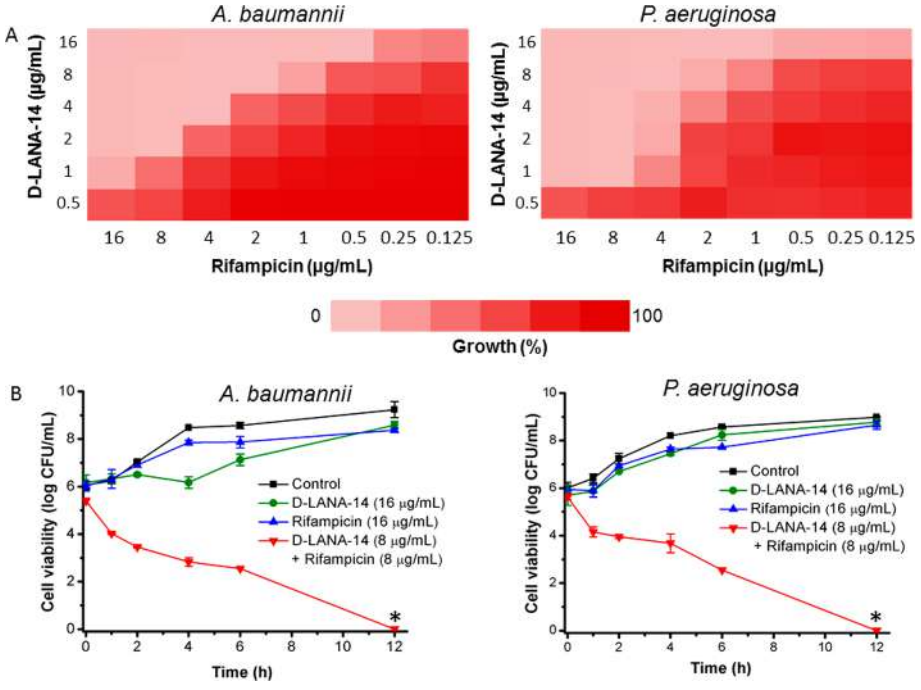

Figure 3. (A) Checkerboard assay showing synergistic activity of D-LANA-14 and rifampicin against *A. baumannii*-R674 and *P. aeruginosa*-R590; (B) Time-kill kinetics of the combined treatment against *A. baumannii*-R674 and *P. aeruginosa*-R590, the symbol (*) represents complete killing, where the detection limit of the experiment is 50 CFU/mL.

(MIC = 0.5 μg/mL, 8 μg/mL against *A. baumannaii*, *P. aeruginosa*, respectively). Rifampicin also displayed low to moderate activity (MIC in the range of 32−128 μg/mL) against these clinical isolates, which suggested that these strains were highly resistant to the drug. The MIC values against the MTCC strains were also moderate (MIC = 4 μg/mL, 32 μg/mL against *A. baumannaii*, *P. aeruginosa*, respectively), which further highlighted the nature of intrinsic resistance of Gram-negative bacteria to this antibiotic. Altogether the results therefore suggested that the individual treatment with D-LANA-14 or any of the antibiotics, tetracycline or rifampicin was ineffective in tackling the clinical isolates of *A. baumannaii* and *P. aeruginosa* studied here. Next, we evaluated the efficacy of the combination of D-LANA-14 and the antibiotics. The results demonstrated that D-LANA-14 at its sub-MICs could bring down the MIC values of tetracycline and rifampicin to a significantly lower value (Table 1 and Supporting Information Figure S1−S8). At MIC/4 of D-LANA-14, tetracycline was active at 4 μg/mL against both *A. baumannii*-R674 and *P. aeruginosa*-R590, whereas rifampicin was effective at 2 μg/mL (Figure 3A). Along with the minimum inhibitory concentration of antibiotics at various sub-MICs of D-LANA-14, the fractional inhibitory concentration index (FICI; the summation of FICs of D-LANA-14 and antibiotics) was also determined, and the results are presented in Table 1. A combination with an FICI value of ≤0.5 is considered as a synergistic combination.[20] Thus, the results clearly suggested that combination of D-LANA-14 and the antibiotics tetracycline and rifampicin exhibited a synergistic effect against a broad-spectrum of Gram-negative clinical isolates. In the case of *A. baumannii*, both tetracycline and rifampicin displayed excellent efficacy, wherein the FICI was far below than 0.5 (except for tetracycline a range of 0.56−0.75 was seen for *A. baumannii*-MTCC1425). In the case of *P. aeruginosa*, although tetracycline revealed a moderate FICI (ranged between 0.19 to <0.63 considering all the strains tested), rifampicin displayed excellent FICI values which ranged between 0.09 and 0.38 (considering for all the strains tested). Altogether, the results therefore suggested that the individual treatments of D-LANA-14, tetracycline, and rifampicin were ineffective, whereas the combination could inactivate multidrug-resistant Gram-negative bacteria effectively. Furthermore, the MIC values for ampicillin demonstrated that all tested bacteria were highly resistant to this antibiotic. It did not show activity even at the concentration of 128 μg/mL, except against *P. aeruginosa*-MTCC strain where it showed an MIC value of 64 μg/mL. Also the combination of D-LANA-14 with ampicillin revealed no efficacy, which was possibly due to enzymatic destruction being the major cause of resistance development for this particular antibiotic (Figure 1 and Table 1). This result indeed strengthens our hypothesis and thereby provides further proof that D-LANA-14 sensitizes tetracycline and rifampicin toward critical Gram-negative pathogens via its membrane-active nature.

Time-Kill Kinetics. The study was performed against both *A. baumannii* as well as *P. aeruginosa*. The results clearly suggested that individual treatment of compound or rifampicin was ineffective to prevent the bacterial growth (Figure 3B). The growth curve appeared almost similar to untreated control, even upon individual treatment of 16 μg/mL of D-LANA-14 and rifampicin. In contrast, the combined treatment at lower concentrations of D-LANA-14 and rifampicin revealed potent efficacy, which recalled the synergistic effect toward bacterial killing (Figure 3B). Within even 2 h, the combined treatment (consisting of 8 μg/mL each) could reduce the cell viability by ∼3.5 log and ∼3.2 log (with respect to untreated control) of *A. baumannii* and *P. aeruginosa*, respectively. Although the killing rate was comparatively slower after that, the complete eradication of bacterial cells was seen within 12 h.

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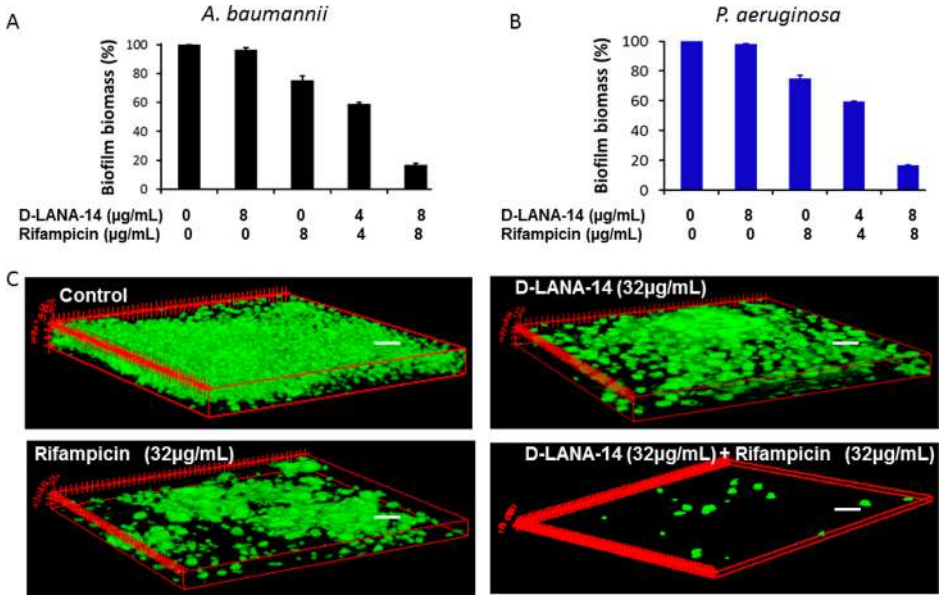

Figure 4. Quantification of biofilm disruption through crystal-violet stating (A) *A. baumannii*-R674; (B) *P. aeruginosa*-R590; (C) Visualization of biofilm disruption of *A. baumannii*-R674 through confocal laser scanning microscopy (CLSM). 3D reconstruction of z-stack images are presented in the figure, and the scale bar is 5 μm.

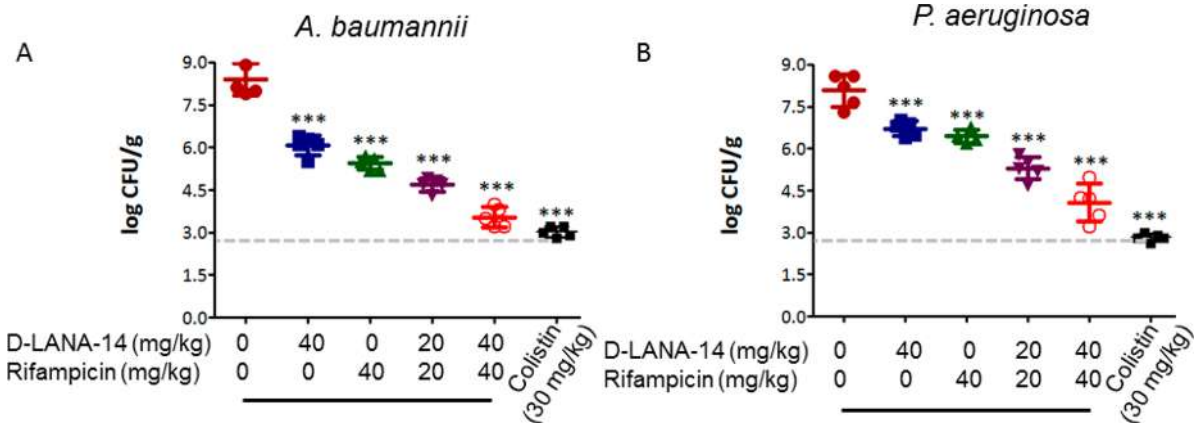

Figure 5. *In vivo* activity in mice model of burn-wound infection against (A) *A. baumannii*-R674 and (B) *P. aeruginosa*-R590. The dotted line corresponds to the detection limit of the experiment. The *P* value was calculated using one way ANOVA (Dunnett's Multiple Comparison Test) between the control group and the treatment groups of mice; the symbol (***) represents $P < 0.0001$, where a value of $P < 0.05$ is considered as significant.

Altogether, the results suggested not only a bactericidal nature of the combination but also complete killing of bacteria.

Antibiofilm Activity. The tendency of bacteria to form biofilms has been renowned as a critical challenge for the treatment of infections.[3−6] Biofilms are surface-adherent structured multicellular communities where the bacteria are present within self-produced extracellular matrix. The majority of the bacterial population is present in a slow-growing dormant state, and the extracellular matrix itself creates a barrier for antibiotics to penetrate inside the biofilms, thereby conferring protection to the entire bacterial population from antibiotics. The biofilm formation also elevates the development of antibiotic resistance through faster transfer of resistance genes among the higher bacterial cell density in the biofilms. Hence, the infections associated with biofilm formation are difficult to treat with conventional antibiotics. The scenario is even more dangerous for multidrug-resistant bacteria, where the antibiotics are inactive to tackle even growing planktonic cells. Herein, the potent activity observed for the combination against the growing planktonic cells of *A. baumanni* and *P. aeruginosa* clinical isolates prompted us to investigate the antibiofilm efficacy. Briefly, the biofilms of both the bacteria were grown on glass coverslip, followed by individual and combined treatment with D-LANA-14 and rifampicin and then the biofilm disruption was quantified by crystal-violet (CV) staining. In general, the results suggested that individual treatment was ineffective as compared to combination. The biofilm treated with 8 μg/mL of D-LANA-14 only showed almost no reduction in biofilm biomass and treatment of the same concentration of rifampicin could disrupt only about 25−30% (Figure 4A and 4B). In contrast, the combination showed higher biofilm disruption even at a lower concentration. About 45−50% reduction of biofilm biomass was seen by the formulation composed of 4 μg/mL of both D-LANA-14 and rifampicin. The combination consisting of 8 μg/mL of both the components demonstrated even better

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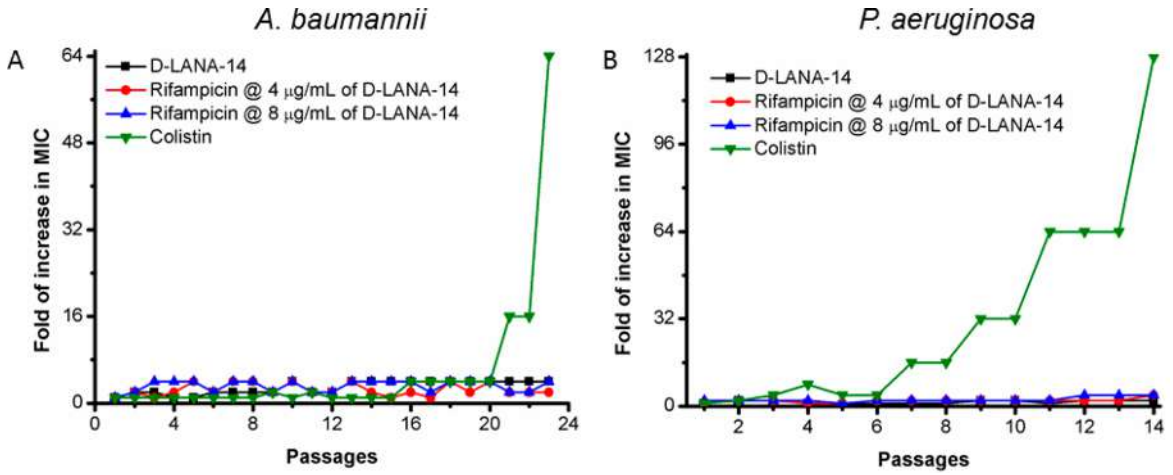

Figure 6. Propensity of resistance development against (A) *A. baumannii*-R674 and (B) *P. aeruginosa*-R590.

biofilm disruption, and >80% reduction in biofilm biomass was noticed for both *A. baumanni* and *P. aeruginosa* clinical isolates. Furthermore, the biofilm disruption was visualized through confocal laser scanning microscopy (CLSM), which also confirmed the better efficacy of the combination compared to the individual treatment (Figure 4C). Altogether, the results therefore suggested that the individual treatments of D-LANA-14 and rifampicin were less effective in treating biofilms, whereas the combination could eradicate biofilm biomass drastically.

***In Vivo* Activity.** The efficacy of the combination was evaluated in a murine model of burn-wound infections. In brief, burn wounds were created on mice and infected with ~10^6 CFU (per mice) of *A. baumanni* or *P. aeruginosa*. Then the infected wounds were left untreated for 24 h, which resulted in increased bacterial count to 10^{7-8} CFU/g. Next, the infected wounds with high bacterial burden were treated with various antibacterial agents for continuous 6 days (single dose after every 24 h). The seventh day after infection, all groups of mice were sacrificed, and the bacterial viability was determined at the wound site. The *in vivo* activity results reflected the *in vitro* antibacterial activity profile. In the case of the control group of mice (that did not receive any treatment of antibacterial agents), the bacterial burden remained high ~10^{8-9} CFU/g. The individual treatment of 40 mg/kg of D-LANA-14 (with 2.3 log and 1.3 reductions for *A. baumannii* and *P. aeruginosa*, respectively) or rifampicin (with 3.0 log and 1.6 reductions for *A. baumannii* and *P. aeruginosa*, respectively) showed lower efficacy (Figure 5A and 5B). The combination even at a lower dose of D-LANA-14 and rifampicin (20 mg/kg each) showed superior activity (with 3.7 log and 2.8 reductions for *A. baumannii* and *P. aeruginosa*, respectively). The combined treatment consisting of 40 mg/kg of D-LANA-14 and rifampicin revealed excellent activity, where a drastic reduction in bacterial burden was seen (with 4.9 log and 4.0 reductions for *A. baumannii* and *P. aeruginosa*, respectively). The antibiotic colistin also showed excellent activity that reduced the bacterial burden to the detection limit (Figure 5A and 5B). Taken together, the results suggested that the combination is highly effective as compared to individual treatment in *in vivo* scenario and thereby holds great potential for being developed as an antibacterial therapeutic agent to treat the infections caused by critical Gram-negative bacteria.

Resistance Studies. In this current state of alarm, where development of antibiotic resistance in *A. baumannii* and *P. aeruginosa* is increasing rapidly, new antibacterial agents with long-lasting antibacterial efficacy are highly essential. Thus, the resistance study of the combination was investigated against clinical isolates of *A. baumannii* and *P. aeruginosa*. D-LANA-14 and the last-resort Gram-negative antibiotic, colistin, were also included in the study. The results demonstrated that D-LANA-14 or the combination was less susceptible to resistance development; hardly a 2−4 fold increase in MIC values was observed (Figure 6A and 6B). On the contrary, both the bacteria developed high level of resistance against colistin. In case of *A. baumannii*, a 64-fold increase in the MIC value was seen after 23 sequential passages (Figure 6A). In the case of *P. aeruginosa*, the propensity of resistance development was even faster. The MIC value of colistin was found to increase rapidly by 128-fold within 14 passages (Figure 6B). Taken together, the results therefore suggested that the membrane-active nature imparted by D-LANA-14 possibly stalls the resistance development of the combined treatment. The results therefore highlight the real potential of the combination as an antibacterial agent with long-lasting activity.

CONCLUSIONS

The rampant emergence of multidrug-resistant *A. baumannii* and *P. aeruginosa* has created an enormous threat toward public health globally. To address this, herein we report the efficacy of a membrane-active molecule D-LANA-14 (D-lysine conjugated aliphatic norspermidine analogue bearing tetradecanoyl chain) to sensitize such critical pathogens toward inactive/obsolete antibiotics, rifampicin and tetracycline. The combined treatment of D-LANA-14 and the antibiotics revealed synergistic activity against the growing planktonic cells and was also able to disrupt the established biofilms of these critical pathogens. Additionally the combination displayed excellent *in vivo* efficacy. More importantly, *A. baumannii* and *P. aeruginosa* showed negligible propensity to develop resistance against the combination therapy. On the contrary, a high-level of resistance development was seen against the last resort Gram-negative antibiotic, colistin. Altogether, the results therefore suggested that this newly developed combination has immense potential for being developed as effective therapeutic agent to counter infections caused by the critical Gram-negative pathogens.

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EXPERIMENTAL SECTION

Materials and Methods. Previously synthesized D-LANA-14 has been used in the present study.[27] However, a brief synthetic protocol including the reaction scheme (Supporting Information Scheme S1), and the details of characterization are provided in the Supporting Information. The antibiotics tetracycline hydrochloride and colistin sulfate were obtained from Sigma-Aldrich. Rifampicin was purchased from Alfa-Aesar. The bacterial strains, *A. baumannii*-MTCC1425 and *P. aeruginosa*-MTCC424 were purchased from MTCC (Chandigarh, India). All the clinical isolates were collected from Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Hosur Road, Bengaluru 560029, India. Nutrient broth (NB) was used to prepare the mid log phase bacterial culture. Mueller Hinton Broth (MHB) was used as the liquid media for all the experiments, and MacConkey Agar was used as the solid media. All the culture media was obtained from HiMedia. Tecan Infinite Pro series M200 Microplate Reader was used for optical density (OD) measurement or fluorescence measurement.

Outer Membrane Permeabilization Assay.[20] The experiment was performed against two bacterial strains, *A. baumannii*-R674 and *P. aeruginosa*-R590. Briefly, the freshly grown bacterial culture (mid log phase, 6 h grown) was centrifuged (at 9000 rpm for 2 min) and then washed with 5 mM HEPES buffer and finally resuspended to $\sim 10^{7-8}$ CFU/mL in 1:1 of 5 mM HEPES buffer and 5 mM glucose. To this bacterial suspension, a working concentration of 10 μM of NPN (*N*-phenyl-1-naphthylamine) was added. Next, 190 μL of cocktail bacterial suspension was placed into the wells of black and clear-bottom 96-well plates and then incubated under dark conditions. After 45 min incubation, the fluorescence of the cocktail bacterial suspension was recorded for 4 min. The emission at 420 nm was recorded by exciting at a wavelength of 350 nm. Then, the dye containing bacterial suspension was treated with 10 μL of D-LANA-14 (8 μg/mL of working concentration), and fluorescence was recorded for another 22 min. In the negative control experiment, 10 μL of Millipore water was added instead of D-LANA-14. These experiments were performed in duplicate, and the average data was plotted in the figure.

Cytoplasmic Membrane Depolarization Assay. By following a reported experimental protocol, the study was performed for *A. baumannii*-R674 and *P. aeruginosa*-R590.[27] The details are provided in the Supporting Information.

Tetracycline Uptake Assay. The experiment was performed by following a reported protocol with a slight modification.[29] Briefly, the freshly grown culture (mid log phase, 6 h grown) of *A. baumannii*-R674 and *P. aeruginosa*-R590 was centrifuged (at 9000 rpm for 2 min) and then washed in 10 mM HEPES buffer. Lastly, the bacteria were resuspended to $\sim 10^{7-8}$ CFU/mL in 10 mM HEPES buffer supplemented with 100 μg/mL of tetracycline. Next, 190 μL of tetracycline containing bacterial suspension was placed into the wells of black and clear-bottom 96-well plates, and the fluorescence was recorded for 4 min. The emission of tetracycline was recorded at 535 nm by exciting at a wavelength of 405 nm. After that, 10 μL of D-LANA-14 (diluted in sterile Millipore water to 8 μg/mL working concentration) was added to the wells, and the fluorescence was monitored for another 22 min. In the negative control experiment, 10 μL of Millipore water was added instead of D-LANA-14. These experiments were performed in duplicate and the average data was plotted in the figure. An enhancement in the tetracycline fluorescence intensity was indicated by the increased uptake of tetracycline in the bacterial cells.

Antibacterial Assay of D-LANA-14 and Antibiotics. The reported experimental protocol was followed to determine the minimum inhibitory concentration (MIC).[26−32] The details are provided in the Supporting Information.

Antibacterial Assay of the Combined Treatment of D-LANA-14 and Antibiotics. The antibacterial efficacy of the combined treatment of D-LANA-14 and the antibiotics was determined by performing the checkerboard assay.[20,29] Briefly, solution of D-LANA-14 and the test antibiotic (25 μL of 2-fold serially diluted solution of each) was added into each well of a 96-well plate. Then 150 μL of freshly prepared bacterial suspension ($\sim 10^5$ CFU/mL) in MHB was added to the well. The plates were then incubated at 37 °C with shaking for 24 h. At the end, the OD values of the plates were measured at 600 nm. The MIC values of the antibiotics were then determined at various sub-MIC concentration of D-LANA-14. At MIC/4, MIC/8 and MIC/16 of D-LANA-14, the MIC of antibiotics were consider as the lowest concentration where the OD was lower than 0.1. The experiment was performed twice and the results were expressed as the concentration range obtained in the experiments.

Time-Kill Kinetic against Growing Planktonic Bacteria.[27,30] To investigate the bactericidal activity of the combined treatment, the time-kill kinetic study was performed against growing planktonic *A. baumannii*-R674 and *P. aeruginosa*-R590. Briefly, the freshly prepared mid log phase ($\sim 10^6$ CFU/mL) of bacterial culture in MHB was treated with 16 μg/mL of D-LANA-14 and rifampicin and physical mixture of D-LANA-14 and rifampicin (8 μg/mL each). As a negative control, the same volume of Millipore water was added instead of any antibacterial agents. The treated samples were then incubated at 37 °C with shaking and bacterial cell viability was assayed at different time points such as 0, 1, 2, 4, 6, and 12 h. The aliquots were 10-fold serially diluted in saline, and 20 μL of each dilution was spot plated on MacConkey agar plate. The plates were then incubated at 37 °C, and bacterial colonies were counted after 24 h. The average results are presented in logarithmic scale with standard deviation for each data point, and the detection limit for this experiment was 50 CFU/mL.

Antibiofilm Assays.[33−35] The antibiofilm efficacy was assayed by both quantification through crystal-violet (CV) staining and visualization through confocal laser scanning microscopy (CLSM). Briefly, the glass coverslips (18 mm of diameter) were sterilized by soaking them in ethanol followed by drying them in the flame. These coverslips were then placed into the wells of 6-well plate, allowing them to cool to room temperature. After that, 2 mL of mid log phase bacterial suspension ($\sim 10^5$ CFU/mL suspended in suitable biofilm forming media) was added to the coverslip containing wells. While *A. baumannii*-R674 suspension was prepared in BM2 media (supplemented with 0.5% glucose, 0.5% casamino acids and 200 μM $FeCl_3$), *P. aeruginosa*-R590 was suspended in nutrient broth (supplemented with 1% glucose and 1% NaCl). The plate was incubated at 30−33 °C under stationary condition to allow the biofilm formation on the coverslips. After 48 h of incubation, the biofilms containing coverslips were washed by using 1× PBS and placed into the wells of 6-well plate containing 2 mL of various antibacterial agents in

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complete biofilm media. In the case of the negative control experiment, 2 mL of medium was added instead of any antibacterial agent. Postincubation of 24 h, the treated coverslips were washed by using 1× PBS and dried. It was then stained with 0.1% (2 mL of 0.1% crystal-violet solution (prepared in sterile Millipore water) and placed into the wells of fresh 6-well plate after washing with 1XPBS. The CV-stained disrupted biofilm was then dissolved in 2 mL of 95% ethanol in water and the OD of the solution was recorded at 520 nm. The experiment was performed in duplicate, and the average percentage of biofilm biomass was calculated by considering 100% for the negative control. To visualize the extent of biofilm disruption, the experiment was performed by following the same protocol described above. The only difference was that instead of crystal-violet staining, the disrupted biofilms were stained with the dye syto-9, and images were captured by using confocal laser scanning microscopy (CLSM).

Mouse Model of Burn-Wound Infection.[34,35] At first, 6−8 weeks-old female BALB/c mice were randomly distributed in groups of five mice in each. The mice were then injected with xylazine-ketamine cocktail intraperitoneally. Once they were anesthetized, the fur on the back was removed by using a trimmer followed by shaving with a razor. After that, a burn wound was created in the shaved area by applying a heated brass bar. The wound was then infected topically with freshly prepared bacterial suspension (~10^6 CFU, 20 μL of 10^8 CFU/mL, of *A. baumannii*-R674 or *P. aeruginosa*-R590 per mice) in sterile saline. Twenty-four h postinfection, the treatment was started with various antibacterial agents (such as, 40 mg/kg of D-LANA-14 or rifampicin, 30 mg/kg of colistin, combination of D-LANA-14 and rifampicin at 20 mg/kg and 40 mg/kg each). 40 μL of the solution of antibacterial agents was applied topically, which were first prepared in sterile Millipore water by considering 20 g as the average body weight of mice. The infected groups of mice were treated with 6 doses at 24 h intervals of the respective antibacterial agents. As a negative control, one group of mice was treated with Millipore water instead of any antibacterial agents. On day 7 (24 h after the last dose of compound treatment), the mice were sacrificed and the skin-tissue (ranged in 0.1−0.2 g) from infected part was collected and transferred into 10 mL of sterile saline. It was then homogenized and the suspension was 10-fold serially diluted in sterile saline. Each dilution was spot plated on MacConkey agar and the plates were incubated for 24 h. The viable bacterial colonies were counted; the results were analyzed and expressed as log (CFU/g) of weight of the tissue collected.

Resistance Studies.[27,29] The propensity of resistance development was investigated for both D-LANA-14 and the combined treatment of D-LANA-14 and rifampicin. In the case of the combined treatment, the MIC value of rifampicin was determined, where the concentration of D-LANA-14 was kept constant. To compare the result, the last resort Gram-negative antibiotic, colistin, was included in the study. By following above protocol described in antibacterial assay, the MIC values of D-LANA-14, colistin and rifampin (in the presence of 4 and 8 μg/mL of D-LANA-14) were determined against *A. baumannii*-R674 and *P. aeruginosa*-R590. The next MIC assay was performed, where the bacterial suspension was then prepared from the bacteria grown at the half-MIC of compound concentration of the first day experiment. By following the same protocol, 23 and 14 subsequent passages were repeated for *A. baumannii*-R674 and *P. aeruginosa*-R590, respectively. To compare the propensity of resistance development, the fold of MIC increased was then plotted against the passages number. The MIC values were calculated by visual observation of the OD values obtained from the triplicate experiment. The fold of increase in the MIC was determined by dividing first MIC value (corresponds to zero passage) from MIC values of every passages.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.9b00221.

Details of synthesis of D-LANA-14, ethics statement, experimental protocol for cytoplasmic membrane depolarization assay and antibacterial assay. Table S1: activity of Meropenem against different *A. baumannii* and *P. aeruginosa*. Figure S1−S8: representation of checkerboard assay showing synergistic activity against different *A. baumannii* and *P. aeruginosa* (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jayanta@jncasr.ac.in. Telephone: +91-80-2208-2565. Fax: +91-80-2208-2627.

ORCID

Jayanta Haldar: 0000-0002-8068-1015

Notes

The authors declare no competing financial interest.

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