Binding Properties of Antimicrobial Agents to Lipid Membranes Using Surface Plasmon Resonance

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In the present study, we examined the interaction of antimicrobial agents with four model lipid membranes that mimicked mammalian cell membranes and Gram-positive and -negative bacterial membranes and analyzed the binding kinetics using our surface plasmon resonance (SPR) technique. The selective and specific binding characteristics of antimicrobial agents to the lipid membranes were estimated, and the kinetic parameters were analyzed by application of a two-state reaction model. Reproducible analysis of binding kinetics was observed. Vancomycin, teicoplanin, erythromycin, and linezolid showed little interaction with the four lipid membranes in the SPR system. On the other hand, vancomycin analogues showed interaction with the model lipid membranes in the SPR system. The selective and specific binding characteristics of vancomycin analogues to the lipid membranes are discussed based on data for in vitro antibacterial activities and our data on the binding affinity of the d-alanyl-d-alanine terminus of a pentapeptide cell wall obtained by SPR. The mechanism of antibacterial activity against Staphylococcus aureus and vancomycin-resistant enterococci could be evaluated using the binding affinity obtained with our SPR techniques. The results indicate that the SPR method could be widely applied to predict binding characteristics, such as selectivity and specificity, of many antimicrobial agents to lipid membranes.

Key words surface plasmon resonance; lipid membrane; antimicrobial agent; vancomycin

The properties and mechanisms of drug interactions with biological membranes serve as critical information in the drug discovery stage to characterize the pharmacokinetics and pharmacodynamics properties of drug candidates. In particular, the affinity of antimicrobial agents for lipid membranes is one of the critical factors influencing their selectivity and potency and plays an important role in the antimicrobial mechanism of action.1–7 In order to accelerate drug discovery and development, various in vitro analytical techniques, such as circular dichroism spectroscopy, fluorescence spectroscopy, differential scanning calorimetry, nuclear magnetic resonance, and fluorescence resonance energy transfer, have been employed.8–10

Recently, surface plasmon resonance (SPR)-based biosensors have been widely used to study the membrane-binding properties of drugs with the aim of gaining insight into their modes of action.11,12 The use of SPR-based biosensors to study the membrane-binding properties of drugs has the advantage of not requiring the presence of labels or chromophores.13 We have already established the evaluation of interactions of various antimicrobial peptides to ligands using SPR biosensors, and demonstrated that the kinetic rate constants and the affinities of ligand interactions can be readily determined by this technique, which in turn provides important insights into the interaction mechanisms.14–16 Also, our SPR techniques have already been used to evaluate the binding properties of an antimicrobial agent (Daptomycin) and antifungal agents (Amphotericin B and Fungizone) to lipid membranes.17–19

Glycopeptide antibiotics are widely used to treat various infections caused by Gram-positive bacteria by inhibiting proper cell wall synthesis, specifically, by binding to the n-alanyl-d-alanine residues on the ends of the peptide chains of the membrane-anchoring cell wall precursor, lipid II.20–24 On the other hand, vancomycin, a glycopeptide antibiotic, cannot bind to lipid II when the last d-alanine residue has been replaced by a d-lactate (d-alanyl-d-lactate). This substitution leads to vancomycin resistance.25,26 Recently, we developed a SPR assay to examine the binding properties of glycopeptide antibiotics, including vancomycin, to dipeptide ligands, mimicking vancomycin-susceptible (d-alanyl-d-alanine) and -resistant (d-alanyl-d-lactate) dipeptides of lipid II.27 In our report, we discussed the correlation of SPR data on the interaction of antibiotics with dipeptide ligands and their antibacterial activity against Staphylococcus aureus and vancomycin-resistant enterococci (VRE).27 The interactions of some vancomycin analogs with lipid II did not show good correlation with their antibacterial activities.27 Some glycopeptide antibiotics are known to perturb bacterial cell membrane potential and permeability.22,28 Therefore, the affinity of antibacterial agents for the lipid membranes was investigated, combining the two data sets on antibacterial activities.

We have demonstrated the interactions of various antimicrobial agents, including glycopeptide antibiotics, with four model lipid membranes which mimicked mammalian cell membranes as well as Gram-positive and -negative bacterial membranes using our established SPR techniques, including analysis of the binding kinetics of lipid membranes.14–16,29,30 Our final target was to predict the mechanism of the antibacterial activity of antibiotic drugs using the SPR system.

This paper describes an investigation of the lipid membrane-binding properties of antimicrobial agents (vancomycin, its four analogs, teicoplanin, erythromycin, and linezolid) with four model lipid membrane systems using the SPR method. The binding kinetics of antimicrobial agents with model lipid
membranes was estimated and good reproducibility was obtained. Vancomycin showed little binding to lipid membranes, but Van-M-02, ΔN-Van-M-02 (vancomycin analogs) and Dimer 1 (vancomycin dimer) could bind to them. The relationship between the interaction of antimicrobial agents to the lipid membranes and the antimicrobial activity is discussed, based on both the data for antibacterial activities in vitro and our reported data on the binding affinity of the d-alanyl-d-alanine terminus of a pentapeptide cell wall by SPR. The mechanism of antibacterial activity against *Staphylococcus aureus* and vancomycin-resistant enterococci could be evaluated using the binding affinity obtained by our SPR techniques. The findings showed that our SPR method could be widely applied as an in vitro system to predict the binding characteristics, such as selectivity and specificity, of many antimicrobial agents to lipid membranes.
MATERIALS AND METHODS

Reagents and Chemicals Vancomycin synthesized in our laboratories was used. Van-M-02, ΔN-Van-M-02, Dimer 1 and Dimer 2 were synthesized at Tohoku University (Fig. 1). Teicoplanin, erythromycin, lincomycin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co., LLC (St Louis, MO, U.S.A.). Distilled water (HPLC grade) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidyl-glycerol (POPG), 1-palmitoyl-2-oleoylphosphatidyl-ethanolamine (POPE) and 1,1′,2,2′-tetraoleoyl cardiolipin (CL) were purchased from Avanti Polar Lipids, Inc. (Alabama, AL, U.S.A.). 3-[3-Cholamidopropyl]-dimethylammoniom propane sulfonate (CHAPS) and chloroform were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Concentrated phosphate buffered saline (PBS) was purchased from GE Healthcare U.K., Ltd. (Buckinghamshire, England).

Apparatus The SPR system used was a BIACORE 3000 analytical system, and a Sensor Chip L1 was used for immobilization of the liposomes (GE Healthcare U.K., Ltd.). The running buffers used were 2×PBS for immobilization of lipid membranes on the chip, and 2×PBS containing 5% DMSO from 20 to 50 µmol/L (20, 25, 30, 35, 40, 45 and 50 µmol/L). The antimi-

Liposome Preparation In order to prepare individual stock solutions, dry POPC, POPG, POPE and CL were dissolved in chloroform. These stock solutions were prepared at the desired ratios: POPC, POPG, POPE/CL (1:1, v/v) and POPE/POPG (4:1, v/v). The solvents were evaporated under a stream of nitrogen, and the lipids were dried under centrifugal evaporation. The lipids were resuspended in 2×PBS at approximately 25°C. The resulting lipid dispersion (1 mmol/L POPC, POPG, POPE/CL (1:1, v/v) and POPE/POPG (4:1, v/v)) was sonicated until it was nearly clear, and extruded 21 times through polycarbonate filters to obtain small unilamellar vesicles (SUV) of 100 nm in size (LipoFast, pore diameter 100 nm). The extruded lipid dispersion was diluted twofold with 2×PBS containing 300 mmol/L sodium chloride (0.5 mmol/L with respect to phospholipid for POPC, POPG, POPC/CL (1:1, v/v) and POPE/POPG (4:1, v/v)).

Formation of Lipid Bilayer Membranes The Sensor Chip L1 surface was washed with injections of 5 µL of 2-propanol/50 mmol/L sodium hydroxide (1:1, v/v) and 20 mmol/L CHAPS at a flow rate of 5 µL/min. The resulting lipid dispersion (1 mmol/L POPC, POPG, POPE/CL (1:1, v/v) and POPE/POPG (4:1, v/v)) was injected at a flow rate of 50 µL/min for liposome capture. To remove any multilamellar structures from the lipid surface, 30 µL of 50 mmol/L sodium hydroxide was injected at a flow rate of 50 µL/min, which resulted in a stable baseline corresponding to the supported lipid bilayer.

Antimicrobial Agent Binding to the Bilayer Membrane Antimicrobial agent assay solutions were prepared by being dissolved in 2×PBS containing 5% DMSO from 20 to 50 µmol/L (20, 25, 30, 35, 40, 45 and 50 µmol/L). The antimicrobial agent solution (50 µL, 100 µL) was injected over the lipid surface at a flow rate of 30 µL/min so as to avoid any limitation by mass transfer. Upon completion of injection, the buffer flow was continued to allow a dissociation time of 700 s. All binding experiments were carried out at 25°C. To remove the antimicrobial agent bound to liposome after dissociation, 10 µL of 50 mmol/L sodium hydroxide as regeneration solvent was injected at a flow rate of 30 µL/min before the next injection of antimicrobial agent over the sensor chip. The affinity of the antimicrobial agent for the lipid bilayer membrane was determined by analysis of a series of response curves collected at seven different antimicrobial agent concentrations injected over each lipid surface in triplicate under the condition of 2×PBS containing 5% DMSO as a running buffer.

Data Analysis The sensorgrams for each antimicrobial–lipid interaction were analyzed by curve fitting using numerical integration analysis (20–50 µmol/L). The data were fitted globally by simultaneously fitting the antimicrobial agent sensorgrams obtained at seven different concentrations using BIA evaluation software (Ver 4.1). The two-state reaction model was applied to the antimicrobial agent binding curves to estimate the association and dissociation rate constants. We chose this model on the basis of results from previous studies on antimicrobial–membrane interactions, in order to estimate the potency of antimicrobial activity induced by disruption of the bacterial membrane of the agents. This model describes two steps, which in terms of the antimicrobial–lipid interaction, would correspond to:

\[
A + L \overset{k_{a1}}{\underset{k_{d1}}{\rightleftharpoons}} AL \overset{k_{a2}}{\underset{k_{d2}}{\rightleftharpoons}} AL^* \]

Here A is an antimicrobial, L is the lipid membrane, AL is the initial complex formed by the lipid membrane and the antimicrobial via, for example, an electrostatic interaction. The complex AL changes to AL*, which cannot dissociate directly to A+L, and which may correspond to the insertion of an antimicrobial agent into the lipid bilayer.

The corresponding differential rate equations for this reaction model have been reported. The association (\(k_{a1}\), \(k_{a2}\)) and dissociation (\(k_{d1}\), \(k_{d2}\)) rate constants and the affinity constant \(K = k_{a1} / (k_{d1} \cdot (k_{a2} + k_{d2}) / k_{d2})\).

RESULTS AND DISCUSSION

Kinetic Parameters and Sensorgrams of Antimicrobial Agents for the Model Lipid Membranes In this SPR study, four different liposome mixtures were used as model lipid systems. POPC was used to mimic mammalian membranes, while POPG and POPC/CL (1:1, v/v) were used as Gram-positive membrane systems. POPE/POPG (4:1, v/v) was used as a negative bacterial membrane system. The response units of lipid membranes immobilized on the L1 chip were confirmed to be approximately 5000–10000 as the same levels.

Various vancomycin analogs have been synthesized and their antimicrobial activities have been estimated and characterized. Van-M-02 (Fig. 1), synthesized as a glycopeptide inhibitor of peptidoglycan synthesis, has activities against Gram-positive bacteria including VRE. ΔN-Van-M-02 (Fig. 1), N-terminal-degraded Van-M-02 was synthesized in order
to assess the contribution of the d-alanyl-d-alanine binding pocket to the activity of Van-M-02.\(^{37}\) Dimer 1 and Dimer 2 (Fig. 1) synthesized as vancomycin dimers exhibit strong activity against vancomycin-resistant bacteria.\(^{38–40}\)

Representative sensorgrams presented in Fig. 2 showed few interactions of vancomycin, Dimer 2, teicoplanin, erythromycin and linezolid with the POPC, POPG, POPG/CL (1:1, v/v) and POPE/POPG (4:1, v/v) liposome membranes immobilized on the Sensor Chip L1 using 2×PBS containing 5% DMSO as a running buffer. Few responses were obtained on calculation of the parameters of the two-state reaction model. On the other hand, Fig. 3 showing representative sensorgrams of the binding of Van-M-02, ΔN-Van-M-02 and Dimer 1 taken under the same conditions, revealed a distinct increase in the response to higher antimicrobial agent concentrations, indicating that the system had not reached saturation.

The association (\(k_{a1}, k_{a2}\)) and dissociation (\(k_{d1}, k_{d2}\)) rate constants and the affinity constant (\(K\)) of vancomycin and its four analogs estimated by the two-state reaction model are listed in Table 1. The reproducibility of each parameter by repeated assay was good. This model was selected based on the multi-step model of antimicrobial–lipid interactions, which may involve an initial binding interaction between the antimicrobial and the head group of the liposome (step 1, characterized by

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**Fig. 2.** Sensorgrams for Vancomycin, Dimer 2, Teicoplanin, Erythromycin and Linezolid of Binding to Liposome Membranes Immobilized on the Sensor Chip L1 Surface (1, POPC; 2, POPG; 3, POPG/CL (1:1, v/v); 4, POPE/POPG (4:1, v/v))

Running buffer, 2×PBS containing 5% DMSO (each compound concentration = 50 µmol/L).

**Fig. 3.** Sensorgrams for Van-M-02, ΔN-Van-M-02 and Dimer 1 of Binding to Liposome Membranes Immobilized on the Sensor Chip L1 Surface

Running buffer, 2×PBS containing 5% DMSO (each compound concentration = 20–50 µmol/L).
values of \( k_{a1} \) and \( k_{d1} \), including an electrostatic interaction, and followed by a reorientation and/or insertion of the analyte into the hydrophobic interior (step 2, characterized by \( k_{a2} \), \( k_{d2} \)), based on the antimicrobial–membrane interaction. 14,17–19) In this model, the difference in the association (affinity for lipid membranes is likely to depend mainly on the POPE/PG (4 : 1, v/v), which mimicked mammalian membrane, than POPC and D-alanyl-D-alanine (step terminal of lipid II ligands). 27) Van-M-02 had stronger activity which are VREs with different phenotypes (VanA and VanB, respectively). 27) Strong \( K \) values of Van-M-02 to POPG and POPG/CL (1 : 1, v/v) membranes, were observed and the affinities of Van-M-02 for D-alanyl-D-alanine and D-alanyl-D-lactate ligands were also stronger than those of vancomycin. This might suggest that the strong antimicrobial activity of Van-M-02 could be attributed to high affinity not only for lipid II but also for lipid membranes. The reason for this difference may be due to the difference in linker chemical structures between Dimer 1 and Dimer 2.

**Table 1. Association (\( k_{a1} \) and \( k_{d1} \)), Dissociation (\( k_{a2} \) and \( k_{d2} \)) Rate Constants and Affinity Constant (\( K \)) Determined by Numerical Integration Using the Two-State Reaction Model (\( n = 3 \))**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lipid type</th>
<th>( k_{a1} ) (1/Ms)</th>
<th>( k_{d1} ) (×10^{-2}, 1/s)</th>
<th>( k_{a2} ) (×10^{-4}, 1/s)</th>
<th>( k_{d2} ) (×10^{-3}, 1/s)</th>
<th>( K ) (×10^{3}, 1/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vancomycin</strong></td>
<td>POPC</td>
<td>—[^a]</td>
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<tr>
<td></td>
<td>POPG</td>
<td>—</td>
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<tr>
<td></td>
<td>POPG/CL</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td></td>
<td>POPE/POPG</td>
<td>—</td>
<td>—</td>
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<tr>
<td><strong>Van-M-02</strong></td>
<td>POPC</td>
<td>197±96.7[^a]</td>
<td>10.1±0.805</td>
<td>4.59±1.00</td>
<td>94.2±31.4</td>
<td>3.00±1.30</td>
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<tr>
<td></td>
<td>POPG</td>
<td>977±108</td>
<td>3.28±0.0854</td>
<td>1.88±0.999</td>
<td>50.1±13.8</td>
<td>41.3±7.65</td>
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<tr>
<td></td>
<td>POPG/CL</td>
<td>1220±122</td>
<td>2.55±0.0231</td>
<td>3.18±0.661</td>
<td>241±62.9</td>
<td>54.4±4.31</td>
</tr>
<tr>
<td></td>
<td>POPE/POPG</td>
<td>176±41.6</td>
<td>5.95±0.286</td>
<td>7.29±2.09</td>
<td>363±29.4</td>
<td>3.53±0.655</td>
</tr>
<tr>
<td><strong>ΔN-Van-M-02</strong></td>
<td>POPC</td>
<td>424±156</td>
<td>7.45±0.544</td>
<td>11.0±2.82</td>
<td>58.4±20.6</td>
<td>20.9±17.9</td>
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<tr>
<td></td>
<td>POPG</td>
<td>453±64.0</td>
<td>3.41±0.122</td>
<td>2.85±0.401</td>
<td>213±101</td>
<td>15.6±3.42</td>
</tr>
<tr>
<td></td>
<td>POPG/CL</td>
<td>601±60.1</td>
<td>3.19±0.230</td>
<td>4.70±1.13</td>
<td>351±209</td>
<td>22.1±4.76</td>
</tr>
<tr>
<td></td>
<td>POPE/POPG</td>
<td>140±57.9</td>
<td>5.00±0.131</td>
<td>6.20±0.321</td>
<td>211±37.5</td>
<td>3.60±1.33</td>
</tr>
<tr>
<td><strong>Dimer 1</strong></td>
<td>POPC</td>
<td>1400±221</td>
<td>1.68±0.0289</td>
<td>19.2±0.200</td>
<td>163±18.8</td>
<td>181±18.9</td>
</tr>
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<td></td>
<td>POPG</td>
<td>611±101</td>
<td>2.19±0.0231</td>
<td>15.0±0.473</td>
<td>236±20.8</td>
<td>45.6±5.52</td>
</tr>
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<td></td>
<td>POPG/CL</td>
<td>875±17.3</td>
<td>1.12±0.0100</td>
<td>3.72±0.918</td>
<td>32.9±4.78</td>
<td>171±39.4</td>
</tr>
<tr>
<td></td>
<td>POPE/POPG</td>
<td>1630±123</td>
<td>1.28±0.220</td>
<td>34.8±7.09</td>
<td>255±62.2</td>
<td>308±29.2</td>
</tr>
<tr>
<td><strong>Dimer 2</strong></td>
<td>POPC</td>
<td>—</td>
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<tr>
<td></td>
<td>POPG</td>
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<tr>
<td></td>
<td>POPG/CL</td>
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<td></td>
<td>POPE/POPG</td>
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[^a]: (1 : 1, v/v), b) POPE/POPG (4 : 1, v/v), c) Not calculated, d) Mean ± standard deviation (\( n = 3 \)).
higher affinities of ΔN-Van-M-02 for the POPG and POPG/CL lipid membranes than those of vancomycin would lead to higher antimicrobial activity against vancomycin-susceptible and -resistant bacteria than vancomycin. This might suggest that the antimicrobial activity of ΔN-Van-M-02 could be attributed to its affinity for the lipid membrane.

Recently developed glycopeptide antibiotics show antimicrobial activity against vancomycin-resistant bacteria by interaction with other targets (lipid membrane, membrane protein (e.g., penicillin-binding protein 2) and cell-wall peptidoglycan), in addition to affinity for the d-alanyl-d-lactate residue. In the present study, we found that Van-M-02 and ΔN-Van-M-02 showed antibacterial activities by interaction with the lipid membrane of vancomycin-resistant bacteria.

**Binding Properties of Other Antimicrobial Agents to the Model Lipid Membranes**

Teicoplanin, a glycopeptide antibiotic, is known to bind to d-alanyl-d-alanine and d-alanyl-d-lactate residues on the end of the peptide chains of lipid II, and inhibit proper cell wall synthesis in Gram-positive bacteria including some VREs. The interaction of teicoplanin with the dipeptide ligands was confirmed in the previous SPR study on the basis of the therapeutic mechanism. We speculated that the binding of teicoplanin to the model lipid membranes would not contribute much to the antibacterial activity. Erythromycin is a macrolide antibiotic that has a wide antimicrobial spectrum and linezolid is an oxazolidinone antimicrobial, which has been approved for the treatment of infections caused by various Gram-positive bacteria, including methicillin-resistant *S. aureus* and VRE. These agents bind to ribosome, and inhibit protein synthesis by their effect on the ribosome function. As we found no binding of erythromycin and linezolid to the model lipid membranes and the dipeptide ligands, we speculated that this would affect their therapeutic mechanisms.

**CONCLUSION**

We examined the interaction of antimicrobial agents with four model lipid membranes that mimicked mammal cell membranes and Gram-positive and negative bacterial membranes and analyzed the binding kinetics by SPR. The selective and specific binding characteristics of the antimicrobial agents to the lipid membranes were estimated and the kinetics parameters were analyzed; reproducible analysis of binding kinetics was observed. As vancomycin and teicoplanin showed less interaction with the four lipid membranes in the SPR system, we speculate that the low affinity of these antimicrobial agents could be attributed to the therapeutic mechanism in *vivo* for bactericidal activity differing from that of disruption of the bacterial cell membrane. The vancomycin analogs used in this study showed interaction with the model lipid membranes in the SPR system. Vancomycin showed little binding to the lipid membranes, but Van-M-02, ΔN-Van-M-02 and Dimer 1 could bind to them. The selective and specific binding characteristics of vancomycin analogs to the lipid membranes could be clarified by combining data on antibacterial activities in *vivo* and the data we reported on the binding affinity of the d-alanyl-d-alanine terminus of a pentapeptide cell wall by SPR. Furthermore, the mechanism of antibacterial activity against *Staphylococcus aureus* and vancomycin-resistant enterococci could be evaluated using the binding affinity obtained by our SPR techniques. The results indicated that the SPR method could be widely applied for predicting binding characteristics, such as selectivity and specificity, of many antimicrobial agents to the lipid membranes as an *in vitro* system.

**REFERENCES**


