The Activity of Glycopeptide Antibiotics Against Resistant Bacteria Correlates with their Ability to Induce the Resistance System

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Running Title: Structure-Activity Study of Glycopeptide Derivatives

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ABSTRACT

Glycopeptide antibiotics containing a hydrophobic substituent display the best activity against vancomycin-resistant enterococci, and they have been assumed to be poor inducers of the resistance system. Using a panel of 26 glycopeptide derivatives and the model resistance system in *Streptomyces coelicolor*, we confirm this hypothesis at the level of transcription. Identification of the glycopeptide structural features associated with inducing resistance gene expression has important implications in the search for more effective antibiotic structures.

Glycopeptides are an important class of antibiotics active against Gram-positive pathogens but vancomycin and teicoplanin are the only two glycopeptide antibiotics currently used in the clinic. They exhibit important differences in activity which are believed to be related to their structural differences, but to date only the mode of action and resistance mechanism to vancomycin has been characterized in detail. The rapid spread of resistance to these two drugs through pathogenic bacterial populations is an acute public health concern and the discovery of additional natural or semi-synthetic glycopeptides with more effective antibiotic activity has been targeted (1). A broad spectrum of vancomycin and teicoplanin derivatives has previously been generated through chemo-enzymatic synthesis, and their activity toward pathogenic enterococcal strains determined (2-9). Interestingly, derivatives containing a hydrophobic substituent were in general found to be significantly more active against both glycopeptide-sensitive and resistant strains. Dong et al. (8) demonstrated that the key functional difference between vancomycin and teicoplanin is due to the absence or presence of lipidation, and evidence that this is related to differing abilities for inducing the resistance system has been obtained in experiments correlating minimum inhibitory concentrations (MICs) with the activity of VanX enzyme

or the activity of reporter protein in a transcriptional fusion assay (10-13), but a direct effect on transcription of the resistance genes has not been investigated. The important implication of this question, that it is possible to produce glycopeptide structures which are invisible to existing inducible resistance systems but which retain significant antibiotic activity, has now stimulated us to seek a definitive answer. Using the vancomycin resistance system in the harmless bacterium *Streptomyces coelicolor* as a model, we assay a panel of different natural and semi-synthetic glycopeptide antibiotic structures for their ability to induce transcription of the *van* gene cluster (14), and the general cell wall stress response sigma factor *sigE* (15), and relate this to the antibiotic activity they exhibit. *S. coelicolor* does not synthesize any glycopeptide antibiotic, but does possess a cluster of seven genes (*vanRSJKHAX*) conferring inducible resistance to vancomycin but not to teicoplanin (similar to the phenotype shown in VanB-type VRE), and it offers a safe and convenient model system for the study of VanB-type glycopeptide resistance (Fig. 1A) (16-21). *sigE* encodes an extracytoplasmic function (ECF) sigma factor (σ^E) which is part of a signal transduction system that senses and responds to general cell wall stress in *S. coelicolor*. *sigE* is constitutively expressed at a low basal level in *S. coelicolor* but is also generically induced by a wide-variety of agents that stress the cell wall (Fig. 1B) (15).

For this study, we have classified all the glycopeptide derivatives analyzed into 4 different groups according to the substituents located at positions 1 and 3, and the presence or absence of a hydrophobic group (Fig. 2). Group 1 includes vancomycin aglycones that carry either a non-hydrophobic carbohydrate or no sugar at all. Group 2 compounds all possess aromatic amino acid residues that are cross-linked into their core peptide backbone as for teicoplanin but are otherwise similar to Group 1. Group 3 are hydrophobic derivatives of vancomycin possessing either a teicoplanin-type monosaccharide containing a saturated lipid or a vancomycin-type disaccharide carrying a chlorobiphenyl residue. Group 4 includes teicoplanin, dalbavancin and related derivatives all

containing a saturated lipid as a hydrophobic substituent. Table 1 reports the MIC of each compound against *S. coelicolor* in liquid culture. Consistent with the previous observations in VRE strains according to Dong et al. (8), all the glycopeptide derivatives containing a hydrophobic substituent (Group 3 and 4) are significantly more active against both vancomycin resistant (wild type) and sensitive (Δ*vanRS*) *S. coelicolor* strains (14). Among all the hydrophobic derivatives, teicoplanin derivatives (Group 4) generally exhibited greater activity than vancomycin derivatives (Group 3). Interestingly, hydrophobic group 3 vancomycin derivatives with a chlorobiphenyl (CBP) substituent were shown to be more active than those with a lipid substituent. To determine the correlation between the MIC of a derivative and its ability to induce the *van* resistance system, the abundance of *vanH* transcripts in RNA isolated from growing liquid cultures of wild type *S. coelicolor* (M600) treated by addition of 10 μg/ml of each glycopeptide derivative was monitored using quantitative real time PCR (qRT-PCR). Samples taken 30, 60 and 90 min after treatment were compared to a preinduction control taken immediately before addition (T0), as previously as described (21). *sigE* transcription was similarly quantified as a reporter for cell wall stress. Consistent with previous results, *vanH* transcription increased immediately in response to vancomycin and reached a maximum level after 30-60 min before beginning to decline (Fig. 3). With the exception of chloroeremomycin, group 1 compounds were typically the best inducers of *vanH* expression, and all, including chloroeremomycin, also induced a strong peak in *sigE* transcript abundance after 30 min. The derivatives in Group 2 behaved similarly, although the maximum level of *vanH* induction was delayed to 60 min, and the level of expression was generally weaker. Strikingly, the Group 3 and 4 derivatives containing hydrophobic substituents exhibited the lowest MIC and all failed to induce *vanH* transcription - except compound 3a which showed only a very weak induction of *vanH* expression - but produced a strong transcriptional response for *sigE*. The order of the *vanH* induction level starting with the best inducer group can therefore be summarized as Group 1 > Group 2 > Group 3 > Group 4, and this result perfectly

97 correlates with the observed MIC result. This implies that the strong activity of glycopeptide
98 derivatives toward vancomycin resistant bacteria is indeed due to their poor ability to induce the
99 resistance system. The hydrophobic substituent presumably prevents productive interaction with the
100 VanS sensor kinase, the key component for triggering the expression of *van* genes, but has no
101 detrimental effect on antibiotic activity. Assessment of the cell wall stress response by monitoring the
102 level of *sigE* transcription allowed the comparison of MIC values with *vanH* transcription to be set in a
103 useful context. Interestingly, *sigE* was significantly induced following exposure to each compound in
104 Groups 1 to 4, but its transcription was quickly and continuously reduced only in cases where *vanH*
105 expression had also been strongly up-regulated (Fig. 3). In contrast, *sigE* transcription remained high or
106 continued to increase if the compound acted as a poor or non-inducer for *vanH* transcription (i.e.
107 Groups 3 and 4). This result implies that expression of the *sigE* system alone is insufficient to produce
108 a recovery from the cell wall stress created by the glycopeptides. Those compounds which failed to
109 induce transcription of *vanH* therefore cause continuous cell wall stress and damage which is in turn
110 reflected in their improved activity against vancomycin resistant strains. A group of damaged
111 glycopeptide derivatives produced by Edman degradation or reductive hydrolysis and exhibiting a
112 significantly reduced affinity toward the D-Ala-D-Ala dipeptide terminus of peptidoglycan precursors
113 were also analyzed (2). Although the damaged derivatives share virtually identical streochemical
114 structures with their corresponding parent glycopeptides, their biological activities are vastly different
115 due to modification of the binding pocket for the D-Ala-D-Ala dipeptide (22, 23). Similar results were
116 obtained in this study where both damaged vancomycin (D-1a) and teicoplanin (D-4a) exhibited no
117 activity in the MIC tests, and failed to induce transcription of either *vanH* or *sigE*. Interestingly
118 however, the MIC test showed that both damaged versions of CBP-vancomycin (D-3f) and dalbavancin
119 (D-4d) retain significant antibiotic activity despite the damage to their D-Ala-D-Ala binding pockets
120 (Table 1 and Fig. 3). In contrast to D-1a and D-4a, both compounds also induced a low but sustained

increase in *sigE* transcription over the 90 min period of the study (Fig. 3). This indicates that these two derivatives possess a second mode of antibiotic action against cell wall biosynthesis in addition to that mediated by binding to the D-Ala-D-Ala termini of peptidoglycan precursors.

This work clarifies the relationship between glycopeptide structure, antibiotic activity and the ability to induce the VanB-type *van* resistance system. By integrating data from MIC studies with reporters for transcription of the *van* resistance (*vanH*) and cell wall stress response (*sigE*) systems in an *S. coelicolor* model, we confirm for the first time that the activity of glycopeptide derivatives previously identified against resistant pathogenic Enterococcal strains can be attributed to an inability to activate transcription of the *van* resistance system. Derivatives with large hydrophobic substituents were shown to be the most successful at evading detection by the VanB-type resistance mechanism while still retaining potent antibiotic activity. Significant activity was also identified in two damaged derivatives whose structures render them incapable of interacting normally with their D-Ala-D-Ala target groups. Such structure-activity data has the potential to inform the future design and production of novel, more effective glycopeptide antibiotic structures.

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FIGURE LEGENDS

FIG 1 A model illustrating organization and regulation of the vancomycin resistance system (A) and the SigE system (B) in *S. coelicolor*.

FIG 2 Chemical structure of glycopeptide derivatives used in this study.

FIG 3 Induction of *vanH* and *sigE* transcription in *S. coelicolor* M600 in response to glycopeptide derivatives. Total RNAs were extracted from each sample and analyzed using qRT-PCR. The X-axis indicates time (min) after addition of the treatment, and the Y-axis shows the fold change in expression relative to the level at time 0. Raw qRT-PCR data are presented in Table S1 and S2. For the detailed experimental procedure, see the experimental section in the supplemental material.

238 **TABLE 1** MIC (μg/ml) of glycopeptide derivatives against *S. coeliicolor* in liquid culture. For

239 experimental details, see the experimental section in the supplemental material.

compounds	*Streptomyces coelicolor*	
	Sensitive (Δ*vanRS*)	Resistant (wild type)
Group 1		
1a vancomycin	0.2	>100
1b vancomycin pseudoaglycone	0.4	>100
1c vancomycin aglycone	<0.3	>100
1d epi-vancomycin	0.2	>100
1e vancomycin + putrescine	<0.1	15
1f chloroeremomycin	<0.1	20
1g balhimycin	0.1	45
Group 2		
2a glucosylated teicoplanin aglycone	<0.1	20
2b teicoplanin aglycone	<0.3	20
2c teicoplanin pseudoaglycone	<0.1	20
2d epi-vanco-Glc teicoplanin	<0.1	10
Group 3		
3a 2-aminodecanoyl-Glc vancomycin	0.3	10
3b 6-aminodecanoyl-Glc vancomycin	0.4	5
3c 6-aminodecyl-Glc vancomycin	<0.1	1
3d C6-CBP vancomycin	<0.1	0.2
3e C6-amino CBP vancomycin	<0.1	<0.1
3f CBP vancomycin	<0.1	<0.1
3g CBP vancomycin + putrescine	<0.1	0.2
Group 4		
4a teicoplanin	<0.1	0.2
4b 2-aminodecanoyl-Glc teicoplanin	<0.1	3
4c 6-aminodecanoyl-Glc teicoplanin	<0.1	0.2
4d dalbavancin	<0.1	<0.1
Damaged glycopeptide derivatives		
D-1a damaged vancomycin	>100	>100
D-4a damaged teicoplanin	>100	>100
D-3f damaged CBP-vancomycin	2	10
D-4d damaged dalbavancin	2	18

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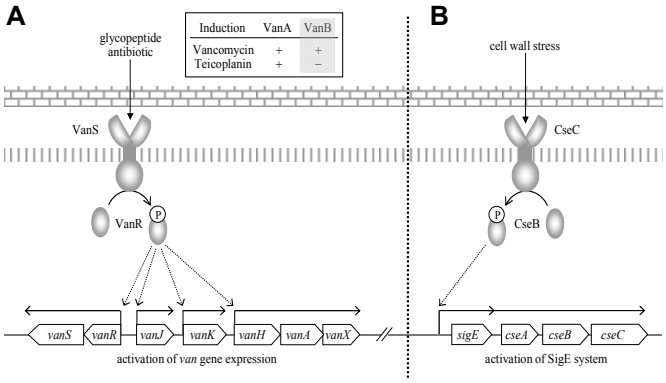


FIG 1 A model illustrating organization and regulation of the vancomycin resistance system (A) and the SigE system (B) in *S. coelicolor*.

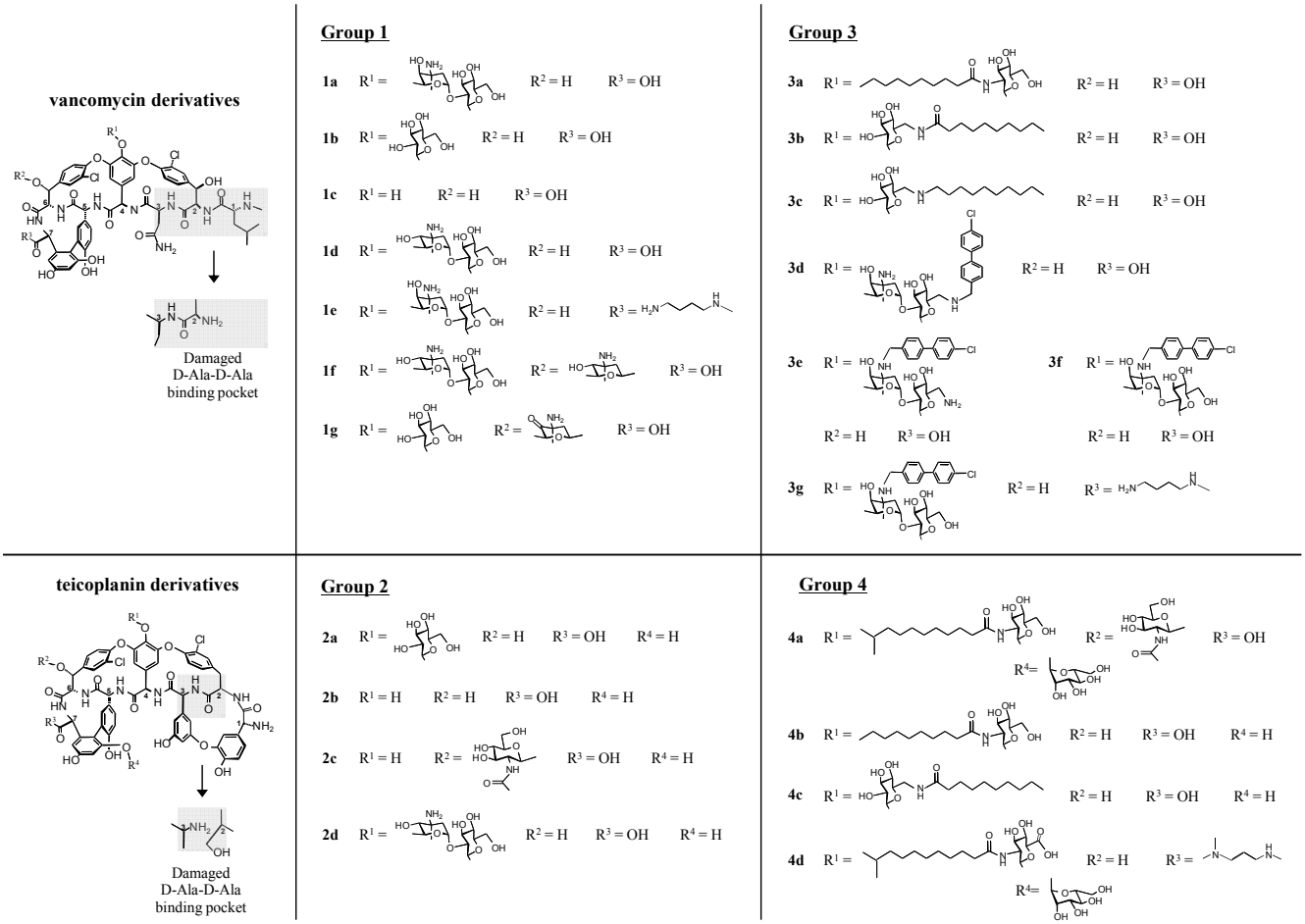
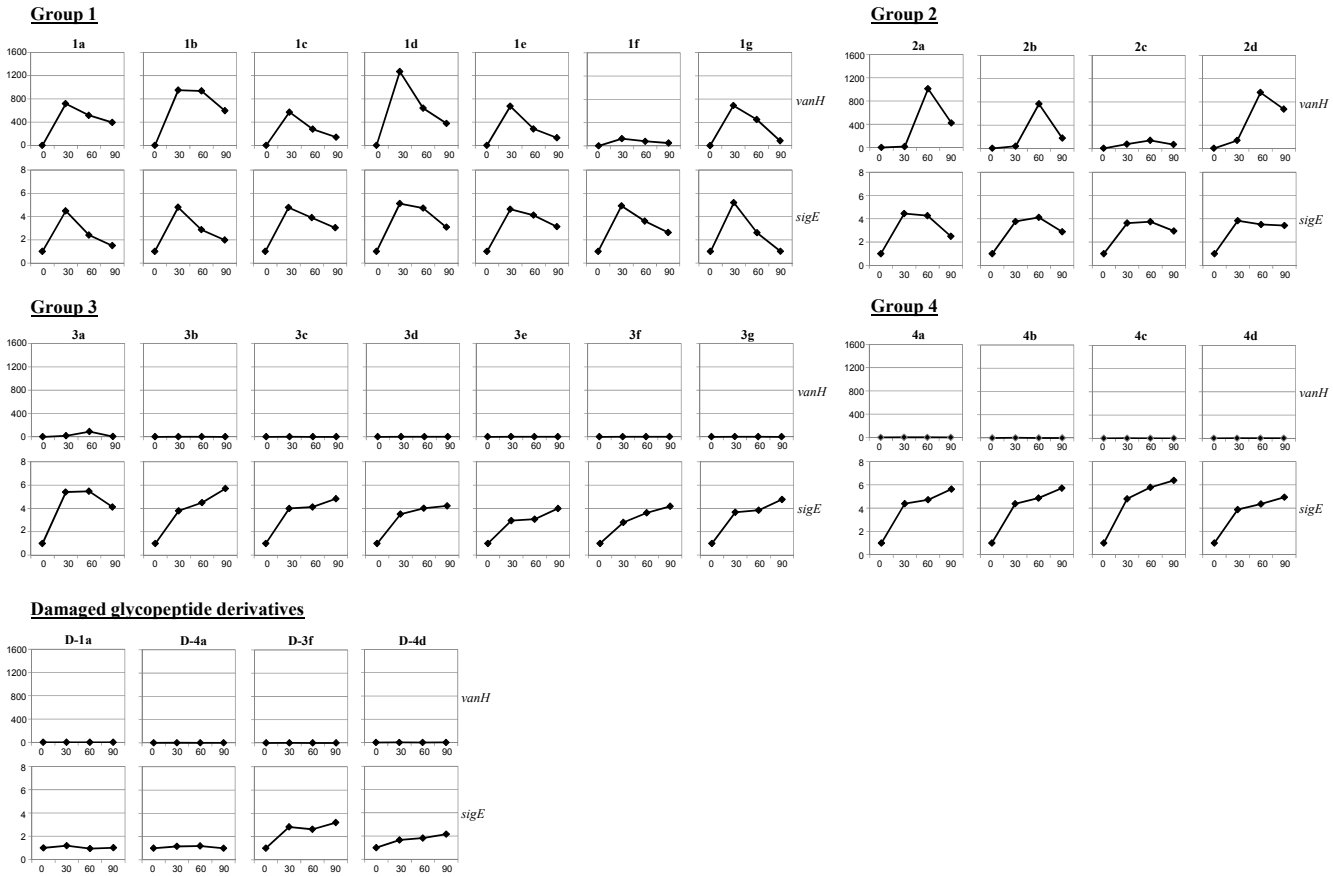


FIG 2 Chemical structure of glycopeptide derivatives used in this study.



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