VIP Antibiotics Very Important Paper

Deutsche Ausgabe: DOI: 10.1002/ange.201601621
Internationale Ausgabe: DOI: 10.1002/anie.201601621

A Vancomycin Derivative with a Pyrophosphate-Binding Group: A Strategy to Combat Vancomycin-Resistant Bacteria

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Abstract: *Vancomycin, the drug of last resort for Gram-positive bacterial infections, has also been rendered ineffective by the emergence of resistance in such bacteria. To combat the threat of vancomycin-resistant bacteria (VRB), we report the development of a dipicolyl–vancomycin conjugate (Dipi-van), which leads to enhanced inhibition of cell-wall biosynthesis in VRB and displays in vitro activity that is more than two orders of magnitude higher than that of vancomycin. Conjugation of the dipicolyl moiety, which is a zinc-binding ligand, endowed the parent drug with the ability to bind to pyrophosphate groups of cell-wall lipids while maintaining the inherent binding affinity for pentapeptide termini of cell-wall precursors. Furthermore, no detectable resistance was observed after several serial passages, and the compound reduced the bacterial burden by a factor of 5 logs at 12 mg kg^{-1} in a murine model of VRB kidney infection. The findings presented in this report stress the potential of our strategy to combat VRB infections.*

Vancomycin, a clinically important glycopeptide antibiotic, has been the antibiotic of last resort for the treatment of drug-resistant Gram-positive bacterial infections, particularly of those resistant to β-lactam antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA).[1] The increased use of vancomycin against prevailing MRSA strains has led to the emergence of vancomycin-intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA).[2] Furthermore, vancomycin-resistant *Enterococci* (VRE) have become one of the most common hospital-acquired pathogens, and constitute a global health concern.[3] At present, the treatment options for these drug-resistant infections are severely limited, and only a few drugs, such as daptomycin, quinupristin/dalfopristin, and linezolid, are available.[4,5] Bacteria have already started acquiring resistance even to these last-line antibiotics in clinical settings.[4,5] This poses a major health problem and has stimulated efforts to develop various strategies to combat drug-resistant pathogens.[6–12]

Vancomycin inhibits the cell-wall biosynthesis of Gram-positive bacteria by specifically binding to the D-Ala-D-Ala terminal of the cell-wall precursor pentapeptide, thus inhibiting transpeptidase-catalyzed cross-linking and maturation of the bacterial cell wall.[1] Bacteria acquired resistance to vancomycin by remodeling their cell-wall precursors that terminate in the D-Ala-D-Lac (lactate) depsipeptide, which reduces the vancomycin binding affinity by a factor of about 1000 and allows for unimpeded peptide-chain cross-linking (VanA and VanB phenotypes of vancomycin-resistant bacteria (VRB)).[13] Several strategies have been developed to combat bacterial resistance towards vancomycin. These involve either enhancing the binding affinity of the drug for bacterial ligands or installing membrane disruption properties, an additional mechanism of action, to vancomycin.[14,15]

The bacterial cell wall (peptidoglycan) is a three-dimensional network of long aminosugar strands (*N*-acetylmuramic acid, NAM; *N*-acetylglucosamine, NAG) cross-linked by a pentapeptide that is located on the exterior of the cytoplasmic membrane. Peptidoglycan subunits are assembled at the extracellular surface on a membrane-embedded polyisoprenoid anchor, such as bactoprenol pyrophosphate and lipid II (undecaprenyl-pyrophosphoryl-NAM-NAG-pentapeptide).[1] As these cell-wall lipids are essential for cell-wall biosynthesis, they represent attractive targets for antibacterial compounds, such as bacitracin, glycopeptides, mannopeptimycins, and several lantibiotics.[16] It has been shown that dipicolyl–Zn^{2+} complexes have a high affinity to complexation with pyrophosphates.[17] Generally, the concentration of divalent zinc ions (Zn^{2+}) in bacteria is about 100 μM, and their concentration is expected to increase in response to inflammation at the bacterial infection site as a consequence of Zn^{2+} release from damaged or apoptotic cells (ca. 100 μM).[18] As cell-wall lipids are present in the periplasmic space, we hypothesized that a dipicolyl derivative of vancomycin, Dipi-van, which can chelate Zn^{2+} (for example, from bacterial sources), can result in the formation of Dipi-van–Zn^{2+} complexes with the pyrophosphates of cell-wall lipids. Furthermore, we anticipated that this complex will not only maintain its inherent binding affinity towards pentapeptide termini but also have the ability to bind to the pyrophosphate groups of cell-wall lipids, which may result in enhanced inhibition of cell-wall biosynthesis. Herein, dipicolyl-1,6-hexadiamine (Dipi) was conjugated to vancomycin to yield a dipicolyl–vancomycin conjugate (Dipi-van). Dipi-van was found to be 375-fold more effective than vancomycin against VRE (VanA phenotype). Unlike vancomycin, Dipi-van did not induce the development of bacterial resistance. Compared to vancomycin, Dipi-van showed a higher in vivo antibacterial activity against VRE in a murine kidney infection model with no toxicity observed under the conditions used.

Dipi-van (**4**) was prepared in 77% yield by coupling the carboxylic group of vancomycin to dipicolyl-substituted 1,6-

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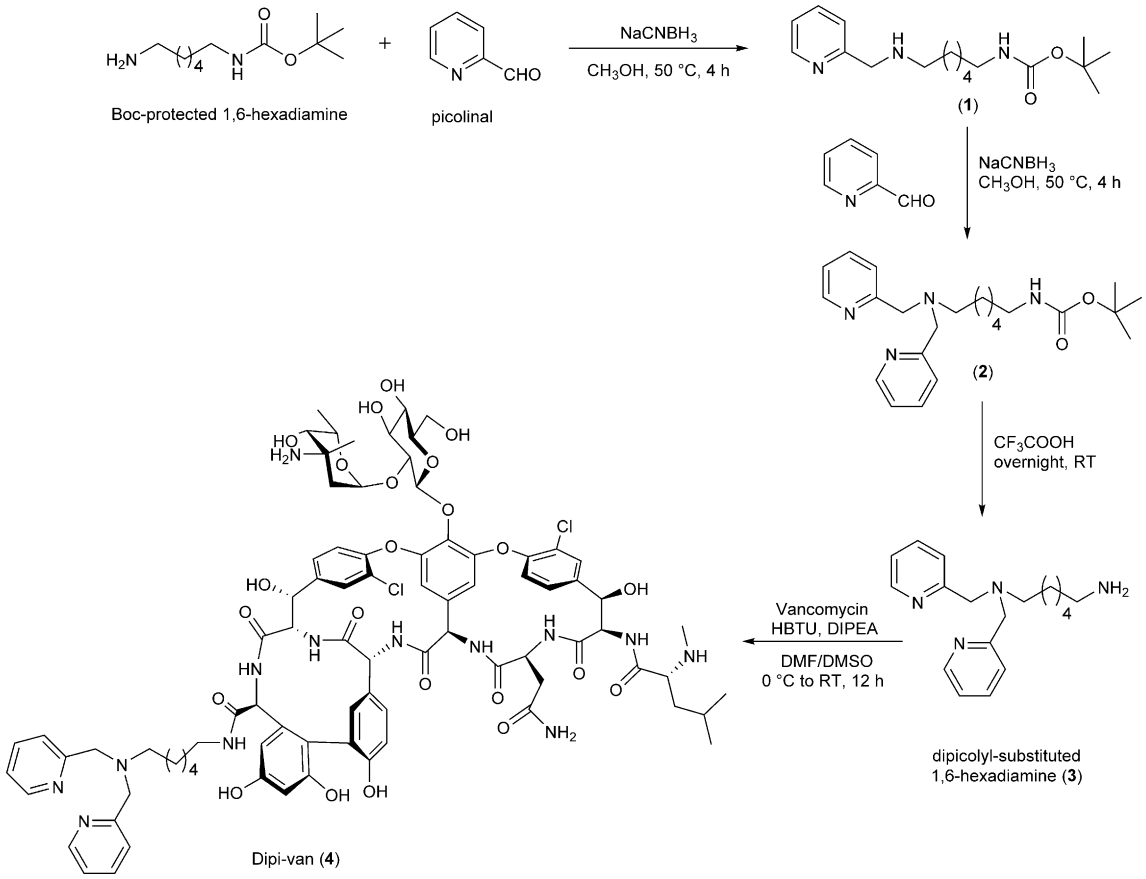
Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201601621.



Scheme 1. Synthesis of Dipi-van (**4**). DIPEA = *N*,*N*-diisopropylethylamine, HBTU = *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

hexadiamine (Dipi, **3**) through an amide coupling with HBTU (Scheme 1). To synthesize **3**, mono-Boc-protected 1,6-hexadiamine was reacted twice with 2-picolinal (pyridine-2-aldehyde) to form the corresponding Schiff bases followed by reduction with sodium cyanoborohydride (compounds **1** and **2**). Then, the Boc group was removed under acidic conditions (Scheme 1) to yield dipicolyl-1,6-hexadiamine (**3**), which was directly used in the coupling with vancomycin. To prepare Dipi-van, vancomycin was dissolved in dry DMF/DMSO (1:1), and a solution of HBTU in DMF was added dropwise at 0 °C. Subsequently, **3** was added to the vancomycin solution, and the reaction mixture was stirred at room temperature for 12 h. It was then purified by reverse-phase HPLC to more than 95 % purity and characterized by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry.

The antibacterial activities of vancomycin and Dipi-van were evaluated by determining the minimum inhibitory concentrations (MICs) against vancomycin-resistant strains of *Staphylococci* (VISA) and *Enterococci* (VRE; VanA and VanB phenotypes). The results are summarized in Figure 1 A. Dipi-van exhibited improved antibacterial activity towards VISA (MIC ≈ 1 μM) compared to vancomycin (MIC of 13 μM). The activity of Dipi-van was found to be approximately 375-fold and 160-fold higher than that of vancomycin against VREm (VanA phenotype, *E. faecium*) and VREs (VanB phenotype, *E. faecalis*), respectively (Figure 1 A). The antibacterial activity of the dipicolyl moiety alone (without vancomycin, compound **3**) was evaluated against VREm (VanA phenotype), and the compound was found to be inactive even at 100 μM. Furthermore, the activity of a physical mixture of vancomycin and compound **3** was determined, and it was found to be inactive against VREm even up to individual concentrations of 50 μM (Dipi-van: MIC = 2 μM).

Next, the activity of Dipi-van against VREm was evaluated in the presence of external divalent zinc ions (added as zinc sulfate, $ZnSO_4$). Under these conditions, the activity of Dipi-van was higher by a factor of two to three than in the absence of external Zn^{2+}. This enhanced activity was attributed to the formation of Dipi-van–Zn^{2+} complexes, which presumably bind to the pyrophosphates of cell-wall lipids that are accessible at the extracellular surface, and thereby interfere with their action in the continuous cyclic process of the formation of peptidoglycan layers. The formation of Dipi-van–Zn^{2+} complexes was confirmed by mass spectrometry (see the Supporting Information, Figure S1).

To validate our hypothesis, we evaluated the ability of Dipi-van–Zn^{2+} to complex with geranyl pyrophosphate (GPP, as a model compound for the cell-wall lipids). Dipi-van–Zn^{2+} and GPP were mixed in a molar ratio of 1:1, and the resulting mixture was allowed to stand for two hours at room temperature. Then the solution was analyzed by mass spectrometry, and a peak corresponding to the Dipi-van–Zn–GPP complex

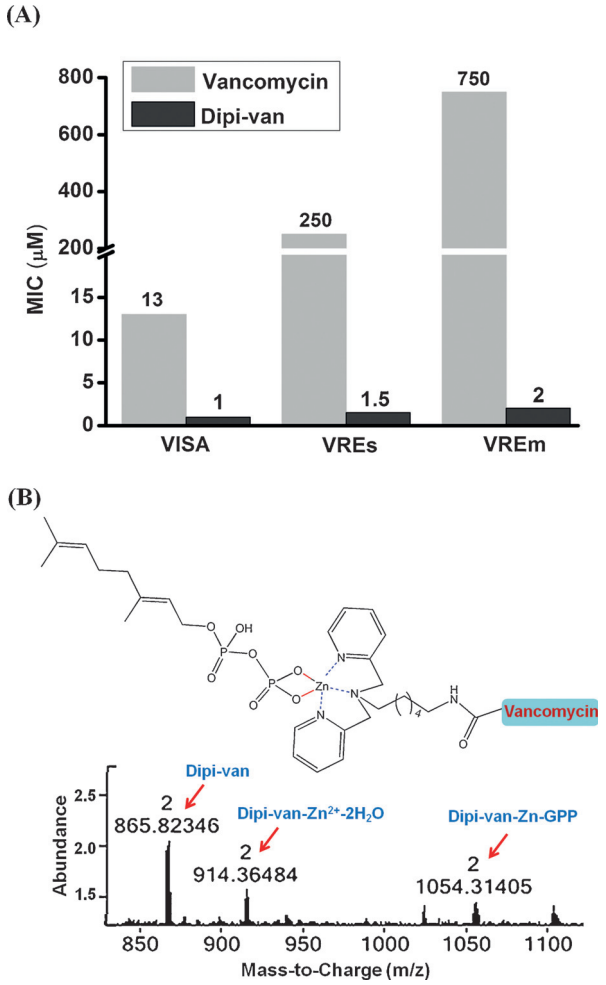


Figure 1. A) Antibacterial activity of Dipi-van and vancomycin against vancomycin-intermediate-resistant *S. aureus* (VISA), vancomycin-resistant *E. faecalis* (VREs, VanB phenotype), and vancomycin-resistant *E. faecium* (VREm, VanA phenotype). B) The complex of Dipi-van–Zn^{2+} with geranyl pyrophosphate (Dipi-van–Zn–GPP) was identified by mass spectrometry as indicated by the peak at *m/z* 1054.31. Dipi-van–Zn^{2+} can also coordinate two water molecules (*m/z* 914.36). In the presence of GPP, these water molecules are replaced by the pyrophosphate of GPP.

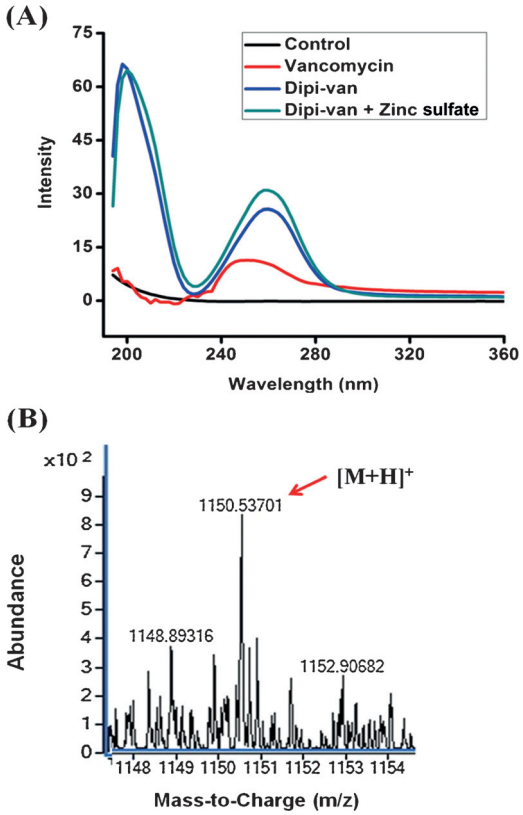


Figure 2. Inhibition of cell-wall biosynthesis: Intracellular accumulation of the cell wall precursor UDPMurNAc-pp after treatment of VREm (VanA phenotype) with vancomycin, Dipi-van, and Dipi-van in the presence of zinc sulfate at 5 μM. Untreated cells were used as the control. A) Identification of intracellular UDPMurNAc-pp by monitoring the absorbance at $\lambda = 260$ nm. B) UDPMurNAc-pp was identified by mass spectrometry (*m/z* 1150.53).

was observed, indicating the ability of the new glycopeptide to bind to pyrophosphate groups in cell-wall lipids (Figure 1 B). We evaluated the MIC of Dipi-van in the presence of molar equivalents of Zn^{2+} and GPP (as an antagonist) against VREm. In the presence of GPP, Dipi-van completely lost its activity owing to the formation of Dipi-van–Zn–GPP complexes. This finding further implies that Dipi-van can bind to the pyrophosphate groups of cell-wall lipids.

To investigate whether the new vancomycin derivative indeed has an inhibitory effect on cell-wall (peptidoglycan) biosynthesis, the accumulation of a UDP-linked peptidoglycan precursor,[19] UDP-*N*-acetylmuramyl-pentadepsipeptide (UDPMurNAc-pp), caused by treating VRE with 5 μM Dipi-van and vancomycin was compared through spectrophotometry (Figure 2 A and B). We also investigated the ability of Dipi-van to inhibit the cell-wall biosynthesis in the presence of external Zn^{2+}. Upon treatment with Dipi-van, a more intense peak was observed at 260 nm than for vancomycin treatment, which corresponds to the accumulation of UDP-MurNAc-pp and was confirmed by high-resolution mass spectrometry (*m/z* calcd for UDPMurNAc-pp ($C_{40}H_{64}N_8O_{27}P_2$): 1150.94 $[M+H]^+$; found: 1150.53). This result suggests that Dipi-van is a better inhibitor of cell-wall biosynthesis than vancomycin. Cell-wall biosynthesis was even more strongly inhibited by Dipi-van in the presence of Zn^{2+}. This result can be attributed to the ability of Dipi-van to bind to the pyrophosphates of cell-wall lipids, which is not possible with vancomycin. The cell-wall lipids are crucial for the formation of peptidoglycans as they translocate cell-wall intermediates (UDPMurNAc-pp) from intracellular to extracellular surfaces. Dipi-van presumably blocks the translocation and enhances the accumulation of UDPMurNAc-pp, which enhances the inhibition of cell-wall biosynthesis.

In the light of the alarming increase in drug resistance in bacteria, the potential emergence of bacterial resistance to this new compound was evaluated. The propensity of bacteria to generate resistance can be evaluated through serial exposure of the organisms to the antimicrobial agents. MRSA was thus exposed to vancomycin and Dipi-van for serial passages, and the changes in MIC values were monitored over a period of 25 days. Even after 25 serial

passages, the MIC of Dipi-van had remained the same (MIC ≈0.5 μM). However, in the case of vancomycin, the MIC value started to increase after seven passages, and the value had increased by a factor of 16 after 25 passages (Figure 3A). Thus bacteria have less propensity to develop resistance against this compound, which emphasizes the suitability of such compounds for clinical applications.

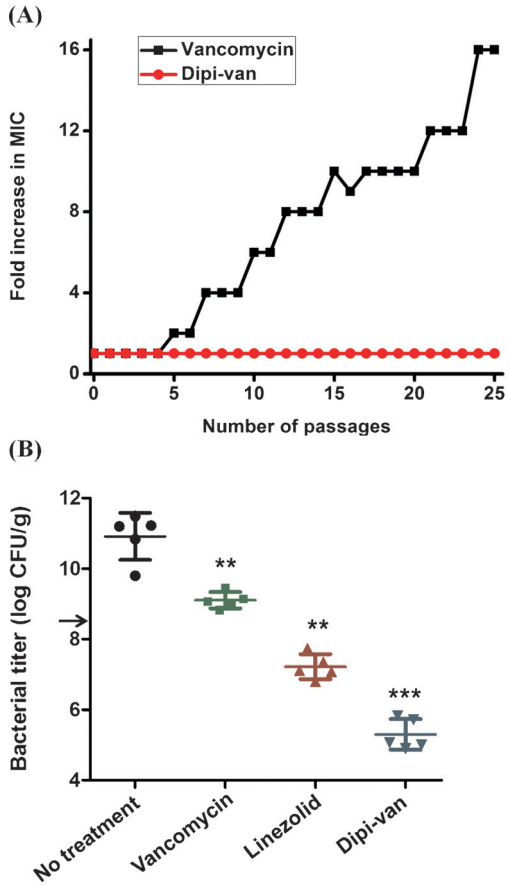


Figure 3. A) Development of bacterial resistance in MRSA towards vancomycin and Dipi-van. B) In vivo antibacterial activity of vancomycin, linezolid, and Dipi-van in a murine renal infection model against VRE at 12 mg kg^{-1}. The black arrow indicates the bacterial pre-treatment titer (ca. 8.0 $\log_{10}$ CFU g^{-1}). Five mice were used in each group. Statistical analysis was performed using Student's *t*-test. Differences are considered statistically significant from the untreated group with a value of $P<0.05$ with 95% confidence intervals (***$P<0.001$; **$P<0.01$).

The toxicity of Dipi-van was studied by measuring its hemolytic activity (HC_{50}; 50% hemolytic concentration) against human red blood cells (RBCs). Dipi-van did not show any significant toxicity towards the RBCs even at a concentration of 1000 μM, which indicates the selective toxicity of Dipi-van towards bacterial cells over mammalian cells. Next, the in vivo systemic toxicity of Dipi-van was assessed after single-dose intravenous (i.v.) administration to mice ($n=5$) at doses of 100 mg kg^{-1}. All of the mice were still alive after 14 days, indicating the high tolerability of Dipi-van in animals with $LD_{50}>100$ mg kg^{-1}.

Infections caused by VRE have been increasing, representing an emerging threat to public health.[3] The in vivo activity of Dipi-van was evaluated in a renal infection model against VRE. Initially, mice were injected intravenously with 0.2 mL of 0.2% λ-carrageenan to increase their susceptibility to bacterial renal infection. After seven days, the mice were infected with VRE (VanB phenotype, ca. 10^8 CFU/mouse; CFU = colony forming unit). After four hours, the mice were treated with three doses (at 24 h intervals) of vancomycin (12 mg kg^{-1}), linezolid (12 mg kg^{-1}), Dipi-van (12 mg kg^{-1}), or saline. 72 h after the initial treatment, the antibacterial activity was determined by measuring the bacterial titer in the infected kidneys. Compared to vancomycin, Dipi-van and linezolid reduced the bacterial titer in the infected kidneys more effectively. Linezolid achieved a CFU reduction of 4 $\log_{10}$ compared to the vehicle-treated control (saline), whereas Dipi-van led to a CFU reduction of approximately 5 $\log_{10}$ (Figure 3B).

In summary, a simple rational strategy has been presented to combat the acquired resistance of Gram-positive bacteria towards glycopeptide antibiotics. The new compound, Dipi-van, was approximately two orders of magnitude more active against vancomycin-resistant bacteria than vancomycin. Furthermore, Dipi-van showed potent in vivo activity against VRE compared to linezolid and vancomycin and displayed no significant toxicity. Our results demonstrate the high therapeutic potential of Dipi-van to address the clinical challenge of vancomycin-resistant bacterial infections.

Acknowledgements

We thank Prof. C. N. R. Rao, FRS (JNCASR) for his constant support and encouragement. S.S. is grateful to the Sheikh Saqr laboratory at JNCASR for a post-doctoral fellowship. We thank Dr. R. G. Prakash (in-house animal facility) for his help with the in vivo studies.

Keywords: antibiotics · bacterial resistance · drug design · multidrug-resistant bacteria · vancomycin

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 7836–7840
Angew. Chem. **2016**, *128*, 7967–7971

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Received: February 15, 2016
Revised: March 7, 2016
Published online: March 24, 2016

