Resistance: How VanA Strains Get Away With It

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IN BRIEF

The longstanding invincibility of vancomycin (VC) – the last line of antimicrobial therapy for life-threatening Gram-positive infections – has been compromised over the past decade. The most common and best understood mode of resistance, the VanA type, consists of the replacement of an ester O for an amide NH in the peptide target recognized by the glycopeptide antibiotic. VC targets the C-terminal D-Ala-D-Ala sequence of the peptide portion of Lipid II, an essential intermediate in bacterial cell wall synthesis; binding of VC eventually results in a compromised bacterial cell wall and ultimately cell death. A single heavy atom substitution by VanA resistant bacteria reduces the binding affinity and efficacy of VC by 1000-fold. A lost hydrogen bond and an attendant repulsive lone pair-lone pair interaction, both of which are brought on by the O for NH substitution, are implicated in the VanA resistance mechanism.

In an effort to determine the relative importance of these effects, McComas *et al.* in a 2003 *JACS* paper measured the binding constants for bacterial peptide analogues to VC having the terminal residue connected to the rest of the peptide by an amide NH, a methylene group, or an ester O. The methylene-containing ligand bound to VC with an affinity 10 times less than the amide-containing ligand, but 100-fold greater than the ester-containing ligand. From these results the authors concluded that the repulsive interaction introduced by the ester O is the most important for VanA-type resistance. Further, a cooperatively-orchestrated enthalpy-entropy compensation effect accentuates the exogenicity imparted by the additional H-bond in the amide-containing ligand, and the endogenicity of the repulsive interaction suffered by the ester-containing ligand. A mixture of advantageous and adverse factors for binding are invoked to rationalize the intermediate binding affinity of the methylene-containing ligand. The differential binding of ligands with such subtle variations illustrates how numerous small forces, working collectively, govern this molecular recognition event.

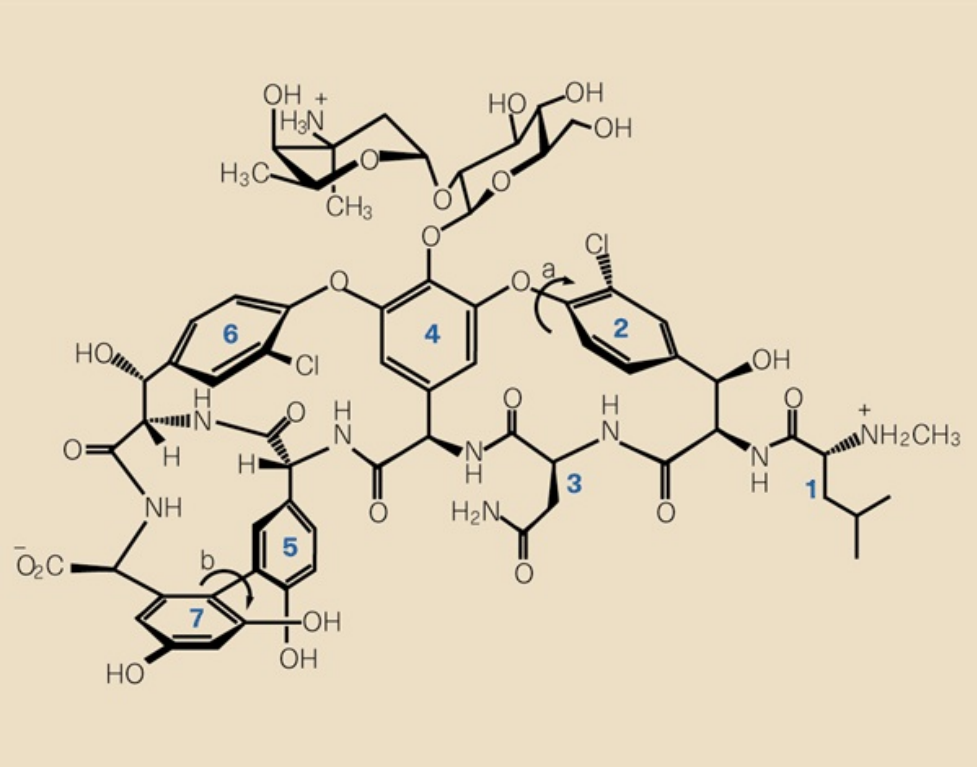

Figure 1. Vancomycin, VC. Figure by permission from Nature 1999, 397, 567-568.

VANCOMYCIN AND VANA RESISTANCE: A PRIMER

Vancomycin (VC), an antibiotic first isolated from soil bacteria inhabiting the jungles of Borneo, has served for decades as the last (and sometimes only) resort of the clinician against life-threatening, opportunistic Gram-positive bacteria.[1-3] VC was named in the 1950s for its ability to vanquish all strains of *Staphylococci*, and the performance of the antibiotic against the 'superbug' methicillin-resistant*Staphylococcus aureus* (MRSA) in subsequent years has been almost legendary.[3] MRSA has evaded, via natural selection, virtually all other clinically approved antibiotics, including cephalosporins, tetracyclines, aminoglycosides, erythromycin and the sulfonamides.[1,2] MRSA infections accounted for 60% of all *S. aureus*infections in U.S. intensive care units as of 2003, and MRSA infections are becoming frequent in non-clinical (i.e., community acquired) settings.[4] In addition to combating the MRSA menace, VC is effective against *Enterococcal* bacterial infections, which are potentially lethal to an expanding population of immunocompromised cancer, AIDS, elderly, and organ transplant recipient patients.[1-3] VC has been used to treat infections for patients allergic to β-lactam antibiotics such as penicillin, post-operative *Clostridium difficile* infections, and is a frontline therapy for endocarditis (inflammation of the tissue lining the heart).[5,6]

The indispensable bastion of effective antimicrobial defense provided by VC is now under siege by the spread of bacterial resistance in recent decades.[2,3] Intense research efforts have sought to elucidate the structure and mechanism of action of VC to enable the development of derivatives that are capable of forestalling the assault by multi-drug resistant bacteria that would return us to the pre-antibiotic era of untreatable, life-threatening infections.[2,3]

VC (Fig. 1), the archetypal glycopeptide antibiotic, consists of a heptapeptide core composed of some non-standard amino acids.[1,3,7] From the N-terminus, VC has β-hydroxychlorotyrosine residues at positions 2 and 6, *p*-hydroxyphenylglycine residues at positions 4 and 5, and a 3,5-dihydroxyphenylglycine residue at the C-terminus. Some glycopeptide antibiotics lack the β-hydroxyl and/or chlorine substituents of residues 2 and 6. Variation among glycopeptide antibiotics also consists in the use of aliphatic or aromatic residues at positions 1 and 3 from the N-terminus. VC employs an N-methylated leucine and an asparagine for residues 1 and 3, respectively. Unlike a β-pleated sheet

strand, the stereochemistry of the peptide backbone of all known glycopeptide antibiotics is not uniformly R or S; from the N to C termini, it is R, R, S, R, R, S, S. This stereochemical pattern along the backbone endows glycopeptide antibiotics with a bowl-shape (PDB Jmol; PDB 1FVM) that is critical to antimicrobial action.[1] The heptapeptide core of all glycopeptide antibiotics is adorned with sugars to varying extents.[1,8] In the case of VC, the side chain of the fourth residue is glycosylated with an L-vancosaminyl-α(1→2)-D-glucopyranosyl disaccharide.[1,8]

Structural rigidity is imparted to VC and other glycopeptide antibiotics by cross-linking of the aromatic side chains of residues 2, 4, and 6 to form a two biphenyl ethers with a shared central ring.[3,7] Hindered rotation about the ether bonds on the chlorinated aromatic rings of residues 2 and 6 locks them into one configuration, endowing VC with two sites of the uncommon phenomenon of atropisomerism.[3] A third site of atropisomerism results from the cross-linking of the aromatic rings of residues 5 and 7 to form a biaryl moiety. Rotation about the biaryl bond is restricted by the substituents of the aromatic rings, as well as the constrained nature of the 12-member macrocycle that contains the biaryl moiety.[3] In fact, the peptide bond spanning residues 5 and 6 assumes the less stable s-*cis* (as opposed to the usual s-*trans*) configuration to accommodate the atropisomer shown for the biaryl unit (PDB Jmol).[3] Consideration of the fact that VC has 18 chiral centers, in addition to 3 elements of atropisomerism, reveals that the antibiotic is a single stereoisomer out of more than 2 million possible structures.[3] As a consequence of these structural features, VC is one of the smallest natural products to exhibit stereospecific molecular recognition.[9]

VC was the first example of an antimicrobial agent that targets a specific bacterial membrane precursor essential for cell wall synthesis.[7] The target, known as Lipid II, is the monomer used to build up the cell wall; VC therefore binds a substrate rather than an enzyme (as many antibiotics) involved in a cellular process critical to survival and replication. All bacterial cell walls are composed of polymeric strands of alternating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), that are crosslinked for mechanical strength via a peptide framework (Fig. 2a).[7,10] Before cross-linking occurs, peptide chains appended to the MurNAc units have the sequence L-Ala-D-Glu-L-Lys-D-Ala-D-Ala.[7,10] In essence, a bacterial cell is effectively jacketed by a single polymeric molecule, known as peptidoglycan, capable of withstanding the positive osmotic pressure of the cell in order to prevent lysis and bacterial death.[10] The disaccharide-peptide monomeric subunit of the peptidoglycan, connected to a membrane-embedded anchor, is transported to the exterior of the plasma membrane for peptidoglycan assembly (Figure 2a).[7,10] The membrane-anchored peptidoglycan precursor subunit (Fig. 2b) is itself Lipid II.[7] In the extracellular space, a transglycosidase enzyme joins the disaccharide of the monomer to the growing glycan strand.[7,10] A transpeptidase enzyme (PBP1b is a “large penicillin binding protein” that contains both the transglycosidase and transpeptidase domain.) then recognizes the C-terminal D-Ala-D-Ala sequence of the pendent peptide of the former monomer, cleaves off the terminal D-Ala, and couples the peptide fragment to an adjacent peptide of the peptidoglycan, thereby freeing the membrane anchor of its cargo.[2,3,7] The survival of bacterial cells depends on the recycling of the membrane anchor that transports precursor monomers for continued peptidoglycan growth.[7]

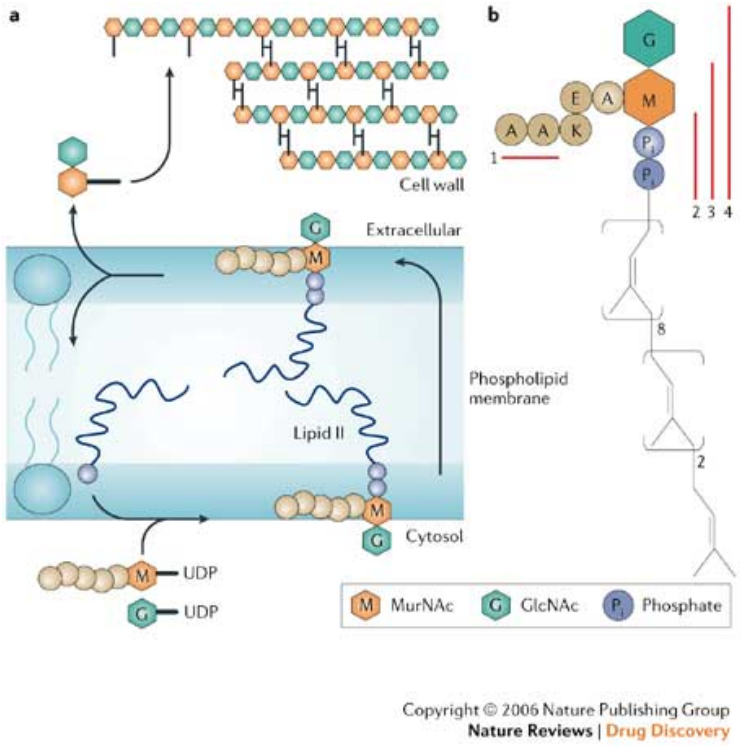

Figure 2. Schematic representation of the bacterial cell wall synthesis cycle. (a) A disaccharide with a pendent pentapeptide is assembled on a membrane-anchored carrier to produce Lipid II, which then transports the monomer for peptidoglycan polymerization from the cytoplasmic to extracellular side of the membrane. The membrane-anchored portion delivers the cargo to the growing cell wall, and is recycled back to the cytoplasmic side to continue the process. (b) Structure of Lipid II. The membrane-anchored carrier is a polyisoprenoid consisting of eight isoprene units in the cis-conformation, followed by two units in the trans-conformation, and the terminal isoprene unit. The cell wall monomer carried by the polyisoprenoid contains a disaccharide with a pendent pentapeptide. The residue 3 lysine of the pentapeptide is coupled to the glutamate at position two via the side chain carboxylate. Figure from [7] and used with permission.

VC easily diffuses through the layers of peptidoglycan that enclose a Gram-positive bacterial cell to reach the site of peptidoglycan polymerization (Fig. 2).[3] Unlike Gram-positive bacteria, Gram-negative bacteria have an outer membrane beyond the peptidoglycan that is impermeable to VC, rendering Gram-negative bacteria intrinsically resistant to it.[10] At the site of cell wall synthesis, the bowl-shaped antibiotic recognizes the C-terminal D-Ala-D-Ala sequence of un-crosslinked peptidoglycan precursor peptides (Fig. 3), and binds with high affinity (K_a = ~10^6 M^{-1}, determined in aqueous buffer with a pH of 4.7-5.1 at 298 K).[6,11] The antibiotic forms five hydrogen bonds as well as an extensive array of hydrophobic contacts (PDB Jmol).[6,10,11] VC sequesters the Lipid II substrate, thereby sterically occluding the approach and action of the transglycosidase/transpeptidase enzyme, and shuts down the recycling of its membrane anchor component.[7,10] Unable to either polymerize and cross-link VC-bound monomeric precursor, or recycle the membrane anchors bearing the VC-precursor complexes to bring new monomeric units to the cell wall, peptidoglycan biosynthesis is halted. Meanwhile, enzymes that remove old layers of peptidoglycan that would ordinarily be replaced continue unabated.[3] As the disassembly of the peptidoglycan decreases its mechanical strength, the bacterial cell becomes susceptible to osmotically-induced lysis.[10]

Roughly at the same time the molecular details of the antibacterial action of VC were finally elucidated after decades of research (see below), the first cases of VC-resistant *Enterococci* were reported in 1988, and of *Staphylococcus aureus* in 1997.[1-3] The most common strains of *Enterococci* and MRSA resistant to VC in the U.S. are of the VanA type, which is the best understood mode of resistance.[3,14] VanA-type bacteria synthesize their peptidoglycan normally in the absence of VC, with precursors for the peptide framework terminating in consecutive D-Ala residues.[1,3] If a VanA cell detects the glycopeptide antibiotic, however, an alarm signal is transduced that results in the

production of a trio of proteins (vanHAX) that work in concert to remodel the peptidoglycan.[3,10] The newly minted enzymes reduce pyruvate to D-lactate, hydrolyze the D-Ala-D-Ala dipeptides of the normal cell wall biosynthetic pathway, and condense D-Ala with D-lactate for use as the C-terminal residues of peptidoglycan precursor peptides.[3,10] Overall, an O is substituted for an NH in the conversion of an amide to ester linkage. This transformation results in a remarkable 1000-fold reduction in the binding affinity of VC.[6] Though not without exceptions, the VC literature has attributed the drastic decline in binding affinity (and antibiotic efficacy) to the deletion of an H-bond. In a review of the VanA mode of resistance in *Enterococci*, Walsh (who, with Courvalin, elucidated the molecular logic of vanA resistance), concludes, "The loss of one H-bond is the elegant and simple solution that can spell life or death to the bacterium and, perhaps, to an infected patient."[10]

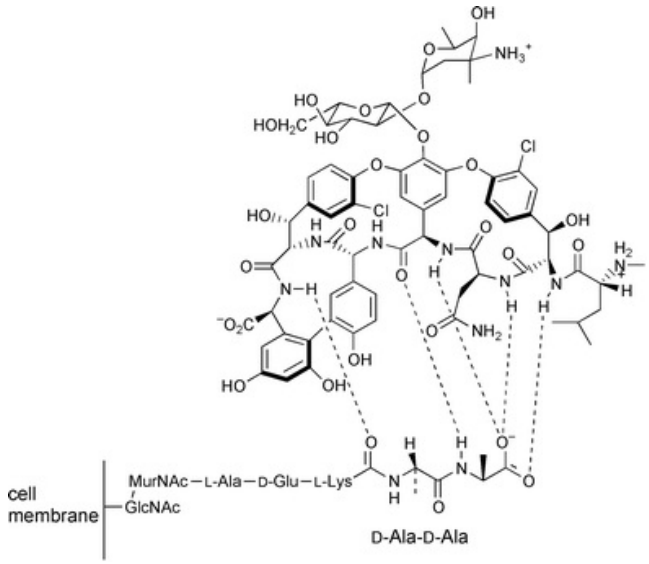

Figure 3. VC recognizes the consecutive D-Ala-terminating sequence of the membrane-anchored disaccharide-pentapeptide intermediate of bacterial cell wall synthesis, and binds by (a) forming five hydrogen-bonds as well as (b) hydrophobic contacts with the methyl groups of the D-Ala residues packed against aromatic rings of the antibiotic. Figure from Nat. Prod. Rep. 2002, 19, 100-107 with permission.

VANCOMYCIN MOLECULAR RECOGNITION EVENTS

An essential step in the molecular recognition of D-Ala-D-Ala by VC in water is desolvation of the ligand, as well as the binding site the ligand will occupy.[9] A computational study, in agreement with some experimental observations, suggested that the amide NH groups of residues 3 and 4 rotate by 180° underneath the bis-diphenyl ether moiety (Fig. 1a) at ordinary temperatures, populating conformers where the NH units of these residues are alternatively on the concave and convex faces of VC.[9] Rotation of the amide NH groups is predicted to distort the macrocycle, and in turn, cause dramatic fluctuations in the shape and size of the binding pocket that destabilize and expel formerly ordered water molecules. This phenomenon of 'macromolecular breathing' is accompanied by the stripping away of H-bonded waters from two of the amide groups needed for molecular recognition, because the NH groups pass through a macrocycle too small for solvating waters to follow along. Experimental evidence suggested that the asparagine sidechain of residue 3 assists in the regulation of binding site solvation by serving as an intramolecular flap.[8] The sidechain acts as a surrogate ligand in the absence of the target peptide to limit solvation of the highly polarized pocket, and then swings out of the way to permit ligand recognition.

In this view, as the amide NH groups of residues 3 and 4 rotate from the convex to concave side of VC, the antibiotic welcomes the D-alanyl carboxylate terminus of a peptidoglycan precursor peptide into a relatively desolvated pocket.[9] The pocket is lined with the partial positive poles of the amide NH groups from residues 2, 3, and 4, which are crowded together and aligned to form fresh H-bonds to the anionic moiety of the ligand (Fig. 3).[1,9] The electrostatic interactions that underlie the three normal H-

bonds formed between VC and the carboxylate terminus of the ligand are strengthened by the folding inward of the isobutyl side chain of the N-methylleucine residue (PDB Jmol).[8] The hydrophobic wall formed by the isobutyl side chain of VC lowers the dielectric constant of the binding pocket and shields the antibiotic-ligand carboxylate H-bonds from direct competition with water.[8] The three amide NH groups of the receptor pocket successfully compete with water to solvate the carboxylate of the ligand, because water molecules, unconstrained by a template, cannot position their oxygen atoms close together without paying an enthalpic price.[1] For the amides of the binding pocket, the enthalpic price for the close proximity of parallel dipoles is prepaid by the expenditure of energy during antibiotic biosynthesis.[1] The inversion in stereochemistry of residue 3 (S), relative to the all R stereochemistries of residues 1, 2, and 4, destabilizes conformations of the peptide backbone relative to the binding conformation, and rigidity within this conformation imparted by side chain cross-links removes a considerable fraction of the adverse entropy that would be incurred upon bimolecular association.[1,8,12]

VC has additional interactions with peptidoglycan precursor peptides terminating in consecutive D-Ala residues.[1] The amide carbonyl belonging to residue 4 in VC accepts an H-bond from the amide NH of the terminal Ala in the ligand (Fig. 3). The amide NH of residue 7 in the antibiotic donates an H-bond to the amide carbonyl of the antepenultimate Lys residue in the ligand (Fig. 3). It is believed that the direct contribution of these two amide-amide H-bonds to the binding affinity of peptidoglycan precursor peptides and their analogues is minimal.[1] *N*-acetylglycine, relative to acetate, can form the first of the amide-amide H-bonds being discussed, and only associates with VC with a Gibbs free energy of binding (ΔG^{o}) more negative by ~0.6 kcal/mol at 303 K in an aqueous buffer of pH 6.[1,13] For comparison, VC binds ligands (e.g., di-acetyl-Lys-D-Ala-D-Ala, Fig. 3b) capable of making most of the interactions of the natural target pentapeptide (Fig. 3a), with a ΔG^{o} of –8 kcal/mol at 298 K in an aqueous buffer of pH 4.7 – 5.1.[6,11] The two amide-amide H-bonds between VC and its natural D-Ala-D-Ala target, however, indirectly play important roles.[1] Both H-bonds orient the bacterial pentapeptide such that the methyls of the Ala residues establish favorable hydrophobic contacts with aromatic rings of the antibiotic (PDB Jmol), and these interactions are believed to promote binding by a factor of 10^3.[1] Furthermore, the two amide-amide H-bonds are thought to tether the ligand to the antibiotic, thereby reducing residual motion and strengthening the H-bonds to the carboxylate. This tethering effect is an example of binding cooperativity that is critical to the antimicrobial activity of VC, as will be discussed later. The complex interplay of H-bonding, hydrophobic contacts, and dynamic motions within an otherwise rigid conformation that is responsible for high affinity binding to the bacterial peptide target attests to the evolutionary fine-tuning of the VC structure. VC is tailored for recognizing its natural ligand, as evidenced by the high amino acid sequence conservation among all known glycopeptide antibiotics; *structural conservation implies functional necessity*.

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