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Glycopeptide Antibiotic To Overcome the Intrinsic Resistance of Gram-Negative Bacteria

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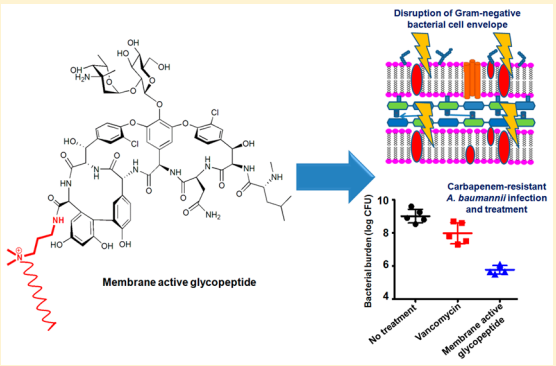
Supporting Information

ABSTRACT: The emergence of drug resistance along with a declining pipeline of clinically useful antibiotics has made it vital to develop more effective antimicrobial therapeutics, particularly against difficult-to-treat Gram-negative pathogens (GNPs). Many antibacterial agents, including glycopeptide antibiotics such as vancomycin, are inherently inactive toward GNPs because of their inability to cross the outer membrane of these pathogens. Here, we demonstrate, for the first time, lipophilic cationic (permanent positive charge) vancomycin analogues were able to permeabilize the outer membrane of GNPs and overcome the inherent resistance of GNPs toward glycopeptides. Unlike vancomycin, these analogues were shown to have a high activity against a variety of multidrug-resistant clinical isolates such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. In the murine model of carbapenem-resistant *A. baumannii* infection, the optimized compound showed potent activity with no observed toxicity. The notable activity of these compounds is attributed to the incorporation of new membrane disruption mechanisms (cytoplasmic membrane depolarization along with outer and inner (cytoplasmic) membrane permeabilization) into vancomycin. Therefore, our results indicate the potential of the present vancomycin analogues to be used against drug-resistant GNPs, thus strengthening the antibiotic arsenal for combating Gram-negative bacterial infections.



KEYWORDS: *intrinsic antibiotic resistance, Gram-negative bacteria, glycopeptide antibiotics, vancomycin, antibacterial activity*

The WHO Global Report on Surveillance of Antimicrobial Resistance 2014 reports that Gram-negative pathogens (GNPs) such as *Escherichia coli* and *Klebsiella pneumoniae* have developed resistance to >50% of commonly used antibacterial drugs.[1,2] More importantly, carbapenem-resistant bacteria such as New Delhi metallo-β-lactamase-1 (bla_{NDM-1}) producing GNPs have become resistant to even the last-line antibiotics such as colistin.[3] In addition to acquired resistance in GNPs, a plethora of Gram-positive antibiotics are left unused due to intrinsic resistance displayed by GNPs toward these antibiotics.[4,5] In fact, only two antibacterial agents, tigecycline and doripenem, have been approved by the U.S. FDA in the past decade for the treatment of Gram-negative bacterial infections.[6,7] The additional outer membrane (OM) and multiple efflux pumps appear to be the main contributors to this intrinsic resistance as these effectively hinder the entry of a variety of drug molecules including glycopeptide antibiotics such as vancomycin.[5]

Antibacterial drugs can penetrate the OM mainly by two pathways: porin channel mediated diffusion by hydrophilic antibiotics and a passive route taken by hydrophobic compounds.[8,9] Glycopeptides are hydrophilic in nature with a complex chemical structure and a high molecular weight (1450−2000 Da). Although they are hydrophilic, they are unable to permeate through porins in the OM to reach the cell wall area because of their high molecular weight and size. Because their site of action lies within the cell wall, GNPs are intrinsically resistant to glycopeptides. Among glycopeptides, vancomycin is the most successful antibiotic for use in the treatment of severe Gram-positive bacterial infections caused by staphylococci, enterococci, and clostridia. Vancomycin inhibits bacterial cell wall biosynthesis by binding to D-Ala-D-Ala found in cell wall precursors.[10−13]

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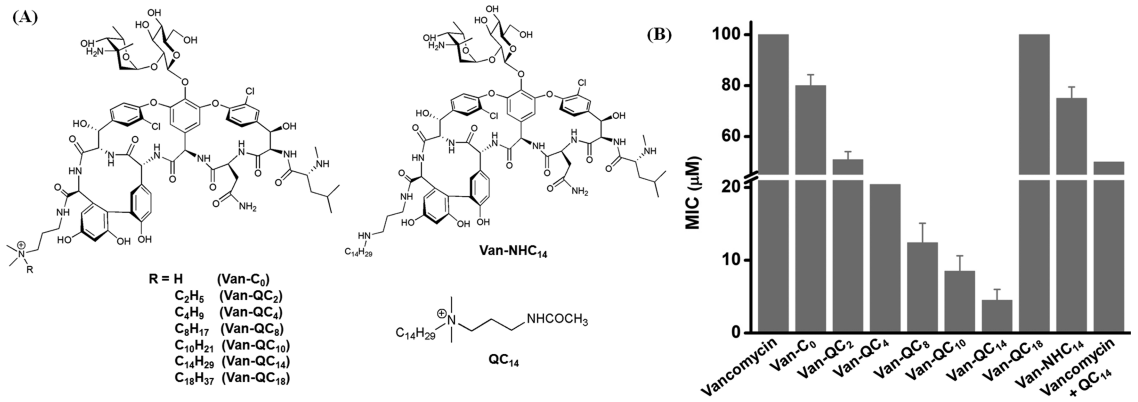

Figure 1. (A) Chemical structures of vancomycin analogues and lipophilic cationic moiety (QC_{14}). (B) Structure–activity relationship study of vancomycin analogues against *E. coli* ATCC 25922.

Table 1. Antibacterial Activities against Various Gram-Negative Pathogens

	minimum inhibitory concentration (μM)								
compound	*E. coli* (ATCC 25922)	*E. coli* (R1747)	*E. coli* (ATCC 35218)	*A. baumannii* (R4942)	*A. baumannii* (R676)	*A. baumannii* (R674)	*E. cloacae* (NDM-1) (R2928)	*K. pneumoniae* (ATCC 700603)	*P. aeruginosa* (R590)
vancomycin	>100	61.3	>100	54.3	>100	>100	>100	>100	>100
Van-QC_8	12.5	14.5	14	7	14.5	16.9	>100	16	25
Van-QC_{10}	8.5	5.3	8.5	4	13.2	8.2	50	14	12.5
Van-QC_{14}	4.5	1.2	4.5	3	5.2	4.8	12.5	9.0	6.1
Van-NHC14	75	ND[a]	>100	ND	>100	>100	>100	>100	>100
QC_{14}	>100	ND	>100	ND	>100	>100	>100	>100	>100
vancomycin + QC_{14}	50 + 50	ND	100 + 100	ND	>100 + >100	>100 + >100	100 + 100	100 + 100	100 + 100
meropenem	2	>50	2.2	>50	>50	>50	>50	2.2	>50
norfloxacin	1	> 0	4	>50	>50	>50	>50	4	>50
colistin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

[a]ND, not determined.

If we target the intrinsic resistance elements of GNPs such as OM with simple chemical modifications of clinically important Gram-positive antibacterial agents such as vancomycin, we can make these drugs active against GNPs, thus broadening the antibacterial spectrum of the drug. Significant strategies have been developed to make vancomycin active against Gram-negative bacteria. Vancomycin-encapsulated fusogenic liposomes and vancomycin–silver composites have been shown to have activity against GNPs.[14,15] Furthermore, there are several studies wherein antibiotic adjuvants have been used to extend the activity of Gram-positive antibiotics toward GNPs.[16] To date, a derivative of vancomycin/glycopeptide that could overcome the intrinsic resistance of GNPs has not been reported.

Generally, antimicrobial peptides (AMPs) and antibacterial peptidomimetics kill bacteria by selectively disrupting the negatively charged bacterial membranes through their facial segregation of positive charges and hydrophobic moieties.[17−28] Furthermore, it has been shown in the literature that membrane damage may nonspecifically affect the other biosynthetic machinery as the bacterial membrane is vital for numerous biosynthetic pathways.[29] Hence, a strategy that installs membrane disruption properties to vancomycin could compromise the OM of GNPs and aid in reaching its specific target and also might affect the other biosynthetic machinery. Recently, we have developed membrane active vancomycin analogues, which demonstrated good antibacterial activity against vancomycin-resistant bacteria due to the incorporation of novel membrane disrupting mechanisms, which overcome the acquired resistance to vancomycin.[30,31] In the present study, we show that these membrane active molecules are also highly active against a variety of multidrug-resistant (MDR) clinical isolates of GNPs, thus overcoming the inherent resistance of GNPs toward vancomycin. The antibacterial activity of these analogues against GNPs is attributed to the installed membrane disruptive properties to vancomycin in addition to cell wall biosynthesis inhibition. Furthermore, as demonstrated in the present study, this class of compound showed good in vivo activity.

RESULTS AND DISCUSSION

Synthesis. We developed vancomycin analogues wherein a lipophilic cationic quaternary ammonium moiety was conjugated to the carboxylic group of vancomycin to produce a series of lipophilic cationic vancomycin analogues with a permanent positive charge and variable lipophilicity from ethyl to octadecyl chain (Figure 1A).[30] Compound Van-C_0 that lacked permanent positive charge and lipophilicity was synthesized by conjugating vancomycin with *N*,*N*-dimethyl-1,3-diaminopropane. Also, a control compound, Van-NHC_{14}, devoid of permanent positive charge but having lipophilicity, was developed by conjugating vancomycin to N^1-tetradecyl-propan-1,3-diamine (Figure 1A).[30] Another important control

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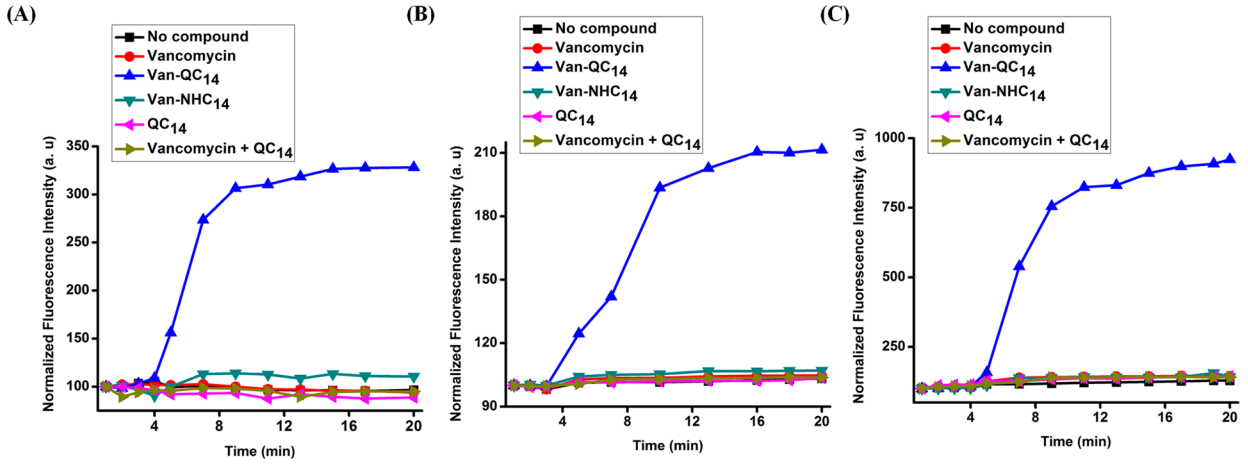

Figure 2. Disruption of bacterial membrane integrity: outer membrane permeability (A), inner (cytoplasmic) membrane depolarization (B), and inner membrane permeability (C) of vancomycin, Van-QC_{14}, Van-NHC_{14}, QC_{14}, and vancomycin + QC_{14} against *E. coli* at 5 μM.

compound, QC_{14}, was synthesized comprising lipophilicity and permanent positive charge without vancomycin.[30]

In Vitro Antibacterial Activity. To test the activity of the compounds, their antibacterial activity was determined against various ATCC strains and clinical isolates of a variety of clinically relevant GNPs of human importance. We determined the minimum inhibitory concentration (MIC) as the lowest concentration required to completely inhibit bacterial growth. Primarily, the in vitro antibacterial activity of these new compounds was evaluated against *E. coli* ATCC 25922 (Figure 1B and Table 1). The MIC of Van-C_0 (devoid of lipophilicity and permanent positive charge) was found to be 80 μM, whereas vancomycin was completely ineffective even at 100 μM. Compounds comprising lower alkyl chain, Van-QC_2 (ethyl chain) and Van-QC_4 (butyl chain), were found to possess moderate activity with MICs of 50 and 21 μM, respectively. Compound Van-QC_8 consisting of octyl chain displayed significant activity against *E. coli* (MIC = 12.5 μM), and a gradual increase in activity was observed with increasing chain length (Van-QC_{10}, MIC = 8.5 μM), with compound Van-QC_{14} (tetradecyl chain) demonstrating an MIC value of 4 μM. However, further increase in lipophilic chain compromised the activity as was observed in the octadecyl analogue of vancomycin, for which the MIC was found to be >100 μM. Our primary results emphasize that lipophilicity from octyl to tetradecyl would be the optimum lipophilic chain length to maintain activity against GNPs (Figure 1B).

Then, we chose the three optimized compounds, Van-QC_8, Van-QC_{10}, and Van-QC_{14}, to evaluate their activities against a variety of multidrug-resistant GNPs including carbapenem-resistant clinical isolates. Table 1 shows the antibacterial activities of Van-QC_8, Van-QC_{10}, and Van-QC_{14} and control compounds (Van-NHC_{14} and QC_{14}) against an array of GNPs such as *K. pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (R4942, R674, and R676), *Pseucomonas aeruginosa* (R590), *E. coli* (ATCC 25922, ATCC 35218, and R1747), and *Enterobacter cloacae* (bla_{NDM-1} R2928). Vancomycin was ineffective even at a concentration of 100 μM against *K. pneumoniae*, *P. aeruginosa*, *E. cloacae*, *A. baumannii* (R674, R676), and *E. coli* (ATCC 25922, ATCC 35218), whereas its MICs were found to be 54 and 61 μM against *A. baumannii* R4942 and *E. coli* R1747, respectively. Against all of these resistant strains Van-QC_8, Van-QC_{10}, and Van-QC_{14} showed good activity, and it was observed that activities increased with increase in the length of the lipophilic chain. Activity was observed against the ATCC strain of *E. coli* (ATCC 35218), Van-QC_{14} being the most active compound (MIC = 4.5 μM) followed by Van-QC_{10} (MIC = 8.5 μM) and Van-QC_8 (MIC = 14 μM). Furthermore, good activity against clinical isolate of *E. coli* R1747 was achieved for Van-QC_{14} with the lowest MIC of 1.2 μM. Next, we evaluated the activity against three clinical isolates of carbapenem-resistant *A. baumannii*. Against all three isolates of *A. baumannii*, again, the best activity was achieved for Van-QC_{14} with MICs ranging from 3 to 5 μM. Also, we noted that Van-QC_{14} was 2−3-fold more active than its shorter alkyl chain homologues Van-QC_8 and Van-QC_{10}. Van-QC_{14} also displayed good activity against MDR clinical isolate of *P. aeruginosa* R590 (MIC = 6.1 μM). Furthermore, Van-QC_{14} demonstrated notable activity against *K. pneumoniae* with the MIC of 9 μM. Next, the activities of these compounds were evaluated against clinical isolate of bla_{NDM-1} GNP, *E. cloacae* R2928 (see Figure S1 for characterization of NDM-1 gene in the Supporting Information). Yet again, Van-QC_{14} was found to be the most active in the series, exhibiting an MIC of 12.5 μM. All of the clinical isolates used in the present study were resistant to meropenem, which is one of the drugs of last resort for MDR GNPs. Therefore, the potent activity of these lipophilic cationic vancomycin analogues against MDR clinical isolates of GNPs is certainly a highlight of this study.

Next, we have established the importance of permanent positive charge over soft charge for potent antibacterial activity and the need to have the lipophilic quaternary ammonium moiety covalently connected to vancomycin as opposed to a physical mixture. We have evaluated the antibacterial activity of Van-NHC_{14} (Figure 1A) having a soft cationic moiety (which is expected to be cationic under physiological conditions) and tetradecyl lipophilic chain (Table 1 and Figure 1B). The MIC of Van-NHC_{14} against *E. coli* ATCC 25922 was found to be 75 μM, whereas in the case of the rest of the GNPs tested, its MIC was found to be >100 μM. In contrast to Van-NHC_{14}, Van-QC_{14} (comprising permanent positive charge and tetradecyl chain) showed potent activity with an average MIC of ~5 μM against most of the GNPs tested. Our results emphasize that it is the installed permanent positive charge along with lipophilicity in vancomycin that affords the potent activity against GNPs. Then, the activity of QC_{14} (without vancomycin) was determined. The MIC of QC_{14} was found to be >100 μM against all GNPs tested. We also evaluated the activity of the

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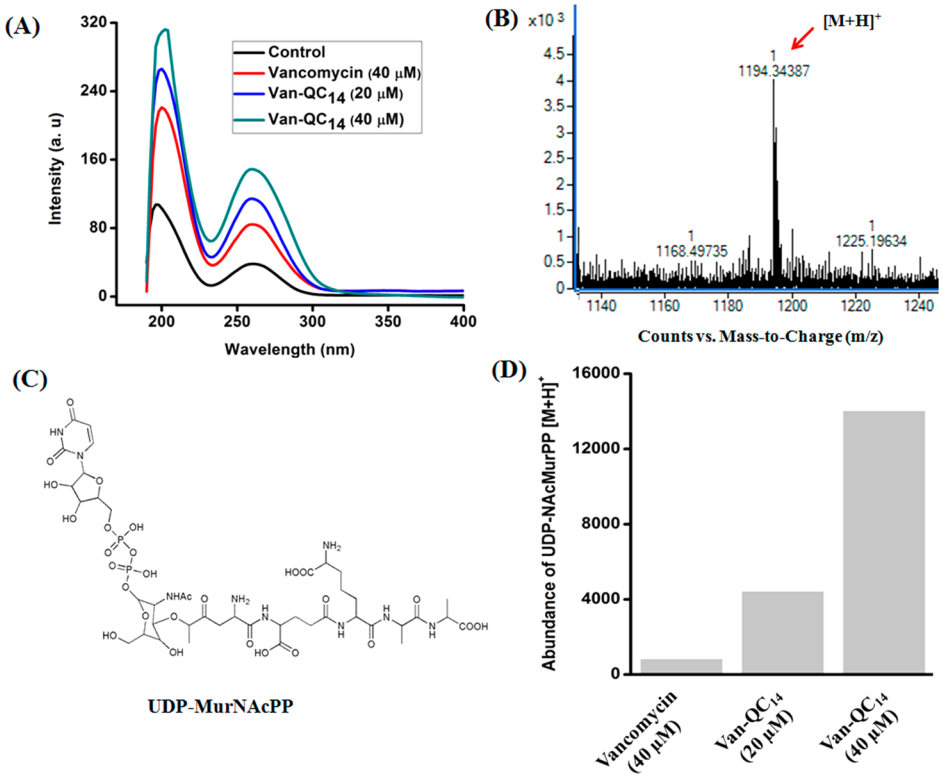

Figure 3. Intracellular accumulation of the cell wall precursor UDP-MurNAcpentapeptide (UDP-MurNAcPP) after treatment of *E. coli* with vancomycin and Van-QC_{14}: (A) identification of intracellular UDP-MurNAcPP by monitoring absorbance at 260 nm wavelength; (B) identification of UDP-MurNAcPP by mass spectrometry as indicated by the peak at m/z 1194.34; (C) chemical structure of UDP-MurNAcPP; (D) mass peak intensity of UDP-MurNAcPP after treatment with the test compounds. Untreated cells were used as control.

physical mixture of vancomycin and QC_{14}. Against most of the GNPs tested, the physical mixture showed antibacterial activity at either 100 or >100 μM of their individual concentrations except in the case of *E. coli* ATCC 25922, wherein the individual concentrations of 50 μM showed activity.

To test the influence of nonspecific interactions on the lead compound, Van-QC_{14} antimicrobial testing was performed in the presence of 4% bovine serum albumin (BSA) against *E. coli* ATCC 25922 and *A. baumannii* R674. The activity of Van-QC_{14} was reduced by ~2-fold in the presence of 4% BSA with the MIC of 8 μM. This slight reduction in activity might be attributed to the expected protein binding efficiency of Van-QC_{14}.

Disruption of Bacterial Membrane Integrity. *Outer Membrane Permeabilization.* Outer membrane (OM) permeabilization refers to disruption of membrane integrity, which facilitates the uptake of exogenous molecules.[4,5,9] This is an important step in the mode of action of many antibacterial agents as the OM plays a vital role in the intrinsic resistance of GNPs. We studied the OM permeabilizing abilities of compounds Van-QC_{14}, Van-NHC_{14}, vancomycin, QC_{14} alone, and its physical mixture with vancomycin (QC_{14} + vancomycin) on *E. coli*. Here, we used 1-*N*-phenyl-naphthylamine (NPN) as a fluorescent probe. Generally, OM acts as a permeability barrier and excludes hydrophobic substances such as NPN but, once damaged, it can allow the entry of NPN to the phospholipid layer, resulting in prominent fluorescence. Hence, this probe could be used to identify the kinetic traits of OM permeabilization associated with new compounds. Our results suggest that treatment with Van-QC_{14} caused a time-dependent rise in fluorescence intensity due to an increased membrane permeabilization of bacteria and consequent uptake of NPN. The rise in fluorescence signal was rapid and high for Van-QC_{14} (Figure 2A). However, vancomycin, Van-NHC_{14}, QC_{14}, and QC_{14} + vancomycin were ineffective.

Inner (Cytoplasmic) Membrane Depolarization. To investigate the interactions of these derivatives with the bacterial cytoplasmic membrane, we monitored the effect of compounds Van-QC_{14}, Van-NHC_{14}, vancomycin, QC_{14}, and QC_{14} + vancomycin on the membrane potential of *E. coli* using the $DiSC_3(5)$ assay. An increase in fluorescence was observed upon dissipation of membrane potential after the addition of test compounds. The membrane potential dissipation caused by the compound Van-QC_{14} was rapid after compound addition (the cells were equilibrated for 5 min prior to addition of the test compound), whereas vancomycin and other control compounds Van-NHC_{14}, QC_{14}, and QC_{14} + vancomycin remained ineffective at 5 μM (Figure 2B).

Inner (Cytoplasmic) Membrane Permeabilization. The observed effect of the cationic vancomycin derivative Van-QC_{14} on membrane potential prompted us to further examine the cytoplasmic membrane permeability. Kinetics of bacterial cytoplasmic membrane permeabilization by the compounds Van-QC_{14}, Van-NHC_{14}, vancomycin, QC_{14}, and QC_{14} + vancomycin on *E. coli* was studied by measuring the uptake of the fluorescent probe propidium iodide (PI). This dye can enter only membrane-compromised cells and fluoresces upon binding to nucleic acids. Unlike vancomycin, which did not cause membrane permeability, Van-QC_{14} showed a strong ability to permeabilize the cytoplasmic membrane (Figure 2C). Yet again, the control compounds Van-NHC_{14}, vancomycin,

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QC_{14}, and QC_{14} + vancomycin were ineffective in permeabilizing the cytoplasmic membrane of *E. coli*.

Our membrane disruption studies therefore suggest that neither vancomycin nor its physical mixture with lipophilic cationic moiety (QC_{14}) shows any effect on bacterial membrane. Also, the lipophilic vancomycin derivative Van-NHC_{14} (having a secondary amine that becomes cationic under physiological conditions) did not show any membrane disruption properties. This implies that permanent cationic charge along with lipophilic moiety needs to be chemically conjugated to the carboxylic group of vancomycin to impart membrane active properties toward GNPs, and they provide the necessary lipophilicity and initial electrostatic attraction to interact with the negatively charged bacterial cell membrane.

Intracellular Accumulation of Cell Wall Precursor, UDP-*N*-acetyl-muramyl-pentapeptide. To investigate whether new vancomycin derivatives interfere with peptidoglycan biosynthesis, we determined the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentapeptide (UDPMurNAc-pp), after treating bacteria with the new vancomycin derivative (Van-QC_{14}) and vancomycin. Accumulation of the cell wall precursor was quantified using UV spectroscopy (Figure 3A) and mass spectrometry (Figure 3D). In the case of Van-QC_{14} an intense peak was observed at 260 nm, which corresponds to UDPMurNAc-pp, and confirmed by high-resolution mass spectrometry (m/z 1194.34 (calcd), 1194.34 (obs) for $[M + H]^+$) (Figure 3B). The peak intensity increases with increase in concentration of Van-QC_{14} from 20 to 40 μM, whereas vancomycin (40 μM) showed negligible accumulation of UDPMurNAc-pp. Our results suggest that, unlike vancomycin, our new vancomycin derivative was able to inhibit cell wall biosynthesis in *E. coli*.

In Vitro Toxicity (Hemolysis and Cytotoxicity). In our previous paper,[30] the cytotoxicity and hemolytic activity of Van-QC_8 and Van-QC_{14} were determined. None of the derivatives showed significant toxicity up to 100 μM (CC_{50}; 50% cytotoxic concentration and HC_{50}; 50% hemolytic concentration were found to be >100 μM) concentration, which is much higher than their corresponding MIC values against GNPs. In contrast, the HC_{50} and CC_{50} of the permanent positively charged lipophilic compound (QC_{14}) were 125 and 27 μM, respectively. The selectivity (HC_{50}/MIC against *E. coli* ATCC 25922) of QC_{14} was found to be $\leq$1, whereas the corresponding vancomycin analogue Van-QC_{14} showed selectivity of >22, which indicates the selective toxicity of compound Van-QC_{14} against GNPs.

Propensity To Induce Bacterial Resistance. Emergence of rapid resistance in bacteria to conventional antibiotics is a major problem and is one of the major hurdles for the introduction of new antibiotics in clinical settings. Because we have explored the possibility of vancomycin analogues toward the treatment against MDR clinical isolates of GNPs in this paper, it is vital to investigate if the compounds themselves have any tendency to trigger bacterial resistance. To evaluate the potential of these compounds as long-lasting antibacterial agents, the ability of *A. baumannii* R674 to develop resistance against these compounds was investigated. Van-QC_{14}, the most active compound, was chosen as a model compound for this study. As a positive control, colistin, the drug of last resort for MDR Gram-negative bacterial infections, was used. Starting MICs for Van-QC_{14} and colistin against *A. baumannii* R674 were found to be 5 and 0.5 μM, respectively. The MIC of Van-QC_{14} toward *A. baumannii* did not change even after 20 passages, whereas the MIC of colistin increased by 32-fold (Figure 4). Thus, bacteria are less likely to acquire resistance against Van-QC_{14} compared to clinically used antibiotics such as colistin.

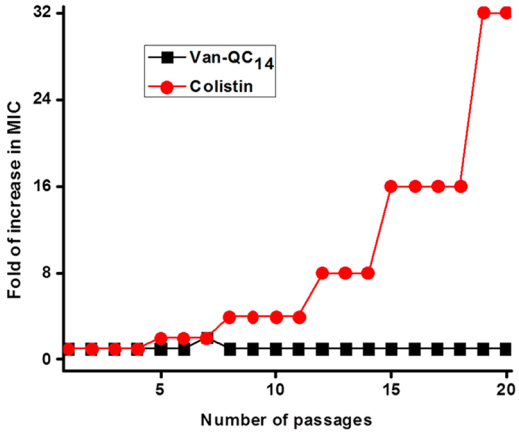

Figure 4. Bacterial resistance studies of colistin and Van-QC_{14} against *A. baumannii* R674. Starting MIC values for colistin and Van-QC_{14} were found to be 5 and 0.5 μM, respectively.

In Vivo Antibacterial Activity. Multidrug-resistant *Acinetobacter* infections have an extremely high mortality rate and occur most frequently in severely ill patients.[32] Treatment options are severely limited, and to our knowledge only carbapenems and colistin are the drugs of choice for most of the drug-resistant infections. A widely used animal model for evaluating antimicrobial activity of preclinical compounds is the thigh burden model, in which the thigh muscle of neutropenic mice is inoculated with bacteria, followed by administration of the antibacterial agents. The in vivo activity of Van-QC_{14} in comparison with colistin against carbapenem-resistant *A. baumannii* R674 is shown in Figure 5. In this study, mice were infected with *A. baumannii* in the thigh. After 1 h of infection, the mice were treated with saline, vancomycin (15 mg/kg), colistin (5 mg/kg), or Van-QC_{14} (15 mg/kg), and five mice were used in each group. After 24 h of treatment, antibacterial activity was determined by finding the bacterial titer in the infected thighs. Vancomycin showed ~0.9 $\log_{10}$

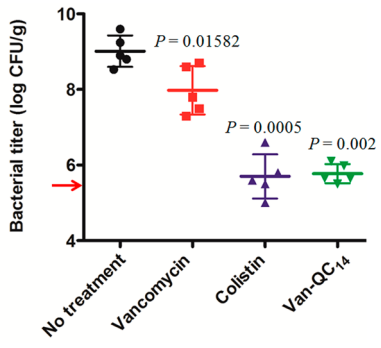

Figure 5. In vivo antibacterial activity of vancomycin, colistin, and Van-QC_{14} in murine thigh infection model against carbapenem-resistant *A. baumannii*. The arrow indicates the bacterial concentration inoculated in the mouse. Five mice were used in each group. Statistical analysis was performed using Student's t test. Differences are considered statistically significant from untreated group with probability $P < 0.05$.

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CFU/g reduction from untreated mice (saline), whereas the in vivo activity of Van-QC_{14} was found to be comparable to that of colistin, wherein they reduced the bacterial titer by ~3 $\log_{10}$ CFU/g compared to control (saline).

In Vivo Toxicity. To evaluate the maximum tolerability of Van-QC_{14}, systemic toxicity study of Van-QC_{14} was performed on BALB/c mice (n = 5). The compound was administered intraperitoneally at two different doses 55 and 100 mg/kg. Then, the animals were observed for mortality for a period of 7 days, and all of the mice had survived at 7 days at 100 mg/kg dosing regimen, indicating the high tolerability of Van-QC_{14} in animals required for therapeutic applications.

CONCLUSIONS

We demonstrated that vancomycin analogues can overcome the inherent resistance of Gram-negative pathogens. The incorporation of lipophilic moiety and permanent positive charge into vancomycin make these compounds distinct from other existing derivatives in their ability to cause strong bacterial membrane disruption, thereby overcoming the inherent resistance of GNPs. We believe, therefore, that our strategy can potentially be a beneficial extension to the antibiotic pipeline for the treatment of infections caused by MDR Gram-negative bacteria.

EXPERIMENTAL SECTION

Materials and Bacterial Strains. New vancomycin analogues were synthesized and purified to >95% purity using HPLC.[30] Vancomycin, meropenem, norfloxacin, colistin, chloramphenicol, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. To evaluate the antibacterial activity of new vancomycin analogues against Gram-negative bacteria, we chose the most common human-relevant pathogens, such as *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter cloacae*. *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 were obtained from the American Type Culture Collection (ATCC). *E. coli* R1747, *A. baumannii* R492, *A. baumannii* R674, *A. baumannii* R676, *P. aeruginosa* R590, and *E. cloacae* R2928 were isolated from clinical samples by 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.[33] *E. coli* ATCC 25922 was procured from MTCC (IMTECH, Chandigarh, India). Luria−Bertani broth was used for *E. coli* ATCC 25922, and nutrient broth was used for the rest of the bacteria.

Animals. Six-week-old specific-pathogen free BALB/c female mice weighing 19−24 g were used for animal studies. Infection studies were performed at the National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI). The mice were housed in individually ventilated cages (IVC) maintained with a controlled environment per the standards. 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 Committee for the Purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi. The toxicology study was performed at the Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR) in accordance with institutional ethical guidelines.

In Vitro Susceptibility Studies. The antibacterial activity of the test compounds was measured in the broth microdilution method, and MIC was calculated per our previously published protocols.[19,28,30,34,35] Also, the MIC of the lead compound, Van-QC_{14}, was performed in the presence of 4% BSA against *A. baumannii* R674 and *E. coli* ATCC 25922.

The combination antibacterial activity of vancomycin and QC_{14} (vancomycin + QC_{14}) was measured using a checkerboard assay as described previously.[36] A 2-fold serially diluted solution of 25 μL each of test compounds was added into each well of a 96-well plate followed by 150 μL of bacterial suspension (~10^5 CFU/mL). The plate was then incubated at 37 °C for a period of 24 h, and the OD value was measured at 600 nm. The MIC from the checkerboard assay was a result of two independent experiments, and each experiment was performed in triplicate.

Outer Membrane Permeabilization Assay. Mid-log phase *E. coli* ATCC 25922 cells were harvested, washed with 5 mM HEPES and 5 mM glucose, and resuspended in a 1:1 solution of the same at a concentration of 10^8 CFU/mL. Measurements were made in a Corning 96-well black plate with a clear bottom with 150 μL of bacterial suspension and 5 μM *N*-phenylnaphthylamine dye.[37] The fluorescence of the dye was monitored using a Tecan InfinitePro series M200 microplate reader at an excitation wavelength of 350 nm and an emission wavelength of 420 nm for 5 min. Then, the test compounds were added to the bacterial suspension at the working concentration of 5 μM. After their addition, the fluorescence intensity of the entire bacterial suspension was monitored (excitation wavelength, 350 nm; emission wavelength, 420 nm) for another 15 min.

Inner (Cytoplasmic) Membrane Depolarization Assay.[38] Bacteria (*E. coli* ATCC 25922) 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). Then, 150 μL of bacterial suspension and 2 μM $DiSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide) were added to the cells in a Corning 96-well black plate with a clear bottom; 0.2 mM ethylenediaminetetraacetic acid (EDTA) was used to permeabilize the outer membrane and allow dye uptake. The fluorescence of the dye was monitored using a Tecan InfinitePro series M200 microplate reader at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Dye uptake, and the resultant self-quenching, was modulated by the membrane potential. After reaching the maximum uptake of the dye by bacteria, which was indicated by a minimum in dye fluorescence, test compounds at 5 μM were added to the cells, and the decrease in potential was monitored by the increase in fluorescence for another 15 min. A control without the test compound served as negative control.

Inner Membrane Permeabilization Assay.[39] Bacteria (*E. coli* ATCC 25922) were harvested, washed, and resuspended in 5 mM HEPES and 5 mM glucose buffer of pH 7.2 (~10^8 CFU/mL). Then, 150 μL of bacterial suspension and 10 μM PI were added to the cells in a Corning 96-well black plate with a clear bottom. The fluorescence of the dye in the entire suspension was monitored using a Tecan InfinitePro series M200 microplate reader at an excitation wavelength of 535 nm and an emission wavelength of 617 nm for 5 min. Then, the test compounds at 5 μM were added to the cells, and the fluorescence of the dye of entire solution was measured for another 15 min as a measure of membrane permeabilization.

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Intracellular Accumulation of UDP-*N*-acetyl-muramyl-pentapeptide. Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool was examined using *E. coli* ATCC 25922 grown in 25 mL of MHB. Cells were grown to an $A_{600\ nm}$ (OD_{600}) of 0.6 and incubated with 130 μg/mL chloramphenicol for 15 min. Then, the test compounds vancomycin (40 μM) and Van-QC_{14} (20 and 40 μM) were added and incubated for another 60 min. Cells were collected and washed with sterile water to remove the antimicrobial agents and then extracted with boiling water. The cell extract was then centrifuged and the supernatant lyophilized.[40−43] Then, the lyophilized powder was dissolved in 2 mL of water and the pH was adjusted to 2.0 with 20% phosphoric acid. Now, the UDP-linked cell wall precursors in the solution were analyzed by RP18-HPLC monitoring the UV absorbance peak at 260 nm and confirmed by high-resolution mass spectrometry (HR-MS).

Propensity To Induce Resistance Development in Bacteria. At first, the MIC value of compounds colistin and Van-QC_{14} was determined against *A. baumannii* R674 in a 96-well plate. Here, the visual end point where there is no bacterial growth was considered to be the MIC of the test compound. For the next-day MIC experiment, the bacterial dilution was made by using the bacteria from sub-MIC concentration of the compounds (at MIC/2). Then, the concentration of this bacterium was adjusted to ∼10^5 CFU/mL on the basis of OD_{600} and subjected to the next MIC assay. After a 24 h incubation period, again bacterial dilution was prepared by using the bacterial suspension from sub-MIC concentration of the compound (at MIC/2) and assayed for the another MIC experiment. The process was repeated for 20 passages, and the fold increase in MIC was determined. The results indicate the fold of increase in MIC every day.[30]

In Vivo Antibacterial Activity.[44] About 6-week-old female BALB/c mice (weight, ∼19−24 g) were used for the experiment. The mice were rendered neutropenic (∼100 neutrophils/mL) by injecting two doses of cyclophosphamide intraperitoneally 4 days (150 mg/kg) and 1 day (100 mg/kg) before the infection experiment. Fifty microliters of ∼10^7 CFU/mL concentration of the bacterial inoculum (*A. baumannii* R674) was injected into the thigh. At 1 h post-infection, the animals were treated with colistin (5 mg/kg), vancomycin (15 mg/kg), or Van-QC_{14} (15 mg/kg) intraperitoneally. The animals were sacrificed to collect the infected thighs at 24 h post-treatment. Then the thighs were placed in 10 mL of sterile saline and homogenized. The 10-fold homogenate dilutions were plated onto nutrient agar plates and incubated at 37 °C for 24 h. The bacterial count was presented as $\log_{10}$ CFU/g of thigh weight.

In Vivo Toxicity.[45] Systemic toxicity of Van-QC_{14} was performed on BALB/c female mice. Each mouse was injected with 0.2 mL of a freshly prepared Van-QC_{14} solution in saline. The compound was administered at two different doses, 55 and 100 mg/kg (n = 5). Animals were directly inspected for adverse effects for 4 h, and mortality was observed for 7 days.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00114.

Details of characterization of *E. cloacae* R2928 for the identification of the NDM-1 gene (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

MIC, minimum inhibitory concentration; CFU, colony-forming units; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; PI, propidium iodide; NPN, 1-*N*-phenyl-naphthylamine; HC_{50}, 50% hemolytic concentration; CC_{50}, 50% cytotoxic concentration

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