Conjugates of Vancomycin and Cell-penetrating Peptides – New Weapons in the Armoury against Drug Resistant Bacteria

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Staying one step ahead in the arms race against antibiotic resistant pathogenic bacteria is an ongoing challenge to the ingenuity of medicinal chemists. Multidrug-resistance has become a menacing threat to the antibiotic armoury used by clinicians to treat nosocomial and community-acquired bacterial infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most notorious of such hospital-acquired pathogens. Until recently, the glycopeptide antibiotic vancomycin (Van) was available as a last resort, to effectively treat patients suffering from life-threatening MRSA and multidrug resistant (MDR) infections. However, the emergence of vancomycin resistance in Enterococcus spp. and subsequently, albeit rarely, of vancomycin resistant (Van^R) *S. aureus* (VRSA), has threatened the usefulness of vancomycin. The more frequent occurrence of strains showing intermediate resistance to vancomycin: heterogeneous vancomycin intermediate *S. aureus* (h-VISA) (Howden *et al*, 2010) gives particular cause for concern. Despite this bleak picture, innovative lab scientists are constantly coming up with new ideas. Two recent publications, from independent research groups, report the effectiveness of conjugates of vancomycin with cell-penetrating peptides (CPPs) in combating strains of Van^R MRSA as well as h-VISA and Van^R enterococci (VRE), and give grounds for optimism.

Vancomycin is a hydrophilic, glycopeptide antibiotic which inhibits cell wall synthesis in Gram-positive bacteria by binding to the C-terminal D-Ala-D-Ala of pentapeptide precursors of peptidoglycan, thus blocking transglycosylation and transpeptidation reactions in the biosynthetic pathway. Resistance to vancomycin was first reported in enterococci in 1988, about 30 years after the antibiotic was first used in the clinic. Several types of vancomycin resistance have been described, of which VanA was the first to be elucidated and is the most common. The transposon-mediated (Tn1546) vanA gene codes for a ligase which replaces the D-Ala-D-Ala with D-Ala-D-Lactate, to which vancomycin cannot bind, and so peptidoglycan synthesis and cell wall formation proceeds (Perichon and Courvalin, 2008).
As a consequence of its hydrophilic nature, vancomycin shows poor penetration of the cell membrane, which hinders its access to intracellular bacteria and capacity to cross the blood brain barrier (BBB).

VANCOMYCIN-HECATE CONJUGATES

The publication by Jelinkova and co-workers (Jelinkova *et al*, 2019) describes the synthesis of a conjugate of vancomycin cross-linked through 1,1'-carbamyldiimidazole (CDI) to the 23-amino-acid-residue peptide Hecate (FALALKALKKALKKLKKALKKAL). Hecate is a synthetic antimicrobial peptide (AMP) derived from the natural peptide, melittin, a venom peptide synthesised by the honey bee, *Apis mellifera* (Arrowood *et al*. 1991). Hecate is highly hydrophobic, with an alpha-helical structure and a net positive charge. A complicating factor with this peptide is its cytotoxicity to eukaryotic cells and haemolytic effect.

The antimicrobial effect of the vancomycin-Hecate conjugate, Van/Hec, against MRSA and VRSA was analysed by measurement of zones of inhibition formed around discs soaked with Van/Hec on lawns of the bacterial strains and by measurement of minimal inhibitory concentration (MIC) of the conjugate required to completely stop bacterial growth as observed in cultures growing in microplate wells (the broth-dilution method).

A strong inhibitory effect of Van/Hec was found for all the bacteria, with diameters of inhibition zones of 25, 24 and 12 mm for Van susceptible *S. aureus*, MRSA, and VRSA, respectively. Van/Hec conjugate showed an MIC for MRSA of 3.5 μM, a little lower than that of Van for this organism (5μM); however these MICs were higher than those for Van susceptible *S. aureus* (Table 1). Notably, the MIC of Van/Hec with VRSA was 5μM, much lower than that of Van for VRSA (>80 μM), indicating the strong bactericidal effect of Van/Hec on this Van^R strain and that vancomycin resistance can be overcome with Van/Hec.

Susceptibility of Van^R *Enterococcus faecalis* (VRE) to Hec, Van and Van/Hec was also tested and it was found that only the Van/Hec conjugate had antimicrobial activity against this organism.

Table 1. MICs of Van/Hec conjugate, Hecate peptide and vancomycin in different strains of *S. aureus*. Hec, Hecate peptide; Van, vancomycin. (From Jelinkova *et al*, 2019).

Bacterial strains/agents	Van/Hec uM	Hec uM	Van uM
S. aureus (Van susceptible)	0.5	>80	0.6
MRSA	3.5	>80	5
VRSA	5	>80	>80

In order to test the ability of Van/Hec to penetrate bacterial cells, a single cell gel electrophoresis assay was performed (also known as the "comet assay" from the characteristic streaks formed by EtBr-stained, disrupted DNA migrating towards the anode from the lysed bacterial cells trapped in the gel). The comet assay clearly showed Van/Hec caused more damage to the bacterial DNA than the positive control, H_2O_2, whereas neither Van alone nor Hec caused disruption of bacterial DNA. Furthermore, microscopic examination revealed that exposure to Van/Hec resulted in disruption of bacterial cell integrity in all tested strains, which was not observed with Van or Hec alone.

The cytotoxicity of Van/Hec was tested by measuring its haemolytic effect in freshly prepared human red blood cells. Remarkably, the conjugate was less cytotoxic than Hec alone. Van/Hec also demonstrated satisfactory biocompatibility, similar to Van, when evaluated in the human cell line PNT1A; neither Van/Hec nor Van caused loss in cell viability at concentrations up to 80 μM, unlike Hec which had a significant apoptotic effect at this concentration.

The work of Jelinkova *et al*. thus indicates that Van/Hec shows promise as an antibiotic agent against VRSA and VRE, showing good penetration of bacterial cells with little cytotoxic effect on host cells.

VANCOMYCIN-TRANSPORTAN10 CONJUGATES

The second report by Ruczynski and co-workers (Ruczynski *et al*, 2019), describes the synthesis and evaluation of four different conjugates of vancomycin with the CPP transportan10 (TP10) which they tested in selected clinical strains of MRSA, h-VISA and VRE (Table 2). Although this group did not test the conjugates in VRSA, as Jelinkova and co-workers did with Van/Hec, they went further towards simulating the pathophysiology of infection in that they tested one of the conjugates in MRSA-infected human cells for its bactericidal effect on intracellular bacteria and in mice following intravenous injection, for its capacity to cross the BBB.

Table 2. Conjugates of vancomycin and transportan10. *Fl*, fluorescein; PEG_4, 4,7,10,13-tetraoxapentadecane linker; PEG_3, 3,6,9-trioxaundecane linker; Tra, 1,2,3-triazole ring. (From Ruczynski *et al*, 2019).

	Compound name	Sequence
	TP10	AGYLLGKINLKALAALAKKIL-amide
I	Van-PEG_3-TP10	Van-NH-PEG_3-Tra(1,4)-C(O)-AGYLLGKINLKALAALAKKIL-amide
II	Van-PEG_4-TP10	Van-C(O)-PEG_4-Tra(1,4)-C(O)-AGYLLGKINLKALAALAKKIL-amide
III	TP10-Ala(PEG_4-Van)	AGYLLGKINLKALAALAKKIL-Ala(Tra(1,4)-PEG_4-C(O)-Van)-amide
IV	[Lys^7(PEG_4-Van)]TP10	AGYLLGK^7(C(O)-Tra(1,4)-PEG_4-C(O)-Van)INLKALAALAKKIL-amide
IVa	*Fl*-[Lys^7(PEG_4-Van)]TP10	*Fl*-AGYLLGK^7(C(O)-Tra(1,4)-PEG_4-C(O)-Van)INLKALAALAKKIL-amide

Transportan10 (TP10) is a 21-amino-acid-residue amphipathic chimera of the N-terminal residues of the neuropeptide galanin, linked to the full-length wasp venom peptide mastoparan. TP10 possesses antimicrobial properties and can transport cargoes across membranes; it disturbs membrane integrity and binds to DNA (Fanghänel et al, 2014).
Estimation of the BBB-penetrating capacity of *Fl*-[Lys^7(PEG_4-Van)]TP10 was carried out qualitatively by fluorescence microscopy of brain slices from mice which had been injected with the conjugate.

(Abstract graphic: Fluorescence microscopy images showing penetration of *Fl*-[Lys^7(PEG_4-Van)]TP10 in mouse brain sections. Left, lower part of right brain hemisphere with olfactory tract. Right, middle part of right hemisphere with striatum).

Quantitative estimation of the amount of [Lys^7(PEG$_4$-Van)]TP10 penetrating mouse brains following i.v. injection carried out by an LC/MS method demonstrated 200 times the amount of [Lys^7(PEG$_4$-Van)]TP10 in mouse brain homogenates compared to Van (Table 3).

Table 3. Concentrations of [Lys^7(PEG$_4$-Van)]TP10, Van and TP10 in mouse brain homogenates. ND, not detected; *Statistically significant ($p < 0.05$) compared to Van. (From Ruczynski *et al*, 2019.)

Treatment (i.v.)	Brain concentrations, nM (Mean ± SD)		
	Van	TP10	[Lys^7(PEG$_4$-Van)]TP10
Saline	ND		ND
Van	11 ± 2		ND
TP10		ND	
[Lys^7(PEG$_4$-Van)]TP10	ND		2611 ± 120*
Van + TP10	ND	ND	

Experiments with HEK293 cells infected with h-VISA demonstrated that treatment with [Lys^7(PEG$_4$-Van)]TP10 caused a 71% reduction in survival of intracellular h-VISA compared to control (no treatment). This compared to a 26% reduction in survival rate when the h-VISA infected cells were treated with TP10 and a 5% reduction when treated with vancomycin. The MICs of all conjugates with h-VISA were in the range of 0.8-1.6 μM, clearly demonstrating their superiority to Van alone (MIC 4 μM). MIC values for two of the conjugates with VRE were 6.3 μM – a marked improvement on the MIC values of Van alone (25 μM) with these strains.

Despite the fact that mastoparan has a marked haemolytic effect, none of the conjugates of Van with TP10 demonstrated toxicity to sheep blood erythrocytes at the concentrations used to achieve a bactericidal effect.

The authors concluded that conjugation of Van with TP10 increases the antibiotic's antibacterial potency mainly against clinical strains of MRSA with improved access to the infected host cells and brain tissue and without significant increase in toxicity.

Conclusion

The results of these two studies indicate that the idea of using CPPs conjugated to vancomycin shows promise as an effective strategy for combating MRSA and Van^R pathogens.

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