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Aryl-alkyl-lysines: Membrane-Active Small Molecules Active against Murine Model of Burn Infection

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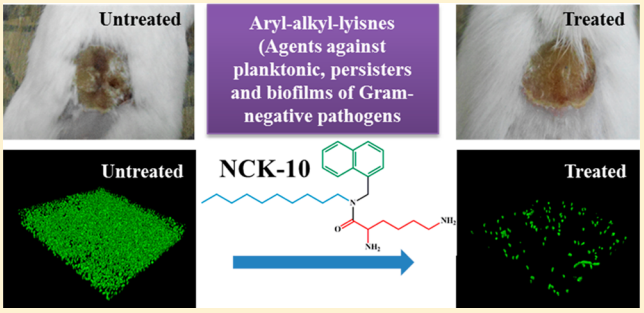
Supporting Information

ABSTRACT: Infections caused by drug-resistant Gram-negative pathogens continue to be significant contributors to human morbidity. The recent advent of New Delhi metallo-β-lactamase-1 (*bla*NDM-1) producing pathogens, against which few drugs remain active, has aggravated the problem even further. This paper shows that aryl-alkyl-lysines, membrane-active small molecules, are effective in treating infections caused by Gram-negative pathogens. One of the compounds of the study was effective in killing planktonic cells as well as dispersing biofilms of Gram-negative pathogens. The compound was extremely effective in disrupting preformed biofilms and did not select resistant bacteria in multiple passages. The compound retained activity in different physiological conditions and did not induce any toxic effect in female Balb/c mice until concentrations of 17.5 mg/kg. In a murine model of *Acinetobacter baumannii* burn infection, the compound was able to bring the bacterial burden down significantly upon topical application for 7 days.



KEYWORDS: *antimicrobial peptides, antibiotics, antimicrobial resistance, Gram-negative, persisters, biofilm, burn infection*

The emergence of drug resistance in bacteria has directed substantial research efforts, in various approaches, toward the identification and development of novel antimicrobial agents.[1] More importantly, it has prompted several important health agencies to initiate programs to expedite antimicrobial drug development. Notable among those are "the '10' × '20' initiative" and ReAct (Action on Antibiotic Resistance).[2] However, despite such efforts, only three antibiotics with novel mechanisms of action have been introduced in the clinics since 2000.[3]

Reports of resistance of New Delhi-metallo-β-lactamase-carrying bacteria to colistin and tigecycline, the drugs of last resort, have set alarm bells ringing in every health agency.[4,5] Substantial efforts have been put toward the development of compounds against NDM-1-expressing pathogens,[6−8,9] but because the number of active compounds is limited, there is a great need to develop antibacterial agents against such bacteria.

Bacterial infection consists of several complications, one of them being the presence of dormant nondividing cells.[10] Most of the antibiotics require bacteria to be in a metabolically active state to carry out their functions and consequently require a higher concentration to clear chronic infections.[11] More importantly, during antibiotic treatment, some of the cells can down-regulate their metabolic processes to escape antibiotic treatment; these cells can also revert to being normal after antibiotic stress is removed.[12] These cells, called persister cells, are responsible for severe chronic infections that are very difficult to cure. Thus, it is extremely important to find compounds that are active against such persister cells.

Natural membrane active agents such as antimicrobial peptides (AMPs) and lipopeptides have received significant attention in recent years. Some of them are already used in clinics, and some are under development as potential drugs.[13−15] Several synthetic membrane-active agents have also been developed, as an alternative to natural host defense peptides, primarily to circumvent the problems of in vivo toxicity, protease instability, and large cost of manufacture.[16] These design strategies include α-peptides,[17] β-peptides,[18,19] oligoacyl lysines,[20] oligoureas,[21] α-AApeptides,[22] arylamide foldamers,[23] antimicrobial polymers,[24−30] cationic amphi-

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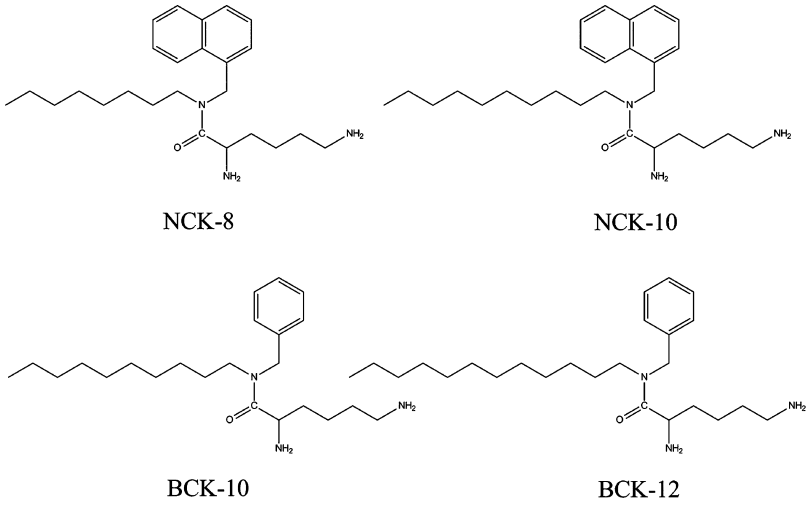

Figure 1. Chemical structures of compounds used in the study.

philes,[31,32] $\beta^{2,2}$-amino acid derivatives[33] and those based on aryl scaffolds.[34,35] In fact, drugs such as vancomycin have also been successfully modified to bring about membrane-active properties in addition to their primary mechanism of action to overcome the problems of drug resistance.[36−38]

Recently, we developed a series of small molecular membrane-active antimicrobial compounds with selective broad-spectrum activity.[39] These compounds were synthetically very simple and represent a compact ensemble of an aromatic core, an L-lysine moiety, and a variable lipophilic chain (the molecular structures are represented in Figure 1). Herein we report the activity of these compounds against clinical isolates of NDM-1-producing Gram-negative pathogens, activity against persister cells of *Escherichia coli*, and their ability to disrupt biofilms formed by *E. coli* and other Gram-negative pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. We have also studied the propensity of bacteria to develop resistance against such compounds. The ability to perturb and infiltrate the cell membrane of planktonic and persister cells was further investigated. Furthermore, to understand the optimal conditions that enhance the potency of these molecules and to gauge the possible applications of these compounds, we have studied their activity in different physiological conditions such as serum and whole blood, as well as various pH and salt concentrations. The toxicity of the compounds was studied in mice by monitoring survival after dosing with different concentrations. The in vivo potency of the compound was studied in a murine model of burn infection caused by *A. baumannii*.

RESULTS AND DISCUSSION

Synthesis. Compounds have been synthesized and characterized following a previously reported protocol.[39] For simplicity we have named the molecules containing a naphthalene core as NCK-8 (with an octyl chain appendage) and NCK-10 (with a decyl chain appendage). Similarly, the compounds in the benzene series are BCK-10 and BCK-12 (Figure 1). In this particular study we have performed the biological assays with four compounds: two from the NCK series (NCK-8 and NCK-10) and two from the BCK series (BCK-10 and BCK-12). The structures of the compounds are provided in Figure 1.

Antibacterial Activity. The compounds displayed potency against all of the Gram-negative pathogens tested (Table 1).

Table 1. Activity of Compounds against Gram-Negative Pathogens (Including Clinical Isolates)

	minimum inhibitory concentration (μg/mL)			
bacterial strain	NCK-8	NCK-10	BCK-10	BCK-12
K. pneumoniae ATCC 700603	21	9	>50	4.5
K. pneumoniae R3421	24	4.5	25	5
A. baumannii MTCC 1425	20	9	21	9
A. baumannii R674	11	1.5	12	1.5
A. baumannii R676	10	2.3	10	4
P. aeruginosa MTCC 424	8.5	4.5	6.5	6.3
P. aeruginosa R590	48	11	25	11
P. aeruginosa R3324	24	11	12	11
E. coli MTCC 443	8	6	11	8
E. coli R250	5	1.5	5	1.5
E. coli (colistin resistant)	10.4	4.5	10	2.4

Both NCK-10 and BCK-12 were active against the ATCC strain of *K. pneumoniae* at minimum inhibitory concentrations (MICs) of 9 and 4.5 μM, respectively. Against the clinical isolate R3421, however, both NCK-10 and BCK-12 were equally potent with MICs of 4.5 and 5 μM, respectively. NCK-8 and BCK-10 were less active against *K. pneumoniae* R3421. The activity of NCK-10 and BCK-12 against *A. baumannii* was a highlight of these compounds. Against a clinical isolate, R674, both compounds displayed a superior MIC of 1.5 μM. Again, both NCK-8 and BCK-10 were 7-fold less active than their higher long-chain homologues. Against the MTCC strain too, the compounds displayed very good activity with NCK-10 and BCK-12 displaying an activity of 9 μM. Because the activity of these sets of compounds toward a laboratory strain of *P. aeruginosa* (MTCC 424) was very good (MIC ranged from 4.5 to 8.5 μM), two clinical isolates were selected for the study. Both strains of *P. aeruginosa* were MDR strains, and the compounds displayed very good activity against them. Again, NCK-10 and BCK-12 turned out to be the most potent compounds. *P. aeruginosa* R590 was a little less susceptible to the compounds; both NCK-10 and BCK-12 were active only at 11 μM. Although BCK-10 displayed a MIC of 25 μM against

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R590, NCK-8 was active only at 48 μM. Against the laboratory strain of *E. coli* (MTCC), the activity of the compounds varied from 6 to 11 μM. However, against the clinical isolate *E. coli* R250, both NCK-10 and BCK-12 displayed a superior activity of 1.5 μM. The compounds displayed good potency against a colistin-resistant strain of *E. coli* as well (MICs ranged from 2.4 to 10.4 μM). Due to the excellent efficiency displayed by these compounds toward inhibiting the growth of MDR clinical isolates, we were motivated to study their activity against New Delhi-metallo-β-lactamase 1-producing Gram-negative bacteria.

Carbapenem-resistant New Delhi-metallo-β lactamase was isolated from patients of the National Institute of Mental Health and Neurosciences, Bangalore. Characterization of the bla_{NDM-1} gene (475 bp) was carried out using PCR and gel electrophoresis (Supp. Figure 1).[9] In this experiment, *K. pneumoniae* (ATCC-BAA-2146) was used as a positive control, whereas *E. coli* (ATCC-25922) was used as negative control. The presence of the bla_{NDM-1} gene was confirmed in *K. pneumoniae* R3934, *E. coli* R3336, *P. aeruginosa* R596, and *E. clocae* R2928 by gel electrophoresis as shown in Supp. Figure 1. MICs of meropenem against these strains were >50 μg/mL.

The activity of these compounds was evaluated against carbapenem-resistant bla_{NDM-1} containing Gram-negative pathogens (Table 2). All of the compounds studied were

Table 2. Activity of Compounds against NDM-1-Producing Gram-Negative Pathogens

	minimum inhibitory concentration (μg/mL)			
compound	*E. coli* R3336	*K. pneumoniae* R3934	*P. aeruginosa* R596	*E. cloacae* R2928
NCK-8	20	20	20	10
NCK-10	4.5	4.5	4.5	4.5
BCK-10	20	20	10	10
BCK-12	4.7	4.7	4.7	4.7
meropenem	>50	>50	nd[a]	>50
tetracycline	>50	>50	nd	>50

[a]nd, not determined.

active against this group of bacteria at low concentrations. Against *E. coli* R3336, NCK-8 and BCK-10 were active at 20 μM, and both NCK-10 and BCK-12 were 4-fold more active with MICs of 4.5 μM each. A similar activity profile was observed against *K. pneumoniae*; NCK-10 and BCK-12 were around 4-fold more active than NCK-8 and BCK-10. The compounds were found to be very active against *P. aeruginosa* R596 with NCK-10 and BCK-12 displaying comparable activities (MIC 4.5 μM). Against *E. cloacae*, too, NCK-10 and BCK-12 were equally active (MICs of around 4.5 μM), whereas the activities of the other two compounds were 2-fold less (MIC of 10 μM).

Bactericidal Time–Kill Kinetics. To obtain a deeper understanding of the nature of the activity of these compounds against bla_{NDM-1}-containing pathogens, the kinetics of bactericidal activity of NCK-10, the most active compound, was studied (Figure 2). To do the experiment, we chose to treat the bacteria with three concentrations of the compounds: MIC/2, MIC, and 2× MIC. As expected, at MIC/2, no activity was observed. However, a >3 log reduction in colony-forming units (CFU) was observed within the first 30 min upon treatment of compound even at its MIC.

Activity against Stationary Phase Bacteria and Persister Cells. As mentioned earlier, most antibiotics require

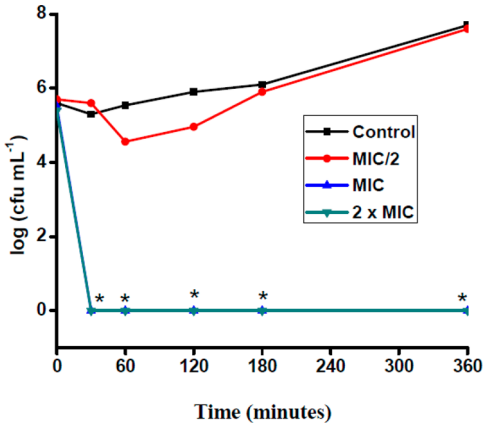

Figure 2. In vitro time–kill kinetics of different concentrations of NCK-10 against *E. coli* R3336 containing the bla_{NDM-1} gene. The detection limit for the experiment is (<50 CFU/mL or 1.69 log). * indicates that no colony was observed.

very high concentrations to have sufficient activity against stationary phase bacteria.[11] NCK-10 when treated against *E. coli* cells in stationary phase showed complete killing even at its MIC. Whereas in the control, the bacterial cell count was maintained at 5 log CFU, no colonies were observed in NCK-10-treated cultures (the limit of detection in this experiment is 50 CFU/mL).

Persister cells of *E. coli* are notorious in evading antibiotics in the case of chronic infections. Thus, there is a huge demand for compounds that act against such cells. NCK-10, at its MIC, was sufficient to completely lyse persister cells of *E. coli* in 2 h. Again, while the untreated control maintained a steady count of cells, no visible colony was observed in persister cells treated with the compound.

Ability To Disrupt Biofilms Formed by Gram-Negative Bacteria. Two experiments were performed to evaluate the ability of NCK-10 to disrupt preformed biofilms of Gram-negative bacteria. In the first experiment, against a representative strain of all Gram-negative bacteria reported, we have evaluated the effective concentration of the compound at which 50% of preformed biofilms (formed on 96-well plates and stained with crystal violet) are dispersed (EC_{50}). NCK-10 was the most active compound against all of the bacteria tested. Specifically, NCK-10 displayed EC_{50} values of 30, 20, 26, and 19 μM against *A. baumannii* (MTCC 1425), *E. coli* (MTCC 443), *K. pneumoniae* (ATCC 700603), and *P. aeruginosa* (MTCC 424), respectively (Table S1). The activity of the other compounds was not as good as that of NCK-10. Furthermore, to visualize the anti-biofilm effect of the compound, confocal microscopic images of untreated and NCK-10 treated (at EC_{50}) biofilms of *E. coli* (formed on glass coverslips and stained with SYTO-9) were taken. The images are shown in Figure 3). The images clearly show that in the untreated case a thick layer (12.6 μm) of *E. coli* biofilm was formed, whereas upon treatment with the compound, hardly any cells were left and the biofilm was completely disrupted. The thickness of the biofilm treated with the compound was 3.5 μm.

Propensity To Induce Bacterial Resistance. As mentioned earlier, the current crisis in antibiotic drug development is the rapid rate at which bacteria develop resistance toward drugs. Because in this paper we have explored the possibility of these compounds as antibacterial agents toward treatment against MDR clinical isolates of pathogens, it was imperative to

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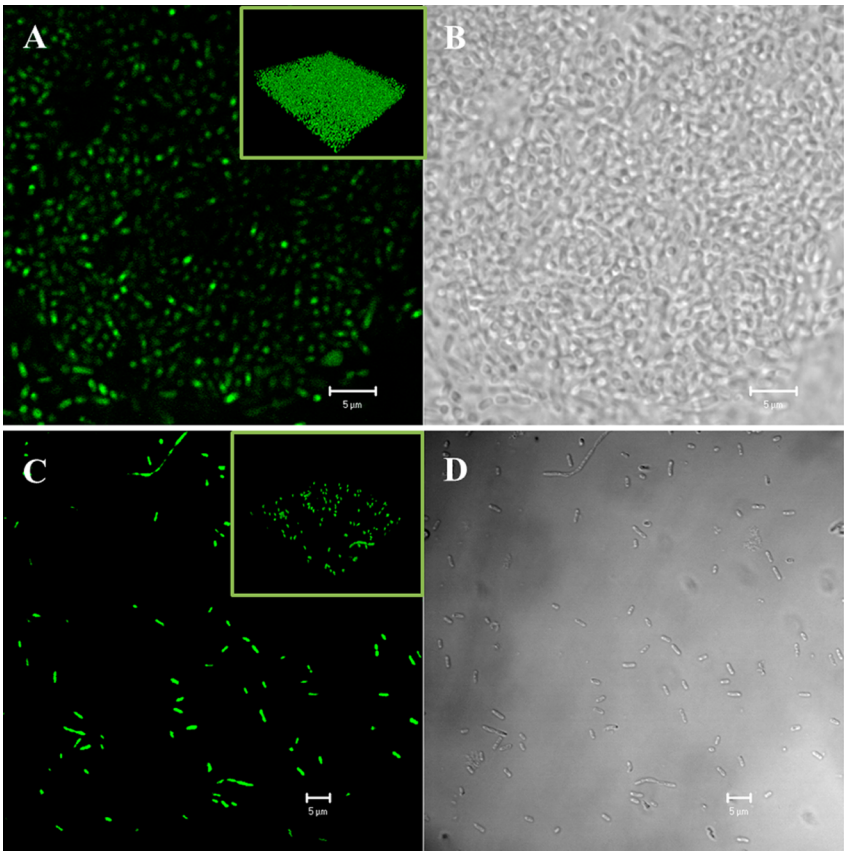

Figure 3. Confocal microscopic images of *E. coli* biofilms: (A) fluorescent images of untreated control ((inset) three-dimensional view of the biofilm); (B) bright-field images of untreated control; (C) fluorescent images of NCK-10-treated samples ((inset) three-dimensional view of the biofilm); (D) bright-field images of NCK-10-treated samples. NCK-10 was used at its EC_{50} value.

study if the compounds themselves possess any propensity to induce bacterial resistance. Thus, to evaluate the potential of these compounds as long-lasting antibacterial agents, the ability of *E. coli* to develop resistance against these compounds was studied (Figure 4). NCK-10, the most active compound, was chosen as a model compound for this study. As a positive control for *E. coli*, colistin was used. The MIC of colistin increased on the fourth day of the experiment. The MIC of NCK-10 toward *E. coli* did not change even after 20 passages, respectively, whereas the MIC of colistin increased by 250-fold.

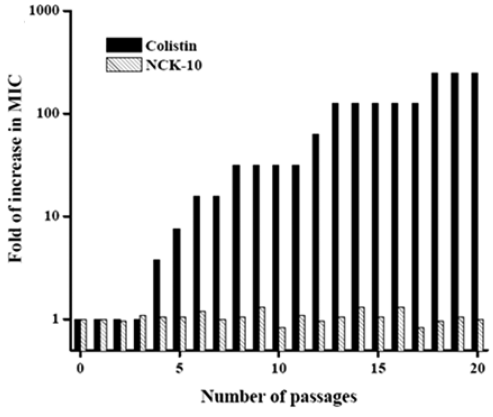

Figure 4. Propensity to induce resistance development in bacteria. Fold of increase in MICs of colistin and NCK-10 against *E. coli*.

Frequency of Resistance Study. To generate mutants resistant to either NCK-10 or colistin, *E. coli* (MTCC 443), at different concentrations ranging from 10^9 to 10^5 CFU/mL, was plated on nutrient agar containing 8× MIC of either colistin or NCK-10. After incubation for 24 h, no mutants were observed on NCK-10 containing plates even at the highest concentration of bacteria (10^8 cells on the plate) proving that frequency of resistance against this compound was $>10^{-8}$. However, the frequency of resistance to colistin was 1.9×10^{-5}. This proved that bacteria were unable to develop resistance against these compounds easily, and it was safe to assume that the longevity of such compounds was greater than that of the clinically used drug colistin.

Activity at Different pH Values. We further studied the effect of different environmental conditions on the antibacterial activity of NCK-10 toward *E. coli*. In the first study, the effect of pH on the antibacterial effect of NCK-10 was determined to understand if the compounds lose activity at any particular pH (Table S1). To do the experiment, first, bacterial growth media at different pH values (3.5, 4.5, 5.5, 6.5, 7.4 and 8.5) were made. Second, compound dilutions were added to the media (of a particular pH) containing bacteria to check the effect of pH on the antibacterial activity of the compounds. The activity was initially determined at pH 7.4, and the effect at other pH values was considered with respect to that.

No bacterial growth was observed at pH 3.5; thus, the activity of the compound at that pH could not be determined. However, at pH 4.5 and 5.5 the MIC of the compound against *E. coli* was 10 μM, whereas at pH 6.5 a 2-fold greater potency

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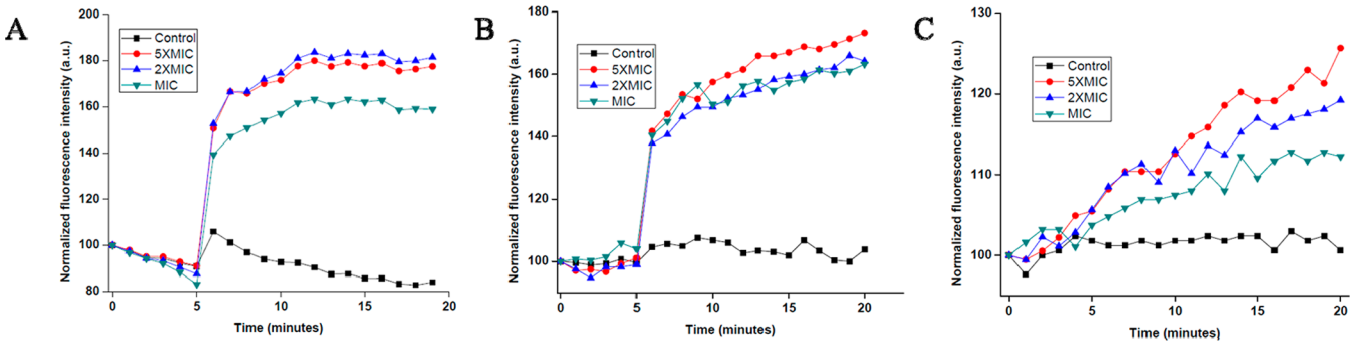

Figure 5. Mechanism of action against persister cells: (A) depolarization of the membranes of *E. coli* persister cells; (B) outer membrane permeabilization of *E. coli* persister cells; (C) inner membrane permeabilization of *E. coli* persister cells.

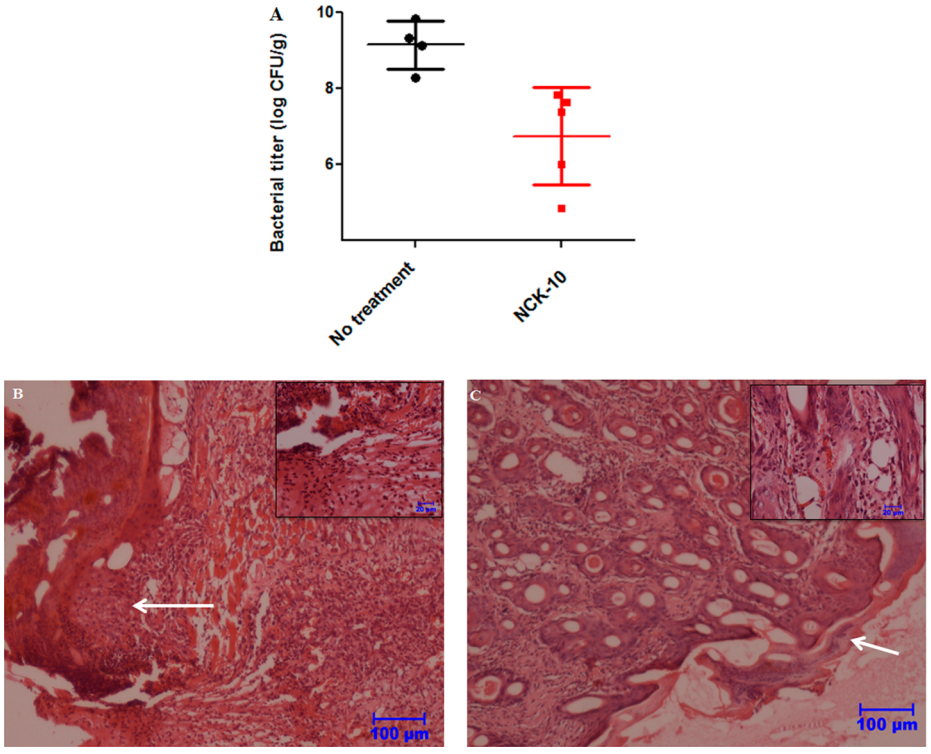

Figure 6. *A. baumannii* burn model of infection: (A) NCK-10 was able to reduce bacterial burden significantly (P value = 0.01) in comparison to control; skin histopathology of (B) tissue section of untreated mice ((inset) magnified picture of a part of the section); (C) tissue section of mice treated with NCK-10 ((inset) magnified picture of a part of the section).

was observed against *E. coli*. At an alkaline pH of 8.5, the compound retained its activity against *E. coli* (MIC of 5 μM).

Activity at Different Salinity. To check if the compounds lose activity in higher salinity, we studied the antibacterial effect of NCK-10 in different percentages of NaCl (0.5, 1, 1.5, and 3%). This experiment would also give us an idea of the nature of the compounds and possible clinical application. The activity at different concentrations of NaCl is furnished in Table S2. Against *E. coli* the activity of the compound was reduced by 2-fold at the highest concentration of 3% NaCl. Antimicrobial peptides are known to lose their activity in the presence of divalent cations such as Ca^{2+} and Mg^{2+}.[40] Thus, we studied the antibacterial effect of the NCK-10 in different concentrations of $MgCl_2$ (Table S1). The concentration of $MgCl_2$ was varied from 20 to 80 mM. Even at 20 mM the activity of the compound dropped to 19 μM in the case of *E. coli*, whereas at higher concentrations the compound was not active until 50 μM.

Activity in the Presence of Serum. Activity in serum was checked by incubating NCK-10 with serum for 3 h and then checking its MIC against *E. coli* in minimum essential media (MEM). NCK-10 retained its activity in 50% serum (MIC of 2.5 μM). In fact, all of the other compounds also retained their activity in the presence of serum, for example, NCK-8, BCK-10, and BCK-12 were all active at 5, 2.7, and 2.5 μM, respectively. The values obtained for MIC when performed in MEM (without serum) were also the same.

Activity in Whole Blood. Due to the presence of various proteins and other ions, compounds might lose activity in whole blood. It was thus imperative to understand the nature of the activity displayed by such compounds in whole blood. NCK-10 at concentrations of 5 and 10 times its MIC, when incubated in whole blood containing bacteria, was able to bring the bacterial count down by >3 log in 3 h against *E. coli* (Supp. Figure 2). The starting concentration of bacteria was 7.1 log (CFU/mL), and in 3 h it increased to a value of 8.3 log (CFU/

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mL) in the control case. However, when treated with compound, the bacterial count was brought down by 3.4 log (CFU/mL) and 4.4 log (CFU/mL) in 5× and 10× MIC, respectively. The activity of the compound was also checked at its MIC and 2× MIC. No activity was observed at its MIC, and only 1 log reduction was obtained at 2× MIC (data not shown).

Mechanism of Action. *NDM-1 Positive E. coli.* To understand how these compounds acted on multidrug-resistant pathogens, mechanistic studies were performed on *E. coli* R3336 containing bla_{NDM-1} with the most active compound NCK-10. Treatment with NCK-10 caused depolarization of the bacterial membrane within a minute after addition (Supp. Figure 3A). NCK-10 was also able to permeabilize the outer membrane of the bacteria (Supp. Figure 3B); however, the inner membrane of the bacterial cell remained intact (Supp. Figure 3C).

Persister Cells. Membrane-active agents are hypothesized to retain a similar mechanism of action against persister cells.[12] Herein, we studied the ability of NCK-10 to depolarize and permeabilize the membranes of *E. coli* persister cells. The concentrations chosen to do the experiment were the MIC, 2× MIC, and 5× MIC. The compound was able to rapidly depolarize the membranes of *E. coli* persister cells (Figure 5A). The compound was able to infiltrate the outer membrane of *E. coli* as well as the inner membrane (Figure 5, panels B and C, respectively). However, the ability to permeabilize the inner membrane was greatly diminished at lower concentrations.

In Vivo Toxicity Studies. To gauge the toxicity of the compounds in mice, we sought to determine the LD_{50} of the compound. LD_{50} determination of the compound was achieved by injection of different concentrations of the drug, intravenously, into mice via the tail vein. Compound NCK-10 was injected at concentrations of 55, 17.5, and 5.5 mg/kg, and survival of the mice was monitored for 14 days. Mice injected with 55 mg/kg succumbed to death within 5 min of treatment. The mice could tolerate 17.5 and 5.5 mg/kg of the drug with no visible toxicity. We followed the Spearman−Karber method to determine the LD_{50} of the compound. The LD_{50} of the compound was determined to be 30 mg/kg.

In Vivo Infection Studies. Infections caused by *A. baumannii* are a growing problem with growing reports of resistance against even drugs of last resort, such as colistin.[41] A serious problem associated with burns is infection caused by *A. baumannii*.[42] We thus chose to address this serious problem by checking the activity of our compounds against a murine model of burn infection caused by *A. baumannii*. To do the experiments, a burn infection was induced on the skin at the back of the mice. Because the application of the compound was topical, we chose to treat the wound at a concentration of 40 mg/kg, slightly higher than the LD_{50} (the LD_{50} was determined by intravenous injection) of the compound, and colistin was used as a positive control (concentration used was 5 mg/kg). After treatment with the compound for 7 days (once daily), the bacterial burden was brought down significantly in comparison to control (Figure 6A). Colistin, on the other hand, was extremely effective; no colony was observed within the detection limit (data not shown).

Skin Histopathology. As can be seen from Figure 6B, the burn wound area of the tissue sections of untreated mice were infested with bacterial cells with proteinaceous exudates (arrow). Severe infiltration of inflammatory cells, mainly neutrophils, was observed, which appeared to be degenerating and attached to the exudates (inset). Loss of squamous epithelial cell layer, sweat and sebaceous glands, and hair follicles along with a change in the architecture of skin tissue was also observed. Treatment with compound NCK-10 showed regeneration of stratified squamous epithelial cells along with keratin and proliferation of a fibrous tissue layer over the burn wound (arrow). Infiltration of neutrophils and congestion of blood vessels in the subepithelial layer (inset) were also observed (Figure 6B).

DISCUSSION

Previously we had reported the structure−activity relationship of small molecular aryl-alkyl-lysines.[39] Because these compounds were shown to act primarily by infiltrating the bacterial membrane permeability barrier, it was rationalized that they should be active against every class of bacteria, even those which express proteins to promote resistance to antibiotics. Selectivity of these compounds had already been established in a previous study, so our aim in this work was to exploit the range of antimicrobial potential of these compounds against Gram-negative bacteria, including those that produce NDM-1.

In the current scenario, the development of drugs against extended-spectrum β-lactamase (ESBL) producing Gram-negative species such as *K. pneumonia* and *E. coli* strains has become crucial. Carbapenems were usually effective against such ESBL-producing strains. However, lately, even carbapenem-hydrolyzing enzymes are being expressed by such bacteria.[43] The emergence of new metallo-β-lactamases such as NDM-1 has made the use of antibiotics such as penicillin, cephalosporin, and carbapenems irrelevant. Reports of bacteria developing resistance to the drugs of last resort such as colistin and tigecycline have also been published.[4,5] Once these drugs are rendered useless, the situation will be reminiscent of the preantibiotic era. Hence, any compounds that show suitable activity against such bacteria bear promise as clinically relevant antibacterial agents.

NCK-10 and BCK-12 were the most active compounds against all of the Gram-negative bacteria tested including those producing NDM-1. However, we have chosen NCK-10 for this study because of its lower toxicity. The activity of NCK-10 was around 10−15 times more than that of antibiotics such as meropenem and tetracycline (Table 2). The rapid membrane activity observed for NCK-10 (Figure 5) was reflected in its time−kill kinetics (Figure 2). NCK-10 was able to inactivate NDM-1-producing *E. coli* within 30 min at its MIC. A remarkable attribute of this compound was its inability to select a resistant mutant not only in multiple passages but also in a densely populated bacterial culture. Colistin, a drug of last resort, on the other hand, selects resistant mutants at a frequency of 1.9×10^{-5}. This brings us to a very important point of argument. Because these compounds cause infiltration of bacterial membrane, immediate lysis occurs and, consequently, bacteria find it difficult to develop resistance. This approach rules out the need to make compounds that target the enzymes responsible for promoting resistance. This strategy of targeting the membrane thus seems to be the most effective in terms of both activity and longevity in antimicrobial therapy.

Biofilm-related infections are a bigger source of worry than infections caused by planktonic cells. Bacterial cells survive within the biofilm at stationary phases and can withstand severe stress to survive for longer periods of time.[12] Higher concentrations of antibiotics are required to eradicate stationary phase bacterial cells, which makes such infections extremely

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difficult to handle. The ability of NCK-10 to kill stationary phase and persister cells of *E. coli* raises the importance of such membrane-active agents against biofilm-related infections. The ability of the compound to act on persister cells prompted us to check their ability of disrupt biofilms formed by different Gram-negative pathogens. Our hypothesis was proved correct as the compound was very efficient in dispersing 3-day-old biofilms of *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*.

Several studies in the literature have reported activity against persister cells but few have probed into the mechanism of action.[44−49] The ability of the compounds to depolarize the *E. coli* persister cells and act on their outer membrane was expected. Interestingly, previous investigations into inner membrane permeabilization of metabolically active *E. coli* yielded negative results for such compounds.[39] This was also observed in the case of *E. coli* NDM-1 species (Supp. Figure 3C). This underscores the difference between metabolically active cells and persister cells; additionally, the importance of such membrane-active agents against persister cells is also established.

Another important point that has to be considered in detail in the development of new antimicrobial agents is their activity in different physiological conditions such as pH, salinity, and whole blood. Membrane-active agents can have a variety of applications as antibacterial agents: as topical antibiotics for skin infections,[50,51] as antibiotics for systemic infections,[52] as food preservatives,[53] and so on. Although all of the applications mentioned above are equally important, the compound's performance in different physiological conditions determines its ultimate use.

The pH of the site of infection varies from place to place inside the body. Infections of the vagina are also regulated by changes in pH.[54] In cystic fibrosis, too, it is believed that low pH reduces the antimicrobial activity of airway surface liquid.[55] Thus, it is important to understand the antimicrobial activity of the test compound at different pH conditions. NCK-10 showed sufficient activity in pH ranging from 4.5 to 8.5. Although activity at the lowest pH is slightly less, it is good enough for clinical settings. The reason for losing activity is not clear, but it can be assumed that in completely protonated form the overall conformation of the molecule changes, resulting in a slight loss in activity. The balance of NaCl is critical in different aspects of antimicrobial treatment. Osmotic stress is known to increase capsule formation in *E. coli*, thereby inducing antibiotic resistance in the infecting strain.[56] Many natural membrane-active agents such as the antimicrobial peptide human-β-defensin are inactivated by high concentrations of NaCl.[57] Dicationic metals such as Mg^{2+} and Ca^{2+} are also known to decrease the activity of antimicrobial peptides and aminoglycosides against Gram-negative bacteria.[58,59] Moreover, use of hypertonic saline (concentration of NaCl ≥3%) is common in the treatment of cystic fibrosis for improved mucociliary clearance and sputum expectoration.[60] Several foodborne pathogens are known to have decreased antibiotic susceptibility on exposure to NaCl.[61] NCK-10 maintains almost constant activity at high percentages of NaCl against *E. coli*. This is a promising result, as it opens up a potential of this class of compounds to be used in the treatment of cystic fibrosis and even as a food preservative. In the presence of divalent cations, however, NCK-10 loses activity against *E. coli*. This was not surprising considering the prevalence of Mg^{2+} on the outer membrane of such bacteria.

A very important result is the ability of this compound to show activity in serum and whole blood. Although the compound retains activity in serum, the compound is active only at higher concentration in whole blood. This observation and the fact that a 55 mg/kg dose (iv) of NCK-10 is too toxic for mice led us to choose to use the compound topically instead of targeting systemic infections. Indeed, the efficacy of the compound was established in the murine model of burn infection. Treatment with the compound was effective enough to reduce the bacterial burden in the infection. As could be interpreted from Figures 6B,C, the compound was successful not only in eliminating the bacteria to a large extent but also promoting wound healing. This was evident from the regeneration of stratified squamous epithelial cells and that of the fibrous tissue layer of the burn wound. One important question that needs consideration now is whether the bacterial burden could be further reduced. An increase in concentration of compound is expected to lead to a reduction in bacterial burden. Moreover, if the dosage is increased to twice a day, a more favorable result might be obtained. The toxicity displayed by the compound at the 55 mg/kg dose has left a lot of scope for further optimization of this class of compounds. We firmly believe that this study has given us a start in the positive direction for development of the next generation of membrane-active antibacterial agents.

In conclusion, this paper effectively documents the range of activity of aryl-alkyl-lysines against infections caused by Gram-negative bacteria. In particular, one of the compounds, NCK-10, has emerged as an effective antibacterial agent against several NDM-1-producing Gram-negative bacteria. Its ability to kill planktonic cells and persister cells of *E. coli* at very low concentrations can be attributed to its membrane-damaging properties. A moderate LD_{50} of 30 mg/kg and a good efficacy against murine model of burn infection make it an important lead molecule in antimicrobial therapeutics, especially for treating Gram-negative infections.

MATERIALS AND METHODS

Antimicrobial Agents. The synthesis and characterization of the antimicrobial compounds used in this study have been reported previously.[39] Antibiotics used in the control study were bought from Sigma-Aldrich.

Bacterial Strains. The various bacterial strains used in the study are as follows: *K. pneumoniae* ATCC 700603, *K. pneumoniae* R3421, *K. pneumoniae* R3934, *A. baumannii* MTCC 1425, *A. baumannii* R674, *A. baumannii* R676, *P. aeruginosa* MTCC 424, *P. aeruginosa* R596, *P. aeruginosa* R590, *P. aeruginosa* R3324, *E. coli* MTCC 443, *E. coli* R250, *E. coli* R3336, *E. coli* colistin-resistant (laboratory strain), and *E. clocae* (R2928). Unless otherwise mentioned, the bacterial strains were clinical isolates. Clinical samples were from the Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences, Bangalore, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France, and Gram-negative bacteria were screened for carbapenem resistance using the Kirby−Bauer disc diffusion method (data not shown). *K. pneumoniae* ATCC 700603 was purchased from ATCC (American Type Cell Culture) and *E. coli* (MTCC 443), *A. baumannii* (MTCC 1425), and *P. aeruginosa* (MTCC 424) were purchased from MTCC (Chandigarh, India). Culture media and all of the antibiotics were from HiMedia and Sigma-Aldrich (India),

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respectively. MICs were determined by using the broth microdilution method according to CLSI guidelines.

PCR and Gel Electrophoresis. The experiment was performed as reported previously.[9] The bla_{NDM-1} gene was identified by conventional polymerase chain reaction (PCR) using primers NDM-F (5′-GGG CAG TCG CTT CCA ACG GT-3′) and NDM-R (5′-GTA GTG CTC AGT GTC GGC AT-3′) (Eurofins Genomics India Pvt. Ltd., Bangalore, India), which amplified an internal fragment of 475 bp using conventional polymerase chain reaction. The conditions included an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C and then a final extension step of 5 min at 72 °C. The PCR products were analyzed in 2% agarose gel, containing 0.05 mg/L ethidium bromide, at 100 V for 1 h in 1× Tris acetate EDTA buffer. A 100 bp DNA ladder was used as a molecular weight marker (SRL Biolit, Mumbai, India). Bands were visualized under UV light, and an amplified product corresponding to 475 bp was considered as positive.

In Vitro Susceptibility Studies. The MICs of the compounds against different bacterial strains were determined using a previously published protocol.[39] Briefly, 50 μL of serially diluted compounds was added to wells of 96-well plates containing 150 μL of medium containing bacteria (10^5 CFU/mL). The plate was then incubated at 37 °C for 24 h, and the OD value was measured at 600 nm using a TECAN (Infinite series, M200 pro) plate reader. Each concentration had triplicate values, and the whole experiment was done at least twice; the MIC value was determined by taking the average of triplicate OD values for each concentration and plotting it against concentration. The data were then subjected to sigmoidal fitting. From the curve the MIC value was determined as the point in the curve where the OD is similar to that of control having no bacteria.

Bactericidal Time−Kill Kinetics. Time−kill kinetics were performed according to the protocol published previously.[39] Briefly, bla_{NDM-1} *E. coli* R3336 was grown in nutrient broth at 37 °C for 6 h. Test compounds were inoculated with the aliquots of bacteria resuspended in fresh medium at ∼10^5 CFU/mL. After specified time intervals (0, 30, 60, 120, 180, and 360 min), 20 μL aliquots were serially diluted 10-fold in 0.9% saline, plated on sterile MacConkey's agar plates, and incubated at 37 °C overnight. The viable colonies were counted the next day and represented as $\log_{10}$ (CFU/mL).

Activity against Stationary Phase Bacteria. The experiment was performed as reported previously.[62] *E. coli* (MTCC 443) was grown for 6 h in Luria−Bertani broth and contained ∼10^9 CFU/mL (determined through dilution plate technique by spread plate method). This was then diluted 1000-fold and incubated at 37 °C for 18 h to obtain stationary phase cultures. At the end of 18 h, the cells were centrifuged down, washed twice with M9 medium, resuspended in M9 medium, and diluted to a concentration of 10^5 CFU/mL. The test compound, NCK-10, was then added to the stationary phase bacteria with the working concentration of 15 μg/mL. It was then incubated at 37 °C with shaking at 150 rpm. At the end of 2 h, 20 μL aliquots from that solution were serially diluted 10-fold in saline. Then from the dilutions, 20 μL was plated on nutrient agar plates and incubated at 37 °C. After 24 h, the bacterial colonies were counted and results represented in logarithmic scale, that is, log (CFU/mL).

Isolation of Persister Cells. Persister cells were isolated as reported previously.[63] After growing *E. coli* to their stationary phases using the protocol mentioned above, they were treated with ampicillin (300 μg/mL) for 3 h. Then they were centrifuged down, washed with M9 medium twice, and resuspended again in the same medium. These cells were then diluted to 10^5 cells and treated with the compound (at concentrations of MIC, 2× MIC, and 5× MIC) for 2 h or left untreated. They were then incubated at 37 °C with shaking at 150 rpm. At the end of 2 h, 20 μL aliquots from that solution were serially diluted 10-fold in corresponding media. Then from the dilutions, 20 μL was plated on agar plates and incubated at 37 °C. After 24 h, the bacterial colonies were counted and results represented in logarithmic scale, that is, log (CFU/mL).

Biofilm Experiment. *Determination of EC_{50} (Concentration of Compound at Which 50% of Biofilms Are Dispersed).* The bacteria used in this study were *E. coli* (MTCC 443), *K. pneumoniae* (ATCC 700603), *A. baumannii* (MTCC 1425), and *P. aeruginosa* (MTCC 424). Bacteria were grown to mid log phase for 6 h and diluted to ∼10^5 CFU/mL in their respective media. The media used to form different biofilms are as follows: *E. coli* biofilms were grown in M9 medium supplemented with 0.5% glycerol and 0.02% casamino acid, *K. pneumoniae* biofilms were grown in M9 medium supplemented with 0.1% $FeCl_3$, *A. baumannii* biofilms were grown in LB medium supplemented with 0.1% $FeCl_3$, and *P. aeruginosa* biofilms were formed in nutrient broth supplemented with 1% NaCl and 1% glucose. In a 96-well plate, 100 μL of media containing respective bacteria was added per well. These 96-well plates were kept in an incubator at 30 °C without shaking for 72 h. Care was taken to maintain sufficient moisture inside the incubator. At the end of 72 h, the supernatant was removed and planktonic cells were removed by washing with 1× PBS. To the wells were added serially diluted concentrations of NCK-10 (in respective media) and incubated for 24 h. At the end of 24 h, the supernatant was removed and the residual planktonic cells were removed by washing with 1× PBS. To this was added 100 μL of 0.1% of crystal violet (CV) solution to stain the remaining biofilms and incubated for 10 min at 37 °C. At the end of 10 min, the unused CV was removed and the plate was washed with 1× PBS. Finally, 100 μL of 95% ethanol was added to the disrupted biofilms containing plate and incubated for 15 min. The OD value of the solution was then measured at 520 nm by using a TECAN microplate reader. The percentage of OD value was plotted with increasing concentration of compound with respect to untreated control (no dispersion). Fifty percent dispersal values were obtained from the resultant sigmoidal plot. Plotting was done in Origin Pro8 software.

Confocal Imaging. Coverslips (diameter = 13 mm) were chosen as the substrates for conducting the biofilm disruption study. Sterilized coverslips were placed in wells of a 6-well plate. Two milliliters of mid log phase (6 h grown) culture of *E. coli* (MTCC 443), diluted to approximately 10^5 CFU/mL in a LB broth supplemented with 0.5% glycerol and 0.02% casamino acid, was added to wells containing the coverslips. The plate was incubated under stationary conditions at 37 °C for 72 h. After 72 h, medium was removed and planktonic bacteria were carefully washed with 1× PBS (pH 7.4) and removed. Biofilm-containing coverslips were then placed into another 6-well plate, and 2 mL of medium containing test compound (EC_{50}) was added to it and allowed to incubate for 24 h. In the case of control, 2 mL of complete medium was added without compound. At the end of 24 h, medium was then removed

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and planktonic cells were removed by washing with 1× PBS. The coverslips were carefully removed from the well and placed on glass slides. The biofilms were stained with 10 μL of SYTO-9 (3 μM) and imaged using a Zeiss 510 Meta confocal laser-scanning microscope. The images were prepared using an LSM 5 Image examiner.

Propensity To Induce Resistance Development in Bacteria. The experiment was performed as reported previously.[63] MIC values of the compounds (NCK-10 and antibiotics) were determined against *E. coli* (MTCC 443) as described above. After the initial MIC experiment, serial passaging was initiated by harvesting bacterial cells growing at the highest concentration of the compound, the OD_{620} of the half-MIC well of the previous MIC assay, and inoculating into fresh medium (Luria−Bertani broth in the case of *E. coli*). This inoculum was subjected to another MIC assay. After a 24 h incubation period, cells growing in the highest concentration of the compound from the previous passage were once again harvested and assayed for the MIC. The process was repeated for 20 passages. The MIC value of the compound was plotted against the number of passages, and the fold increase in MIC was determined. The results indicate the fold of increase in MIC every day.

Frequency of Resistance Determination. This experiment was performed following a previously published protocol with modifications.[64] To check the frequency of resistance against NCK-10 or colistin, *E. coli* (MTCC 443), at different concentrations, was plated on agar containing the compounds at 8× MIC. Specifically, *E. coli* (MTCC 443) was grown for 6 h in Luria−Bertani broth. This was centrifuged and resuspended in 1 mL of PBS; the count obtained was 10^9 CFU/mL (determined through dilution plate technique by spread plate method), which was serially diluted 10-fold. One hundred microliters of these solutions was plated on nutrient agar plates containing either NCK-10 (8× MIC) or colistin (8× MIC). Colonies were counted after 24 h of incubation at 37 °C. The frequency of resistance was determined by dividing the number of resistant mutants (number of colonies obtained) by the total number of cells plated.

Antibacterial Activity in Different Physiological Conditions. First, the pH and salinity of the culture medium were brought to the desired pH by adding NaOH (1 N) or HCl (1 N) or to the desired salt concentration by adding NaCl and $MgCl_2$ to the medium. The different pH conditions considered were 4.5, 5.5, 6.5, 7.4, and 8.5. The different percentages of NaCl considered were 0.5, 1, 1.5, and 3%. $MgCl_2$ concentrations were varied from 20 to 40 to 80 mM. Then the antibacterial activity of the compounds was determined in this medium according to the same assay as described above.

Antibacterial Efficacy in Serum. Blood (sodium heparin as anticoagulant) was donated by healthy human donors. Serum was obtained by using SSTTM II Advance serum tubes (BD vacutainer) (ref. 367956) containing human blood and then centrifuging the blood at 3500 rpm for 5 min. *E. coli* (MTCC 443) was grown similarly as above in LB medium for 6 h to give ∼10^9 CFU/mL. This was centrifuged and resuspended in MEM. This was diluted further in MEM to give 10^5 CFU/mL. The test compound NCK-10 was dissolved in sterile water at a concentration of 400 μg/mL. Two hundred microliters of this was mixed with 200 μL of freshly obtained human serum and incubated for 3 h. After 3 h, this mixture was subjected to 2-fold dilution, and then 50 μL of the solutions was added to wells of a 96-well plate. Bacterial suspension (150 μL, 10^5 CFU/mL) prepared in MEM was added individually to the wells containing the test solutions. The plates were then incubated for 24 h at 37 °C, and the antibacterial efficacy of the test compound was determined by evaluating their MIC as mentioned above. The MICs of the compounds were also determined in only MEM to compare the results.

In Vitro Whole Blood Assay. Whole blood assay was performed according to a previously published protocol with slight changes.[37] Briefly, to 30 μL of *E. coli* (MTCC 443) in saline (0.9% NaCl; 10^6 CFU/mL) were added 10 μL of NCK-10 (at concentrations MIC, 2× MIC, 5× MIC, and 10× MIC) and 270 μL of fresh human whole blood and incubated at 37 °C for 3 h. After the incubation period, antibacterial activity was determined by finding the bacterial titer in the infected blood by plating and reading the plates after 24 h.

Mechanism of Action. The assays for determining the mechanism of action of the compounds were performed according to a previously published protocol.[65] Brief descriptions of the assays are provided below.

Cytoplasmic Membrane Depolarization Assay. Mid log phase *E. coli* R3336 cells were harvested, washed with 5 mM HEPES and 5 mM glucose, and resuspended in 5 mM glucose, 5 mM HEPES buffer, and 100 mM KCl solution in a 1:1:1 ratio (10^8 CFU/mL). To this solution was added $DiSC_3$ (5) dye to a final concentration of 2 μM. The bacterial suspension containing the dye (200 μL) was pre-incubated for 40 min in a well of a black 96-well plate with transparent bottom (dye uptake and resultant self-quenching). The fluorescence of the bacterial suspension was measured (excitation wavelength, 622 nm; emission wavelength, 670 nm) and allowed to stabilize for 5 min at room temperature before the addition of 2 μL of NCK-10 (at 10 μg/mL). After addition, fluorescence intensity was measured every 1 or 2 min for 20 min. The resultant plot was obtained by joining the data points.

Outer Membrane Permeabilization Assay. Mid log phase *E. coli* cells R3336 were harvested, washed with 5 mM HEPES and 5 mM glucose (10^8 CFU/mL), and resuspended in a 1:1 solution of the same. *N*-Phenylnaphthylamine dye was added to the bacterial suspension containing the dye (200 μL) to give a final concentration of 10 μM in the bacterial suspension. The suspension containing the dye (200 μL) was then added to the well of a 96-well plate (black plate, clear bottom with lid) and stabilized for 5 min. Then 2 μL of NCK-10 was added to the solution at 10 μg/mL. After addition, fluorescence intensity (excitation wavelength, 350 nm; emission wavelength, 420 nm) was measured every 1 or 2 min for 20 min. The resultant plot was obtained by joining the data points.

Inner Membrane Permeabilization Assay. Mid log phase *E. coli* cells R3336 were harvested (4000 rpm, 4 °C, 10 min), washed, and resuspended in 5 mM HEPES and 5 mM glucose, pH 7.2. To this solution was added propidium iodide (PI) dye to a final concentration of 15 μM. The suspension containing the dye (200 μL) was then added to the well of a 96-well plate (black plate, clear bottom with lid), and then 2 μL of NCK-10 was added to the solution to a final at concentrations of 10 μg/mL. Fluorescence intensity was measured at an excitation wavelength of 535 nm (slit width = 10 nm) and an emission wavelength of 617 nm (slit width = 5 nm). The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner membrane permeabilization.

Persister cells were isolated as described above and diluted to 10^8 cells in respective media. Membrane depolarization studies

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using $DiSC_3$ (5) dye, membrane permeabilization studies using PI, and outer membrane permeabilization studies were all conducted in the same procedure described above, the only difference being isolated persister cells were used instead of mid log phase bacteria. The concentrations used in this study were MIC, 2× MIC, and 5× MIC.

In Vivo Systemic Toxicity Studies. The mice were housed in individually ventilated cages (IVC) maintained with controlled environment per the standards, which include housing in a pathogen-free conventional caging system and bedding material (corn cob). The husbandry conditions were as follows: light/dark cycle, 12:12 h; animal room temperature, 22 ± 2 °C; relative humidity, 30−40%; access to feed and water, ad libitum; and water, RO water. Animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days before the experiment to allow for acclimatization to the experimental conditions. Animal handling and experimentation protocols followed OECD Guidelines for the Testing of Chemicals (OECD 425). All care was taken to cause no pain to the animals. Humane end points were used to avoid unnecessary distress and suffering in animals following an experimental intervention that would lead to death.

The experimentation protocols for the determination of dosage, number of animals per groups, etc., were followed OECD Guidelines for the Testing of Chemicals (OECD 425). Female Balb/c mice (6−8 weeks, 18−22 g) were used for systemic toxicity studies. Mice were randomized into control and test groups with four or five mice per group. Control groups received 200 μL of sterilized PBS (pH 7.4). Different doses (5.5, 17.5, and 55 mg/kg) of the test drug was used as per the OECD guidelines. Two hundred microliters of the test drug solution in sterilized saline was injected into each mouse (5 mice per group) through intravenous (iv) (tail vein) route of administration. The mice in the high-dose group (55 mg/kg)-immediately post-injection of the drug, showed clinical signs of tremors, recumbence, severe distress, and convulsions, which were indicative of impending death or moribund condition. Therefore, some of the mice in moribund condition were humanely euthanized using isoflurane (Halothane) inhalant anesthetic. Then onward, they were monitored daily for 14 days. During the observation period of 14 days, no onset of abnormality was found.

In Vivo Infection Studies. Animal studies were performed according to the protocols approved by the Institutional Animal Ethics Committee (IAEC) of the National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI). The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of the National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru (881/GO/ac/05/CPCSEA), and carried out per the guidelines of the Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi, India. The mice were housed in (IVC maintained with controlled environment per the standards. They were housed in pathogen-free conventional caging system, and the bedding material used was corn cob. The husbandry conditions were as follows: light/dark cycle, 12:12 h; animal room temperature, 22 ± 2 °C; relative humidity, 30−40%; access to feed and water, ad libitum; and water, RO water. Animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days before the experiment to allow for acclimatization to the experimental conditions. Animal handling and experimentation protocols followed OECD Guidelines for the Testing of Chemicals (OECD 425). All care was taken to cause no pain to the animals. Humane end points were used to avoid unnecessary distress and suffering in animals following an experimental intervention that would lead to death.

***A. baumannii* Burn Infection.** This experiment was done following a previously published protocol with modifications.[66] Female Balb/c mice (6−8 weeks, 22−25 g) were anesthetized with a ketamine−xylazine cocktail, and their dorsal surface was shaved and cleansed. Around 6 mm burn wounds were created by applying a 120 s heated brass bar for 10 s. Immediately after injury, burn wounds were infected with a mid log phase bacterial inoculum of 10^7 CFU of *A. baumannii* (MTCC 1425) prepared in PBS. Treatment was started 4 h post-infection. Burn wounds were treated every 24 h for 7 days. NCK-10 and colistin was dissolved in saline. Burn wounds were treated with 40 μL of solutions containing NCK-10 (40 mg/kg) or colistin (5 mg/kg), whereas only saline was used as untreated control. Mice were euthanized 7 days post-injury; the wounded muscle tissue was excised, weighed, and homogenized in 10 mL of PBS. Serial homogenate dilutions were plated on MacConkey agar (Himedia, India), and the results were stated as log (CFU/g) of tissue. P value was calculated using unpaired Student's t test (two-tailed two samples assuming equal variances) between the control group and the treatment group, and a value of $P < 0.05$ was considered significant.

Skin Histology Studies. The experiment was performed as reported previously.[66] The portion of skin was collected and fixed in 10% formalin (10 mL of 40% formaldehyde added to 90 mL of water). The tissues were fixed for 48 h and washed for 1 h in running tap water. Then dehydration of the tissues was performed with increasing concentrations of ethanol (70, 90, and 100%; each for 1 h). Then the tissues were cleared in xylene for 1 h for two changes. Paraffin embedding was carried by keeping the tissues in melted paraffin at 56 °C for three changes. Longitudinal and transverse sections (5 μm) were prepared with semiautomatic microtome and placed on a glass slide coated with Meyer's egg albumen. Tissue sections were dried by incubating them for 2 h at 40 °C. Rehydration of fixed sections was carried in decreasing grades of alcohol (100, 90, 70, and 50%; each for 1 h) and then water. Subsequently, the sections were stained with hematoxylin and eosin. Then the sections were covered with DPX (SRL, India) mounting medium with cover glass and observed under a light microscope (Nikon, Japan) to study the histopathological changes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00092.

Anti-biofilm activities of the compounds, the activity of the compound in different physiological conditions, characterization of NDM-1 gene and mechanism of action of the compounds are provided as Tables and Figures (PDF)

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Notes

The authors declare the following competing financial interest: JNCASR has filed a patent application based on the work described.

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■ ABBREVIATIONS

AMP, antimicrobial peptide; MIC, minimum inhibitory concentration; CFU, colony-forming units; NDM-1, New Delhi-metallo-β-lactamase

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