

A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor

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Inducible resistance to the glycopeptide antibiotic vancomycin requires expression of *vanH*, *vanA* and *vanX*, controlled by a two-component regulatory system consisting of a receptor histidine kinase, VanS, and a response regulator, VanR. The identity of the VanS receptor ligand has been debated. Using a synthesized vancomycin photoaffinity probe, we show that vancomycin directly binds *Streptomyces coelicolor* VanS (VanSsc) and this binding is correlated with resistance and required for *vanH*, *vanA* and *vanX* gene expression.

The emergence of resistance to the glycopeptide antibiotic vancomycin (**1**) has had an enormous impact on the health care sector. Vancomycin binds to the acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala) side chain of the muramyl pentapeptide component of bacterial peptidoglycan, thereby interfering with cell wall biosynthesis. The near ubiquity of the D-Ala-D-Ala terminus in bacterial peptidoglycan and the fact that the antibiotic binds to a substrate rather than an enzyme (which could be susceptible to mutation) provided some confidence to the infectious disease community that vancomycin resistance would be slow to emerge. Nevertheless, glycopeptide resistance did indeed emerge in pathogenic enterococci in 1988 (ref. 1). This consequence followed a significant increase in use of oral vancomycin and its lipoglycopeptide analog teicoplanin for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA), along with the widespread use of an analog (avoparcin) in animal feed. Twenty years later, vancomycin resistance has become a serious clinical problem, with a growing prevalence in enterococci and more recently in the more virulent staphylococci².

High-level resistance to vancomycin in pathogens is the result of expression of the *vanH*, *vanA* and *vanX* genes. The expression of these genes results in the formation of alternate peptidoglycan precursors that terminate in the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) rather than D-Ala-D-Ala. Incorporation of D-Ala-D-Lac into the bacterial cell wall confers resistance to vancomycin and other glycopeptides, which only bind to D-Ala-D-Ala-containing precursors^{3,4}. The genes *vanH*, *vanA* and *vanX* respectively encode enzymes responsible for D-Lac synthesis from pyruvate, ATP-dependent D-Ala-D-Lac ligation, and cleavage of endogenous D-Ala-D-Ala that continues to be produced by the cell's constitutive peptidoglycan biosynthetic machinery. The origin of this three-gene cassette is likely a reservoir of vancomycin-resistant bacteria in the environment⁵, as these genes are also found in several nonpathogenic bacteria, including members of the actinomycetes that produce glycopeptide antibiotics⁶ and even some that do not produce these compounds⁷.

Inducible expression of the *vanH*, *vanA* and *vanX* cassette in both pathogens and nonpathogens is controlled by a two-component regulatory system consisting of a transmembrane receptor histidine

kinase, VanS, and a cytoplasmic response regulator, VanR^{8–10}. Most effort has been concentrated on the VanR and VanS systems associated with the clinically important enterococcal VanA strains (resistant to vancomycin and the lipoglycopeptide teicoplanin) and VanB strains (resistant to vancomycin but not teicoplanin). More recently, there has been analysis of VanR and VanS systems found in actinomycetes, the order of bacteria that make all of the known glycopeptides¹⁰. The VanS proteins from enterococcal VanA and VanB strains are only distantly related (16% overall identity), and their sensor domains are not related in amino acid sequence. The enterococcal VanS_A and VanS_B proteins are also very diverged from their actinomycete equivalents¹⁰.

Exposure of cells to vancomycin triggers autophosphorylation of VanS on a cytosolic histidine residue and subsequent transfer of this phosphoryl group to an aspartate residue of VanR, resulting in transcriptional activation of the *vanH*, *vanA* and *vanX* resistance cassette (Fig. 1a). Though it has been known for some time that vancomycin and other glycopeptides can trigger *vanH*, *vanA* and *vanX* expression through the action of VanR and VanS⁸, there has been no agreement on whether this is the result of direct binding of the antibiotic to the VanS receptor or whether VanS activation is triggered by cell wall intermediates that accumulate as a result of antibiotic action. Indeed, several indirect studies—including the use of reporter gene constructs under control of *van* promoters, measurement of VanX dipeptidase activity, and monitoring of Lac-containing precursors or induction of vancomycin resistance in pretreated cultures—have supported the latter model rather than direct binding of the antibiotic, though there has been little consensus on the precise nature of the hypothesized stimulatory intermediates^{7,10–18}.

To directly address this question, we prepared the vancomycin photoaffinity probe (VPP, **3**; Fig. 1a, Supplementary Scheme 1, Supplementary Methods and Supplementary Results). VPP includes a biotin tether for purification of complexes and visualization by antibiotin antibodies, and a benzophenone photoaffinity probe¹⁹ for covalent labeling of protein-probe complexes. We linked the benzophenone to the primary amino group of the vancosamine sugar since it is known that second-generation vancomycin derivatives in clinical trials such as oritavancin and dalbavancin tolerate alkylation at this site²⁰. Similarly, based on the dalbavancin precedent and other similar known molecules, we predicted that modification at the C terminus by the biotin tether would not impact antibiotic activity. Indeed, VPP retained comparable antibiotic activity against glycopeptide-sensitive Gram-positive bacteria but not vancomycin-resistant strains (Table 1). Furthermore, *in vitro* titration analysis using isothermal titration

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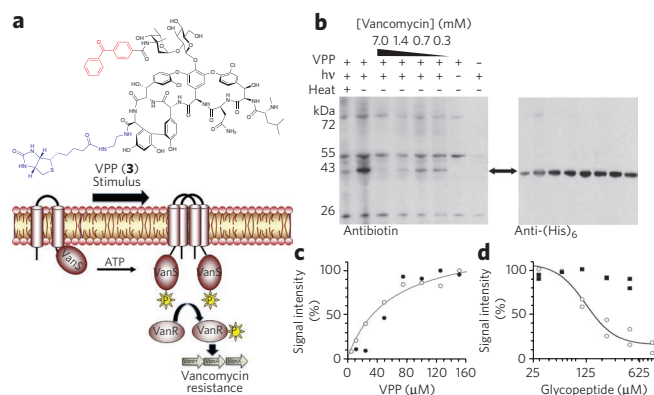


Figure 1 | VPP covalently labels VanS. (a) Structure of VPP and organization of the VanR and VanS two-component system. VPP consists of the vancomycin backbone (black) linked to a benzophenone photoaffinity label (red) and a biotin linker (blue). Vancomycin and VPP trigger phosphorylation of the two-component histidine kinase VanS. Phospho-VanS in turn phosphorylates the transcriptional activator VanR. Phospho-VanR induces the transcription of the vancomycin resistance genes *vanH*, *vanA* and *vanX*, which convert the cell wall structure to one terminating in glycopeptide antibiotic-resistant D-Ala-D-Lac. (b) Covalent labeling of VanSsc by VPP. A membrane protein sample of *E. coli* BL21 cells expressing histidine-tagged VanS from *S. coelicolor* was incubated with VPP (10 μM) and irradiated at 365 nm followed by separation of proteins by 4–12% gradient SDS-PAGE. Biotinylated proteins were visualized by antibiotin antibody (left panel) and identified a strongly labeled 43 kDa protein corresponding to the predicted molecular mass of His₆-VanSsc. Equivalent loading of protein in each well was confirmed with anti-His₆ antibody (right panel). Addition of vancomycin decreased incorporation of VPP. (c) VPP labeling of His₆-VanSsc is saturable with apparent K_d of 56 ± 12 μM; solid and hollow circles represent independent replicates. (d) Quantitative competition experiments show that vancomycin competes with VPP with an IC_{50} of 124 ± 12 μM (hollow circles), but teicoplanin does not (solid squares).

calorimetry (Supplementary Fig. 1) showed that VPP bound di-*N*-Ac-Lys-D-Ala-D-Ala with a K_d of 17.7 ± 2.6 μM, which compared well to benzophenone-vancomycin (compound 2; Supplementary Scheme 1; 26.0 ± 3.7 μM) and vancomycin (10.5 ± 1.9 μM). VPP is therefore a valid probe of vancomycin activity.

We chose to study the interaction of VPP with VanS proteins from the actinomycetes *Streptomyces coelicolor* (VanSsc) and *Streptomyces toyocaensis* (VanSst) as examples of tractable systems resistant and sensitive to vancomycin. *S. coelicolor* does not produce a glycopeptide antibiotic but nevertheless is resistant to vancomycin but not the lipoglycopeptide teicoplanin⁷ (the VanB phenotype). On the other hand, *S. toyocaensis* produces the glycopeptide antibiotic A47934, to which it is resistant, yet it is sensitive to both vancomycin and teicoplanin²¹. As expected for a vancomycin analog, *S. coelicolor* is resistant to VPP whereas *S. toyocaensis* remains sensitive to this compound (Table 1). Similar results were obtained with compound 2; therefore, modification of the vancosamine

sugar with benzophenone does not recapitulate a teicoplanin-like activity as it does with other alkylations, such as by biphenyl²².

We expressed recombinant *Streptomyces* VanS proteins as His₆-tagged proteins in *Escherichia coli* to provide a sensitive tag to measure the presence of the histidine kinases. We were careful not to significantly overexpress the proteins in order to enrich in membrane proteins in native conformation and noted that all VanS was associated with purified membranes as detected by anti-His₆ antibodies. Incubation of membranes isolated from *E. coli* expressing VanSsc with VPP followed by irradiation labeled a membrane protein of 43 kDa with biotin (the mass of His₆-VanSsc is 41,687 Da and the mass of VPP is 1,923 Da) (Fig. 1b). At least two other proteins were labeled by VPP in *E. coli* membranes, one at 55 kDa and the other at 72 kDa; we did not explore these further, but note that interaction of vancomycin with cell wall assembly proteins of similar size range has been previously reported in this vancomycin-insensitive bacterium²³.

The labeling of VanSsc was dependent on VPP concentration and saturable with apparent K_d of 56 ± 12 μM (Fig. 1c). Furthermore, photolabeling was blocked by the addition of free vancomycin (Fig. 1d; half-maximal inhibitory concentration (IC_{50}) = 124 ± 12 μM), or by boiling of the membranes. Neither the lipoglycopeptide teicoplanin (Fig. 1d), which does not induce the vancomycin resistance phenotype in *S. coelicolor*⁷, nor the β-lactam antibiotic penicillin G (not shown) had any effect on photolabeling of VanSsc by VPP. Tryptic digestion of the labeled protein followed by mass spectrometry unequivocally identified the labeled protein as VanSsc (Supplementary Fig. 4c). These results demonstrate that VanSsc specifically binds the vancomycin analog VPP and is covalently labeled by this photoaffinity probe.

We next explored labeling of VanSst from the vancomycin-sensitive *S. toyocaensis* (Supplementary Fig. 2). We were able to detect VPP labeling of the protein overexpressed in *E. coli* with an apparent K_d of 55 ± 17 μM (Supplementary Fig. 2a). However, labeling still occurred in boiled membranes (Supplementary Fig. 2b), and neither vancomycin nor a glycopeptide biosynthetic product of *S. toyocaensis*, desulfo-A47934, competed with VPP (Supplementary Fig. 2c,d). We therefore conclude that this labeling is not biologically relevant—that is, that it does not trigger VanS autophosphorylation. This is consistent with the observation that vancomycin does not induce glycopeptide antibiotic resistance in *S. toyocaensis*²¹.

These results using recombinant proteins in *E. coli* were confirmed using membranes isolated from *S. coelicolor* and *S. toyocaensis* where a band consistent with the size of VanS (~40 kDa) was labeled in the former but not the latter (Fig. 2a). Labeling of the *S. coelicolor* protein was inhibited by the addition of vancomycin, consistent with experiments with recombinant VanSsc. Furthermore, photolabeling of a protein of similar size was not detected in a strain of *S. coelicolor* where the gene encoding VanSsc was deleted (Fig. 2b). VPP is therefore a VanSsc ligand.

S1 nuclease mapping of *vanH*, *vanA* and *vanX* transcript production in the presence and absence of VPP revealed that the photolabel induces gene expression in *S. coelicolor* but not *S. toyocaensis*

Table 1 | Minimum inhibitory concentration of glycopeptide derivatives against selected bacteria

Antibiotic	Vancomycin-resistant				Vancomycin-sensitive		
	VRE VanA	VRE VanB	<i>S. coelicolor</i> (M600)	<i>S. coelicolor</i> Δ <i>vanS</i> (J3200)	<i>S. coelicolor</i> Δ <i>vanSR</i> (J3201)	<i>S. toyocaensis</i>	<i>Micrococcus luteus</i>
VPP	>512	64	64	4	1	<0.25	0.4
Vancomycin	>512	32	32	4	1	<0.25	0.4
Teicoplanin	512	<1	<0.25	4	0.5	<0.25	<0.25

The minimum inhibitory concentration (MIC, μg ml⁻¹) is defined as the lowest concentration of the drug necessary to inhibit bacterial growth. The compounds were tested against *Enterococcus faecalis* VanA clinical isolate, donated by A.K. Petrich (Department of Pathology and Molecular Medicine, McMaster University), *E. faecalis* ATCC51299 (VanB), *S. coelicolor* M600 (wild type), *S. coelicolor* (Δ*vanS*), *S. coelicolor* (Δ*vanSR*), *S. toyocaensis* NRRL 15009 and *Micrococcus luteus*.

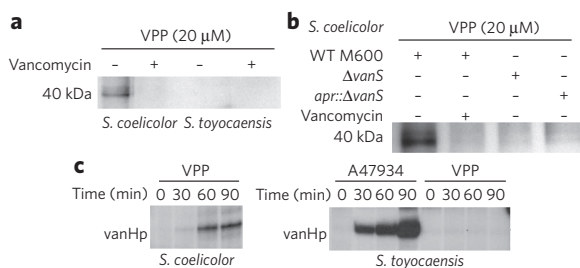


Figure 2 | VPP binding to VanS correlates with *vanH*, *vanA* and *vanX* gene expression.

(a) VPP labels membrane fractions from wild-type *S. coelicolor* but not from wild-type *S. toyocaensis* when treated with VPP (20 μM) in the presence or absence of vancomycin (2.5 mM). Biotinylated proteins were affinity purified with streptavidin-agarose beads and resolved by 12% SDS-PAGE. (b) VPP labels an ~40-kDa protein in membrane fractions from wild-type *S. coelicolor* but not in equivalent membrane fractions from two Δ vanS deletion strains. Images of uncropped gels can be found in **Supplementary Figure 3**. (c) Induction of the *vanH* promoter (*vanHP*) in wild-type *S. coelicolor* and wild-type *S. toyocaensis* in response to VPP and A47934, the teicoplanin-class glycopeptide produced by *S. toyocaensis*. Germinated spores were grown at 30 °C in NMMP liquid medium⁷ to an optical density at 450 nm of ~0.4 before the addition of the glycopeptide derivatives (10 μg ml⁻¹). RNA was extracted from samples taken immediately before (*t* = 0 min) and at 30 min intervals after the addition of glycopeptide and analyzed by an S1 nuclease protection assay. In both species, the *vanH* transcript was detected using a 0.26-kilobase PCR probe generated using a ³²P 5' end-labeled oligonucleotide primer.

(Fig. 2c). Coupled with the drug resistance phenotype (Table 1), we conclude that VPP is a bioactive VanS ligand. VanSsc therefore can bind glycopeptide antibiotics (or possibly their lipid II complexes) and induce resistance *in vivo*.

Hydropathy analysis predicts a topology model of VanS with an N-terminal extracytoplasmic receptor domain lying between two transmembrane helices and a C-terminal intracellular histidine kinase domain (Fig. 1a). We reasoned that the N terminus, which is exposed to the cell exterior, was the likely site of labeling by VPP, which we predicted was membrane impermeant. Several attempts were made to identify VPP-labeled peptides in full-length His₆-VanSsc by mass spectrometry, but these were unsuccessful. However, though we were easily able to obtain tryptic and chymotryptic peptide coverage for the majority of the protein, residues 22–43 and 77–87 (numbering for the untagged wild-type VanSsc) from the N-terminal region encompassing the two transmembrane helices and the intervening 27-residue predicted extracytoplasmic domain were always absent (Supplementary Fig. 4c). Therefore, to determine which part of VanSsc is modified by VPP, we treated VPP-labeled VanSsc with BNPS-skatole (3-bromo-3-methyl-2-(2-nitrophenyl) thiol-3H-indole) under conditions that favor protein cleavage at Trp–Xxx junctions. There are four tryptophan residues in His₆-VanSsc, at positions 41, 68, 82 and 274. Resolution of the products of BNPS-skatole digestion on 20% (w/v) Tris-tricine gels revealed a single 9-kDa band that was labeled by both anti-His₆ and antibiotin antibodies. Because the histidine tag is N-terminal in His₆-VanSsc, the colabeling of the 9-kDa peptide by both anti-His₆ and antibiotin antibodies (Supplementary Fig. 4b) demonstrates that VPP modifies the N-terminal peptide 1–41. This region includes the putative first transmembrane helix and the first four residues, DQGW, of the predicted extracytoplasmic receptor domain; these four residues were consistently not observed by mass spectrometry of tryptic and chymotryptic digests of VanSsc, either before or after labeling with VPP (Supplementary Fig. 4c).

Understanding the molecular basis for induction of antibiotic resistance is essential for the proper deployment of antibiotics in

the clinic and for the development of new agents. Here we resolve for the first time the nature of an inducing ligand linked to expression of vancomycin resistance genes using a chemical biology strategy. This work resolves a long-standing controversy in the glycopeptide antibiotic resistance field. That is, for at least some VanS sensor kinases associated with the VanB phenotype (vancomycin resistance and teicoplanin susceptibility), the antibiotic itself is a ligand that induces drug resistance. If generally applicable to clinical strains, this discovery provides a simple biochemical assay for direct screening of new antibiotics and agents that could block induction of resistance. Further, it could provide a screen for glycopeptide modifications that block productive interaction with VanS, thereby identifying compounds that would kill vancomycin-resistant bacteria.

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Author contributions

K.K. synthesized the compounds, performed labeling experiments and prepared the figures. H.-J.H. prepared genetic constructs and performed the gene expression studies. X.D.W. prepared genetic constructs. I.N. prepared genetic constructs and assisted in protein preparation. M.J.B. and G.D.W. designed and analyzed the experiments. D.H. performed the NMR. M.J.N. performed the mass spectrometry. G.D.W. wrote the manuscript, and all authors edited it.

Competing financial interests

The authors declare no competing financial interests.

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