Binding of glycopeptide antibiotics to a model of a vancomycin-resistant bacterium

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Background: The vancomycin group of glycopeptide antibiotics is active against a wide range of gram-positive bacteria. The increasing resistance to vancomycin is the result of a change of an amide linkage (D-Ala–D-Ala) to an ester linkage (D-Ala–D-Lactate) in the bacterial cell-wall precursors.

Results: We have used a peptide terminating in the sequence -Lys–D-Ala–D-Lactate linked by its amino terminus to a docosanoyl (C_{22}) acyl chain and anchored in a supported lipid monolayer to mimic the surface of vancomycin-resistant enterococci. Surface plasmon resonance analysis was then used to investigate the binding of glycopeptide group antibiotics to this surface. Vancomycin, which dimerises weakly, bound with low affinity, whereas strongly dimerising antibiotics, such as chloroeremomycin, bound with higher affinities. Antibiotics that have attached hydrophobic groups, such as teicoplanin and biphenylchloroeremomycin (LY307599), bound to the lipid monolayer. This resulted in an enhanced affinity for the lipid-anchored peptide at the surface relative to affinities for an analogous non-anchored peptide in solution.

Conclusions: We have shown that the affinities of glycopeptide antibiotics for a model of the surface of a vancomycin-resistant bacterium are enhanced relative to affinities determined in free solution. We have also shown that antibiotics that have membrane anchors bind tightly to the model surface and that this feature is an important determinant of the ability of an antibiotic to kill vancomycin-resistant enterococci.

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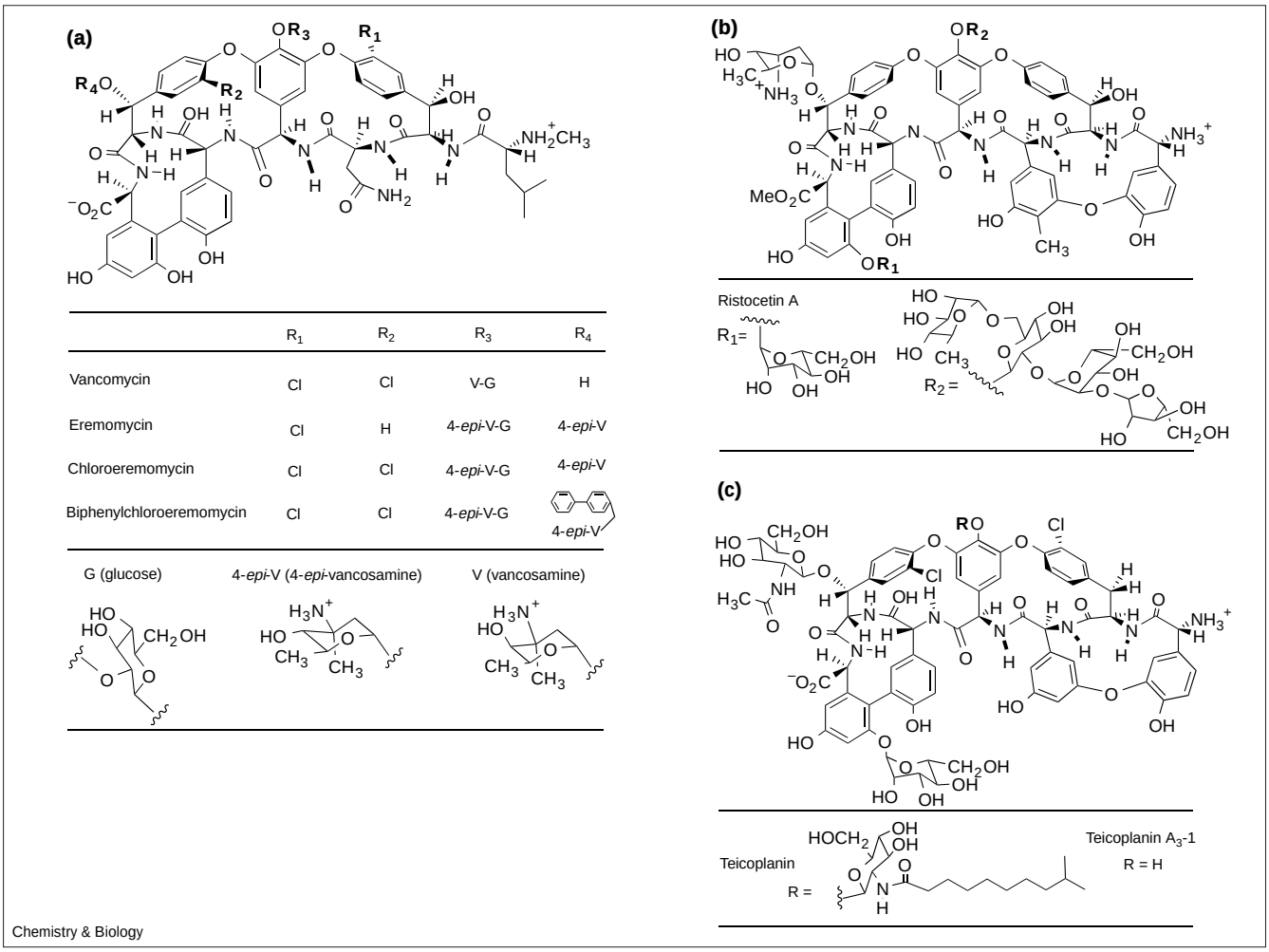
Introduction

The vancomycin group of glycopeptide antibiotics is active against a wide range of gram-positive bacteria, particularly those staphylococci and enterococci responsible for postsurgical infections. The activity of the glycopeptide antibiotics (Figure 1) arises from their ability to bind mucopeptide precursors terminating in the sequence -Lys–D-Ala–D-Ala (-KAA) [1,2]. We have shown previously that, with the exception of teicoplanin, glycopeptide antibiotics dimerise in aqueous solution [3] and that dimerisation plays an important role in their biological activity [4,5]. The molecular details of the vancomycin dimer have been confirmed recently from X-ray crystal structures [6,7]. We have proposed that glycopeptide antibiotics are able to bind cooperatively as dimers to two nascent mucopeptide chains at the bacterial membrane surface, thereby disrupting cell-wall synthesis (Figure 2a).

Unfortunately, resistance to vancomycin is now increasing [8], and the accompanying increase in the number of deaths from bacterial infections has given new urgency to the search for novel antibiotics. This resistance is the result of a deceptively simple change of an amide linkage to an ester linkage in the bacterial cell-wall precursor lipid II (undecaprenyl-pyrophosphoryl-MurNAc-[pentapeptide]-GlcNAc) [9]. The change, conferred by substitution of the terminal D-alanine of the cell-wall precursor with D-lactate, results in a repulsive interaction within the binding pocket of the antibiotic, and a consequent ~1000-fold decrease in affinity, rendering the antibiotic therapeutically useless [8–10]. Work by the groups of Griffin [11] and Whitesides [12] has shown that covalently linked dimers of vancomycin were more active against vancomycin-resistant enterococci (VRE) by a factor of about 100 than was vancomycin itself. Very recently, Uemura and coworkers [13] have shown that a multivalent polymer of vancomycin also shows activity against VRE. Whitesides and coworkers have used self-assembled monolayers terminating in D-Ala–D-Ala [14] and in D-Ala–D-lactate [12] together with surface plasmon resonance (SPR) to show that the covalently linked vancomycin dimer had a much higher binding constant at a surface than in solution.

In previous studies, we have employed sodium dodecyl sulfate micelles as model membranes to study the binding interactions between antibiotic and peptide ligands anchored to the lipid surface [15]. The small size of the micelles allows nuclear magnetic resonance (NMR)

Figure 1



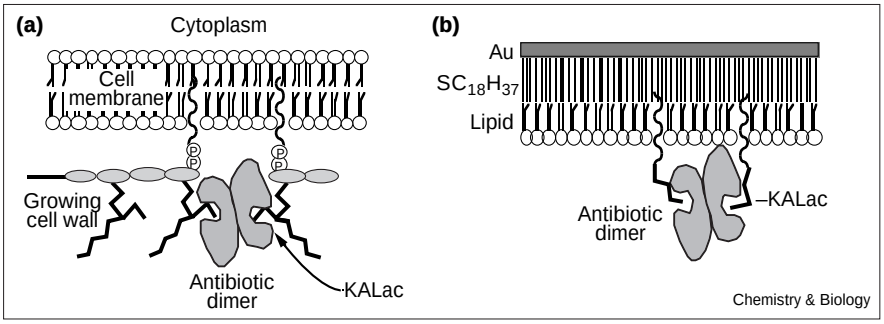
	R_1	R_2	R_3	R_4
Vancomycin	Cl	Cl	V-G	H
Eremomycin	Cl	H	4-*epi*-V-G	4-*epi*-V
Chloroeremomycin	Cl	Cl	4-*epi*-V-G	4-*epi*-V
Biphenylchloroeremomycin	Cl	Cl	4-*epi*-V-G	4-*epi*-V

G (glucose) 4-*epi*-V (4-*epi*-vancosamine) V (vancosamine)

Structures of the glycopeptide antibiotics used in this study.

analysis of antibiotic–peptide–micelle aggregates, but their high radius of curvature and surfactant properties makes them a poor membrane model. In this study, we have used a peptide ligand terminating in the sequence -Lys–D-Ala–D-Lactate, anchored in a supported lipid monolayer by means of a C_{22} hydrocarbon tail (Figure 2b). This peptide–lipid surface models the mucopeptide precursors anchored in the membranes of

Figure 2



(a) Disruption of peptidoglycan synthesis by binding of antibiotic dimer to nascent mucopeptide on the surface of a vancomycin-resistant bacterium. (b) Supported lipid monolayer model of a bacterial membrane with antibiotic dimer binding to the lipoyl peptide *N*-α-docosanoyl-ε-acetyl-Lys–D-Ala–D-Lactate (doc-KALac).

vancomycin-resistant bacteria. Supported lipid monolayers can be formed on an alkane-thiol self-assembled monolayer, which is in turn mounted on a gold surface [16]. The lipid monolayers formed in this way provide a chemically and physically stable environment that resembles the surface of a cellular membrane, and the gold surface is suitable for SPR analysis [16,17]. Changes in the measured refractive index at the interface, given in response units (RU), are proportional to the amount of material in the immediate vicinity of the sensor surface [18]. Buffered solutions of an antibiotic can be passed over the surface and the affinity of the binding event can be calculated from analysis of the resultant binding curve.

Results and discussion

Formation of lipid and peptide–lipid monolayers

Supported lipid monolayers were formed from extruded phosphatidylcholine vesicles on a hydrophobic self-assembled monolayer, resulting in the deposition of ~2000 RU or 1.84 ng/mm^2 (2.3 pmol/mm^2) of lipid [17]. Coverage of the surface with lipid was confirmed by the lack of nonspecific binding of bovine serum albumin, which binds strongly to the hydrophobic self-assembled monolayer alone [17]. *N*-α-docosanoyl-ε-acetyl-Lys–D-Ala–D-Lactate (doc-KALac) was then inserted into the lipid monolayer by direct injection across lipid surfaces as a dilute solution (data not shown). This resulted in the deposition of ~200 RU of doc-KALac, corresponding to a surface density of ~0.18 ng/mm^2 or 0.28 pmol/mm^2 [17]. Studies using different radiolabelled proteins [18] have shown that there is a linear relationship between the amount of absorbed material and the SPR response. Although the sensitivity of the SPR instrument is lower for absorbed species with lower molecular weights, the instrument used for this work has good sensitivity for molecular weights > 200 Da, and the doc-KALac ligand has a molecular weight of 652 Da.

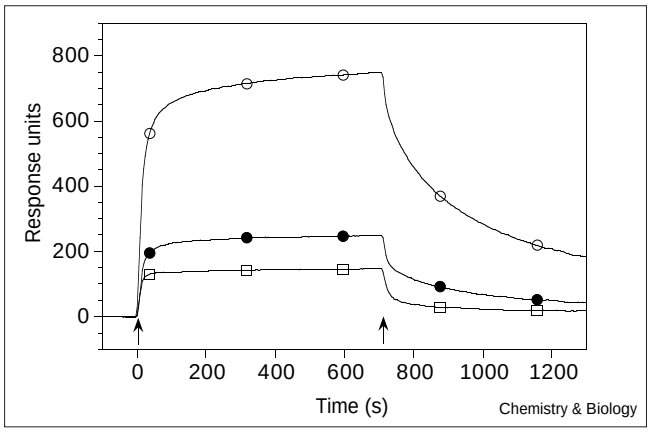
It could not be directly determined whether the doc-KALac ligands inserted in the monolayer were mobile in the plane of the lipid. Attempts to measure the diffusion coefficient of a fluorescently labelled alkyl peptide anchored in the lipid monolayer using fluorescence recovery after photobleaching (FRAP) were unsuccessful, as the fluorescence was quenched by the proximal gold film. Recently, Plant [19] has reported that transferral of lipids from a vesicle to a supported lipid monolayer has an effect similar to that of reducing temperature: lipids in the monolayer were shown to have a similar degree of hydration and temperature-dependent phase transitions to those in a bilayer. Studies using peptides anchored in Langmuir–Blodgett films and supported lipid bilayers have shown that the anchored molecules are homogeneously distributed in the lipid layer and have translational diffusion coefficients in the order of 10^{-8} cm^2/s [20,21]. Furthermore, we have shown previously that doc-KAA inserted into a supported lipid monolayer gives rise to enhanced antibiotic-binding constants consistent with an antibiotic homodimer binding cooperatively to two ligands [22]. The magnitude of the binding enhancements reported in the current work is almost identical to that observed from a competition NMR experiment using a lactate-terminating peptide inserted into lipid vesicles [23]. In the light of these similarities, and of the abundant evidence that molecules are mobile in supported lipid bilayers and Langmuir–Blodgett films, we conclude that it is highly likely doc-KALac is also mobile in the supported lipid monolayer.

The stability of the peptide–lipid layer was assayed by flowing buffer at a flow rate of 20 μl/min across the surface for 2 h (data not shown). The peptide was leached from the lipid monolayer at a rate of 1.3 RU/min — a rate significantly faster than the drift observed for a lipid monolayer alone (0.4 RU/min). Upon repeated injection of chloroeremomycin across a doc-KALac-containing lipid monolayer, the amount of antibiotic bound at equilibrium decreased by 1% per cycle of binding and regeneration. To correct for this effect, in addition to subtraction of data from the flow cell containing lipid alone, data for binding to this surface were further corrected by subtracting data resulting from an injection of buffer across the doc-KALac–lipid surface. This extra correction resulted in a stable baseline before the injection of antibiotic and resulted in reproducible binding levels. We acknowledge that this type of correction does not account for the fact that the surface capacity is changing during the binding event and that this effect can be accurately described by introducing extra terms into the binding algorithm [24]. This was not done because the drift, and the resultant reduction in surface capacity, was very small. In addition, the corrected data were not subjected to kinetic analysis and binding constants were derived solely from examination of equilibrium binding levels at varying antibiotic concentration.

Antibiotics bind specifically to peptide-containing lipid monolayers

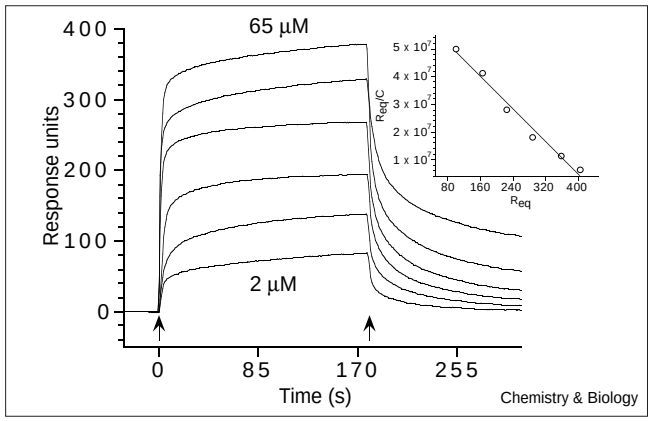
With the exception of the lipophilic antibiotics teicoplanin and biphenylchloroeremomycin (BCE), which bound to lipid, all antibiotics showed extremely weak or negligible binding to the control lipid surface (Figure 3). The rapid change in response at the beginning and end of injection of ristocetin A over the control lipid surface (Figure 3) is associated with a bulk refractive index difference between the antibiotic and buffer solutions. Binding of antibiotics to the doc-KALac-containing surface was generally much weaker than binding to a *N*-α-docosanoyl-lysyl(*N*-ε-acetyl)-D-alanyl–D-alanine (doc-KAA)-containing surface [22] (Figure 3). The affinity of vancomycin for the doc-KALac-containing lipid monolayer ($K_a \leq 10^3$ M^{-1}) was 1000-fold less than for the doc-KAA-containing lipid monolayer ($K_a = 1.4 \times 10^6$ M^{-1}) [22]. The minimum inhibitory concentration (MIC) values of vancomycin against VRE are up to 500-fold larger than for vancomycin-sensitive bacterial

Figure 3



Ristocetin A at 5 μM binding to a lipid monolayer alone (□), a lipid monolayer containing 200 RU of doc-KALac (●) and a lipid monolayer containing 200 RU of doc-KAA (○). The arrows indicate the beginning and end of each injection.

Figure 4



Binding of serial twofold dilutions of ristocetin A (65–4 μM) to a doc-KALac-containing lipid monolayer. Data has been corrected for bulk refractive index changes by subtraction of data for binding to a lipid monolayer alone. The inset is a Scatchard plot of the data: R_{eq} is the response in response units when the binding reaches equilibrium and C is the concentration of vancomycin. A plot of R_{eq} versus R_{eq}/C has a slope of $-K_a$ (see the Materials and methods section for derivation). Equilibrium binding levels for use in the Scatchard plot were determined from extrapolation of binding curves to infinite time.

strains. These observations suggest that the doc-KALac- and doc-KAA-containing lipid monolayers are reasonable models of the surfaces of vancomycin-resistant and vancomycin-sensitive bacteria, respectively.

With the exception of BCE and teicoplanin, data for binding of the antibiotics to a lipid monolayer alone were subtracted from data for binding to the doc-KALac-containing surface to correct for bulk refractive index changes between the antibiotic sample and the running buffer. Regeneration of the free peptide was effected with 10 mM hydrochloric acid without any apparent perturbation of the peptide–lipid monolayer. TA_3-1 and the weakly dimerising antibiotics vancomycin and ristocetin A bound with pseudo-first-order kinetics to the doc-KALac-containing lipid monolayer. The strongly dimerising and lipid-anchoring antibiotics bound in a biphasic manner. Kinetic analysis of this complex behaviour will be discussed in a forthcoming paper and in the present work affinities are derived solely on the basis of analysis of equilibrium binding levels.

Gram-positive bacterial membranes are composed primarily of phosphatidylglycerol, not of phosphatidylcholine. Lipid monolayers were therefore formed both from dimyristoylphosphatidylcholine (DMPC) and from dimyristoylphosphatidylglycerol (DMPG). The affinities calculated using the different types of lipid were very similar (data not shown) and all data presented in this paper result from experiments using phosphatidylcholine.

Correlation of affinities with dimerisation and antimicrobial activity

Affinity constants of antibiotics for doc-KALac were determined by Scatchard analysis of binding levels at equilibrium for varying concentrations of antibiotic (Figure 4). These affinities at a surface showed a moderately good correlation (R = 0.87) with antimicrobial activity against vancomycin-resistant bacteria (Table 1, Figure 5). This correlation is similar to that between antibiotic affinities to a doc-KAA–lipid monolayer and activity against vancomycin-sensitive bacteria (R = 0.85) [17]. There was a poor correlation between antibiotic dimerisation constants (K_{dim}) and activity against vancomycin-resistant bacteria (R = 0.48, data not shown), which is in contrast to the good correlation between K_{dim} and activity against vancomycin-sensitive bacteria (R = 0.90) [5]. This suggests that there are factors other than dimerisation that are important determinants of antibiotic activity against vancomycin-resistant bacteria.

Binding of vancomycin to the doc-KALac–lipid layer was too weak to be observed with the SPR instrument employed, which is capable of measuring affinities between 10^3 and 10^{12} M^{-1} [25]. Vancomycin binds weakly to the surface and has a relatively high MIC value against vancomycin-resistant bacteria because there is less cooperative benefit to be gained from cooperative binding to two peptides at a surface when the antibiotic dimerises to a smaller extent [26]. Chloroeremomycin, which dimerises more strongly, bound with higher affinity to the surface (Figure 5; Table 1). Like vancomycin, chloroeremomycin had a low affinity for ac-KALac in solution, but it is a strong dimeriser and therefore might be able to bind cooperatively to two peptides templated on the surface. The initial binding of peptide to one half of the dimer is intermolecular, whereas binding to the other half of the

Table 1

Minimum inhibitory concentrations, dimerisation constants (K_{dim}) and affinity constants (K_a) of antibiotics for ac-KALac in solution and for doc-KALac in a lipid monolayer.

Antibiotic	MIC (μg/ml) *E. faecium* (207) Van A	K_{dim} (M^{-1})*	K_a (M^{-1}) ac-KALac	K_a (M^{-1}) doc-KALac†	$\rho_{surf/soln}$‡
TA_3-1	1024	< 1	n.d.	$5.9 \pm 0.3 \times 10^4$	n.d.
Vancomycin	512	700	410§	$< 10^3$	< 2.4
Ristocetin A	512	300	n.d.	$1.5 \pm 0.9 \times 10^5$	n.d.
Eremomycin	1024	4.0×10^5	70#	$5.1 \pm 0.9 \times 10^4$	730
CE	128¶	1.6×10^4	240#	$3.1 \pm 0.4 \times 10^5$	1300
BCE	1¶	2.0×10^5	350§	$5.8 \pm 0.3 \times 10^6$	1.6×10^4
Teicoplanin	256	< 1	600	$2.5 \pm 0.3 \times 10^6$	4200

*From [22]; †In the case of teicoplanin and BCE the affinity describes the interaction of the antibiotic with both the lipid monolayer and doc-KALac (see text). ‡$\rho_{surf/soln}$ is defined as K_a (doc-KAA)/K_a (ac-KALac), a measure of the enhancement gained by binding to ligand at the lipid surface compared with binding to ligand in solution. §From [31]. #From [41]. ¶Previously reported [42] average values against six strains of VRE; CE, 32 μg/ml; BCE, 1 μg/ml.

dimer at the surface is effectively intramolecular and results in an affinity 1300-fold greater than that in solution (Table 1). Chloroeremomycin also has a much lower MIC value (Table 1). Using fluorinated peptides and ^{19}F NMR with phosphatidylcholine vesicles, we have previously demonstrated that chloroeremomycin has a much higher affinity for lactate-terminating peptides anchored in a vesicle than for analogous ligands in free solution [27], but the two values show a much smaller variation in the case of vancomycin [23].

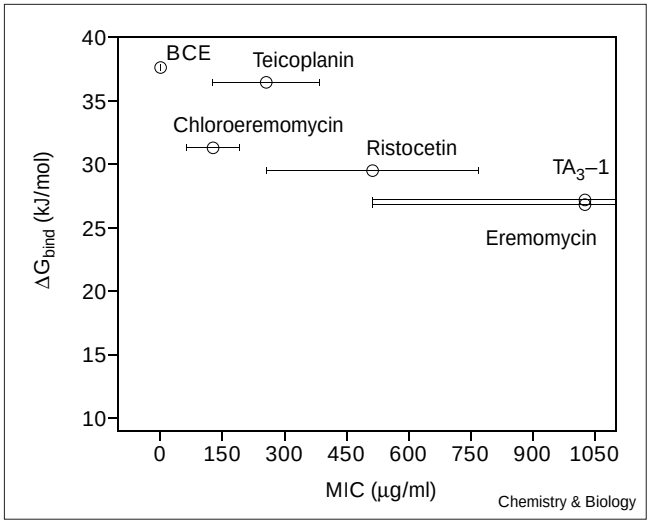
Eremomycin, which also dimerises strongly, bound with only moderate affinity to the doc-KALac-containing surface and bound more weakly than chloroeremomycin to ac-KALac in solution (Table 1). Eremomycin differs from chloroeremomycin in that it lacks a chlorine atom on the aromatic residue 6 ring (Figure 1a). The chlorine atom at this position has been shown in vancomycin to help define the shape of the pocket into which the methyl group of the binding peptide fits [28]. The chlorine atom contributes to both the stability and the specificity of the binding site, which may help explain why eremomycin binds so poorly to the doc-KALac-containing surface and why eremomycin has a 32-fold higher MIC value against VRE than chloroeremomycin.

Ristocetin A, which dimerises weakly, was shown to bind well to the doc-KALac surface (Table 1). The affinity of Ristocetin A for ac-KALac in free solution, however, could not be determined using UV difference spectroscopy or capillary electrophoresis. It is not known why the surface binding constant of this weakly dimerising antibiotic is relatively high, and in the absence of an accurate value for the solution binding constant we are reluctant to speculate further. TA_3-1, which does not dimerise, showed very weak affinity for the doc-KALac-containing surface and the affinity to ac-KALac in free solution could not be determined as no binding isotherm was observed using UV difference spectrophotometry, even upon addition of a 10 mM solution of ac-KALac to a 50 μM solution of the antibiotic. It is likely that the affinities are very weak. Both TA_3-1 and ristocetin A had poor activity against VRE (Table 1).

Lipophilic antibiotics have enhanced affinities for the doc-KALac surface

Teicoplanin is remarkable as it does not dimerise, yet it has a surface-binding constant to doc-KALac 4200-fold higher than that to ac-KALac in free solution. This is because teicoplanin has a C_{11} acyl chain that functions as a membrane anchor to localise the antibiotic at the surface (Figure 1c) [4,15]. Binding to peptide in this case can be

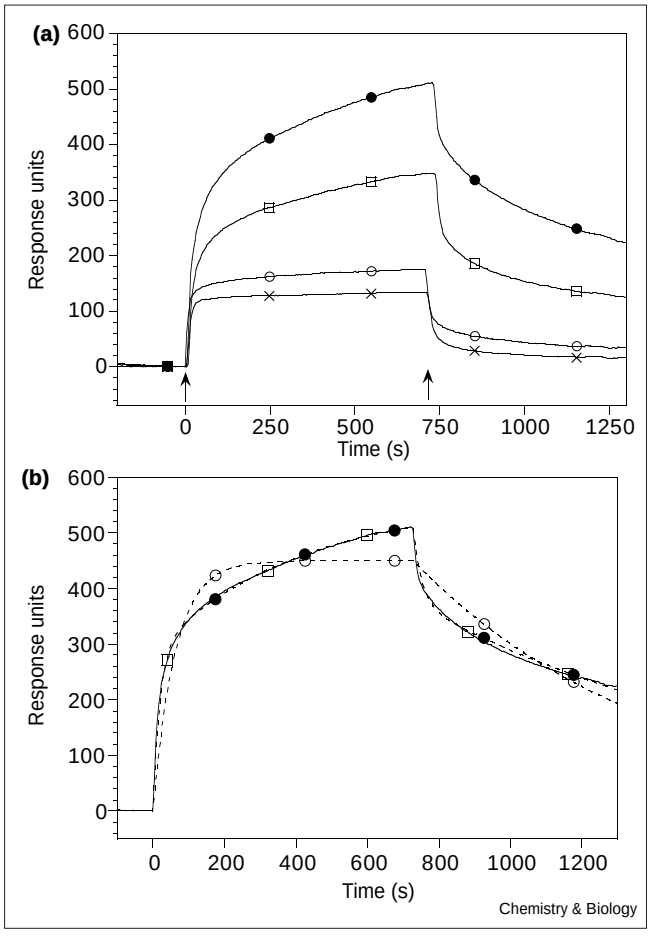
Figure 5



Correlation between antimicrobial activity against *E. faecium* (207) Van A and free energy of binding to doc-KALac in a lipid monolayer, measured using SPR.

cooperative as the antibiotic and peptide are attached to the same template and the event is effectively intramolecular, as is the case with the dimerising antibiotics. The physical interpretation of the higher binding (2.5×10^6 M^{-1}) of teicoplanin to the model VRE surface is not simple, because removing the antibiotic from the surface requires its dissociation from the peptide and removal of the hydrocarbon chain from the model membrane. The high value obtained, however, is entirely consistent with some surface cooperativity between mucopeptide precursor binding and membrane anchoring to improve its MIC value relative to TA_3-1 against VRE (Table 1). Similar arguments apply in the interpretation of the data for the binding of biphenylchloroeremomycin (Table 1, Figure 6; see below).

Figure 6



(a) Binding of BCE at 5 μM to a lipid monolayer alone (□) and a lipid–doc-KALac monolayer (●). Binding of chloroeremomycin at 5 μM to a lipid monolayer alone (×) and a lipid–doc-KALac monolayer (○). (b) BCE at 5 μM binding to a lipid–doc-KALac monolayer (□, solid line) together with the data fitted to the simple 1:1 Langmuir binding algorithm (○, dashed line) and to the more complex two-step binding algorithm (●, dashed line) described in the Materials and methods section.

Several new semisynthetic glycopeptides have been discovered recently that show activity against resistant strains of bacteria in the order of 500 times greater than that of vancomycin [29]. One such antibiotic, BCE (or LY307599), has a biphenyl group attached synthetically to the residue 4 epi-vancosamine sugar (Figure 1a). This modification is remote from the known site of interaction with cell-wall peptides and it is therefore not obvious why it would have such an effect on the ability of the antibiotic to kill bacteria.We have shown, however, that the affinity for the –Lys–D-Ala–D-Ala sequence is not the only factor determining antibiotic efficacy [4,5]. Membrane anchoring in teicoplanin and dimerisation in chloroeremomycin increase the effectiveness of these antibiotics. This enhancement of activity arises through the ability of these features to locate the antibiotic at its site of action—the cell surface. The new antibiotic BCE has been shown to dimerise strongly and the biphenyl moiety has been shown to act as a membrane anchor [30,31]. We have therefore hypothesised that the activity of this antibiotic is due to membrane anchoring and dimerisation acting cooperatively to enhance greatly the low intrinsic affinity for –D-lactate-terminating peptides found at the surface of vancomycin-resistant bacteria.

The lipophilicity of BCE, teicoplanin and their analogues lacking hydrocarbon appendages (CE and TA_3-1) was assayed. Both BCE and teicoplanin bound to a lipid monolayer, whereas CE and TA_3-1 did not bind significantly. BCE showed an enhanced affinity for the doc-KALac-containing surface relative to CE (Figure 6a, Table 1). The affinity, or partitioning coefficient, of BCE for lipid was calculated to be $K_p = 3.6 \times 10^6$ M^{-1}. The affinity of teicoplanin for lipid was $K_p = 2.3 \times 10^6$ M^{-1}. The biphenyl rings of BCE and the C_{11} alkyl chain of teicoplanin were shown, using molecular modelling, to contribute an extra 312 $Å^2$ and 290 $Å^2$ of hydrophobic surface area, respectively, compared with the nonalkylated parent compounds. Using the solvent transfer value of 0.125 kJ mol^{-1} $Å^{-2}$ for the burial of hydrophobic surface [32], this gives theoretical values for the burial of the biphenyl rings of BCE and the acyl chain of teicoplanin in hydrocarbon from water of 6.0×10^6 M^{-1} and 2.3×10^6 M^{-1}, respectively. These values are reasonably close to the K_p values determined experimentally.

BCE and teicoplanin have enhanced affinities for doc-KALac inserted into a lipid monolayer relative to those of the nonacylated analogues CE and $TA_3$1 (Table 1). It is not possible in the case of BCE and teicoplanin to partition what part of the binding energy results from interactions with the lipid layer and what part from interactions with doc-KALac as we can only measure the affinities for the surface as a whole. Affinities were calculated from Scatchard analysis of equilibrium binding levels and not by kinetic analysis because many of the antibiotics did not

bind with first-order kinetics. The deviation from first-order kinetics was especially prominent in the case of the strongly dimerising antibiotics and with BCE (Figure 6a). Although the BCE-binding data could not be fitted using algorithms describing a simple 1:1 Langmuir association, a more complex algorithm describing successive binding to the lipid layer, then to the lipid-bound doc-KALac (equations 4 and 5 in the Materials and methods section), fitted the data well (Figure 6b). The faster association and dissociation components of the biphasic binding curve were described by $K_1 = k_{a1}/k_{d1}$ and the slower phases by $K_2 = k_{a2}/k_{d2}$. This gave $k_{a1} = 4400\ M^{-1}\ s^{-1}$, $k_{d1} = 0.032\ s^{-1}$, $k_{a2} = 3.7 \times 10^7\ mol^{-1}\ cm^2\ s^{-1}$ $k_{d2} = 0.0010\ s^{-1}$ and thus $K_1 = 1.4 \times 10^5\ M^{-1}$ and $K_2 = 3.7 \times 10^{10}\ mol^{-1}\ cm^2$. The second association rate constant (k_{a2}) calculated from equation 5 (see the Materials and methods section) must be expressed with two dimensional units ($mol^{-1}\ cm^2\ s^{-1}$) as the concentration of the initial complex bound to lipid (AL) is calculated from equation 5 in terms of the observed response. For this reason we cannot compare the rate and affinity constants derived from the complex model directly with those derived from a simple 1:1 kinetic analysis, and we have instead used affinities calculated from simple Scatchard analyses throughout this work.

Kahne and coworkers have recently reported data indicating that vancomycin group antibiotics that have lipophilic moieties attached to the disaccharide portion of the antibiotic act primarily by inhibiting transglycosylation, but that vancomycin itself primarily inhibits the subsequent transpeptidation step [33]. Although we cannot preclude that antibiotics carrying putative membrane anchors can act in a different manner to vancomcyin, we note that Reynolds [34] has concluded that vancomycin can inhibit both transglycosylase and transpeptidase action. In addition, Allen *et al.* [35] have employed a strain of *A. viridans* that synthesises, but does not cross-link, peptidoglycan, to show that biosynthesis of non-cross-linked peptidoglycan was inhibited by vancomycin. This is consistent with vancomycin inhibiting the transglycosylation step, a hypothesis that was supported by an observed accumulation of lipid II intermediate.

Kahne and coworkers [33] also showed that a disaccharide portion of vancomycin that has no -D-Ala–D-Ala binding site, which was derivatised with a biphenyl lipophilic anchor, was active against VRE, albeit at a MIC of 128 μg/ml. This is a striking finding, but, in the light of the high MIC value relative to other lipophilic antibiotics such as BCE, it is possible that the observed antimicrobial activity arises from nonspecific inhibition of membrane-associated glycosylases. There are numerous examples of lipopeptides that interact with membranes and have antibacterial activity. Many have surfactant activity and cause nonspecific membrane disruption (e.g., subtilisin and surfactin [36]), whereas others appear to be much more specific in their action (e.g., amphomycin, daptomycin, globomycin and ramoplanin [37]). In the light of the differing conclusions regarding the peptidoglycan synthesis step inhibited by vancomycin, and the possibility of nonspecific activity of compounds lacking the -D-Ala–D-Ala binding site, we note here our alternative hypotheses without further speculation.

We propose that a membrane-anchoring chelate effect can serve to enhance binding of antibiotics to cell-wall precursors at a cell surface because both the antibiotic and its target are attached by membrane anchors to the same template, and the binding is therefore effectively intramolecular [15]. In accordance with the hypothesis that dimerisation and membrane anchoring promote antibiotic activity, it has been shown that these features make the antagonism of antibiotic action by externally added ac-KAA much more difficult [38]. We have also shown that high surface-binding affinities are observed for binding of the strongly dimerising antibiotic chloroeremomycin to lactate-terminating peptides anchored in a supported lipid monolayer (this work) and in lipid vesicles [23]. We therefore believe the remarkable activity of antibiotics such as BCE against VRE is most likely due to two factors: an enhanced ability to bind to -lactate terminating precursors, which results in enhanced inhibition of bacterial transpeptidase, and localisation at the membrane surface, which results in enhanced inhibition of bacterial transglycosylase.

Significance

Studies of cooperative interactions at surfaces have important implications for the study of weak interactions that occur between a drug and a receptor at the surface of a cell membrane in general. We have shown that the affinities of glycopeptide antibiotics for a model of the surface of a vancomycin-resistant bacterium are enhanced relative to those affinities determined in free solution. We have also shown that antibiotics that have membrane anchors bind tightly to the model surface and that this feature is an important determinant of the ability of an antibiotic to kill vancomycin-resistant enterococci.

Materials and methods

Octyl D-glucoside, bovine serum albumin, egg L-α-phosphatidylcholine, dimyristoylphosphatidylcholine and diglycerolphosphatidylcholine were purchased from Sigma-Aldrich (UK). The glycopeptide antibiotics biphenylchloroeremomycin (LY307599), chloroeremomycin (LY264826), eremomycin and vancomycin were a gift from Eli Lilly & Co (Indianapolis, Ind., USA) Teicoplanin and teicoplanin aglycone A3-1 were a gift from MMDR1 Lepetit Research Centre (Gerenzano, Italy). Ristocetin A was obtained from Abbot Laboratories. The synthesis of *N,N*-acetyl-lysyl-D-alanyl-D-lactic acid has been described previously [30]. The SPR instrument was a BIACORE 2000 (Biacore AB, UK) used with a hydrophobic association (HPA) chip which consisted of an octadecane-thiol self assembled monolayer on a gold surface. Each sensor chip contained four flow cells of dimensions $2.4 \times 0.5 \times 0.05$ mm ($l \times w \times h$) with a probing spot for the SPR signal of ~0.26 mm^2 for each flow cell. All experiments were carried out at 25°C with data points taken every 0.5 s.

Determination of solution affinities by UV difference spectroscopy

Binding constants between ac-KALac and antibiotics were carried out on a UVIKON 940 dual beam spectrophotometer. Antibiotic and ligand solutions were buffered with filtered, degassed phosphate buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4). Antibiotic were assayed at 50 μM with titrated ac-KALac solutions between 0.5 and 2 mM. The λ_{max} for the antibiotic–ligand complexes typically fell in the region of 290 nm. The concentration of antibiotic was kept constant during the titration and ac-KALac was added in 10 μl aliquots. The solution was stirred after each addition and the absorbance at λ_{max} and at baseline measured repeatedly until stable. The data at λ_{max} were subtracted from baseline values and the resulting data set analysed using a simplex nonlinear least squares curve fitting program [39], which fits the data to a simple 1:1 binding isotherm.

Organisms, growth media and MICs

E. faecium (207) Van A phenotype was a clinical isolate that was inoculated in *B. stearo* broth and incubated overnight at 37°C. A sample of the culture was then inoculated in fresh broth and incubated at 37°C for 1 h. 200 μl of the resultant mid-log phase cultures diluted to 10^6 cfu/ml were then added to 96-well polystyrene tissue culture plates (Costar). Antibiotics were then serially diluted twofold across the culture-containing wells from 1024 μg/ml to 0.5 μg/ml. The 96-well plates were than covered and incubated with gentle shaking at 37°C overnight. MICs were determined by inspection of broth turbidity after incubation.

Deposition of peptide–lipid monolayers

Phosphatidylcholine lipid monolayers were formed in filtered, degassed phosphate buffer as described previously [17] on the first two cells of an HPA sensor chip resulting in the deposition of ~2000 RU of lipid. *N*-docosanoyl-lysyl(*N*-ε-acetyl)-D-alanyl–D-lactate (doc-KALac) was then inserted into the lipid monolayer on the second flow cell of the sensor chip by injection across the surface as a dilute solution (30 μl, 10 μl/min, 20 μM, freshly prepared from dry powder) in phosphate buffer. The stability of the peptide in the lipid monolayer was assayed by flowing buffer across the surface for 2 h at 20 μl/min.

Antibiotic–peptide binding assay

Serial twofold dilutions (65–2 μM) of the antibiotics vancomycin, teicoplanin aglycone (TA_3-1), ristocetin A, chloroeremomycin, eremomycin, teicoplanin and biphenylchloroeremomycin (LY307599) in phosphate buffer were injected (60 μl, 20 μl/min) across a lipid monolayer alone and then across a doc-KALac–lipid monolayer with the BIACORE instrument in multiflow cell mode. The antibiotic–peptide complexes were allowed to dissociate for 4 min, then the free peptide was regenerated by injection of hydrochloric acid (10 μl, 10 mM). Five dummy runs of binding and regeneration were performed before data acquisition. All assays were carried out at 25°C in duplicate.

Molecular modelling

The different antibiotic structures were modelled using MacroModel 3D GLX running on a Silicon Graphics Indy work station. Structures were minimised *in vacuo* using the MM2 force field [40] and the hydrophobic and hydrophilic surface areas of each were then calculated by applying a 1.4 Å water probe to the van der Waal surface of the relevant subsets of each molecule.

SPR data analysis

Data were prepared for analysis by adjusting the response prior to injection to zero and adjusting the time of each injection to zero. With the exception of data for BCE and teicoplanin, which bound significantly to lipid, data from the flow cell containing lipid alone was subtracted from corresponding data obtained from the peptide-containing flow cell to correct for bulk refractive index changes.

Affinities were calculated from analysis of equilibrium binding levels at varying antibiotic concentration. When a binding curve had not reached complete equilibrium, the equilibrium value was determined by careful extrapolation of the binding curve. In the SPR flow cell, the antibiotic is being continually added to and removed from the system so the concentration will remain at the initial value, C. The total amount of ligand present is expressed in terms of R_{max}, the maximum possible response. The amount of complex formed is proportional to R_t, the observed response. Thus after a time, t, the concentration of antibiotic will still be C, and the amount of free ligand will be given by $R_{max}–R_t$:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \qquad (1)$$

t = 0:	C	R_{max}	0
t = t:	C	$R_{max}–R_t$	R_t

The association and dissociation rates, k_a and k_d, for formation of a homogeneous binary complex of antibiotic A and ligand B in a SPR flow cell are thus given by:

$$\frac{dR}{dt} = k_a C(R_{max} - R_t) - k_d R_t \qquad (2)$$

By measuring the resonance units attained at equilibrium (when by definition dR/dt is zero), as a function of analyte concentration, affinities can be determined from a Scatchard analysis using the equation:

$$\frac{R_{eq}}{C} = K_a R_{max} - K_a R_{eq} \qquad (3)$$

where R_{eq} is the response at equilibrium and K_a is the association constant (M^{-1}). A plot of R_{eq}/C versus R_{eq} thus has a slope of $–K_a$.

The binding of an antibiotic to lipid (L) followed by binding to lipid-bound ligand (B), is described by two response elements: R_1, the response due to the lipid-bound complex (AL) and R_2, the response due to the complex bound to both lipid and ligand (ALB), with both elements contributing to the total observed response: $R_t = R_1 + R_2$:

$$A + L + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AL + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} ALB \qquad (4)$$

at t = 0:	C	R_{max1}	0	R_{max2}	0	
at t = t:	C	$R_{max1}–R_1–R_2$	R_1	$R_{max2}–R_2$	R_2	

The kinetic rate constants are described by:

$$\frac{dR_1}{dt} = [k_{a1} \bullet C(R_{max1} - R_1 - R_2) - k_{d1} \bullet R_1] - [k_{a2} \bullet R_1(R_{max2} - R_2) - k_{d2} \bullet R_2]$$

$$\frac{dR_2}{dt} = k_{a2} \bullet R_1(R_{max2} - R_2) - k_{d2} \bullet R_2 \qquad (5)$$

where C is the concentration of antibiotic, R_{max1} is the maximum binding capacity of the lipid layer and R_{max2} is the maximum binding capacity of the ligand inserted in the lipid layer.

Supplementary material

Supplementary material including synthesis and ^1H NMR assignments for the peptide doc-KALac used in this study is available at http://current-biology.com/supmat/supmatin.htm.

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