



# Interactions of oritavancin, a new lipoglycopeptide derived from vancomycin, with phospholipid bilayers: Effect on membrane permeability and nanoscale lipid membrane organization

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## ABSTRACT

Antibiotics acting on bacterial membranes are receiving increasing attention because of widespread resistance to agents acting on other targets and of potentially improved bactericidal effects. Oritavancin is a amphiphilic derivative of vancomycin showing fast and extensive killing activities against multi-resistant (including vancomycin insusceptible) Gram-positive organisms with no marked toxicity towards eukaryotic cells. We have undertaken to characterize the interactions of oritavancin with phospholipid bilayers, using liposomes (LUV) and supported bilayers made of cardiolipin (CL) or phosphatidylglycerol (POPG) and phosphatidylethanolamine (POPE), all abundant in Gram-positive organisms. Changes in membrane permeability were followed by the release of calcein entrapped in liposomes at self-quenching concentrations, and changes in nanoscale lipid organization examined by Atomic Force Microscopy (AFM). Oritavancin caused a fast (<5 min) and complete (>95%) release of calcein from CL:POPE liposomes, and a slower but still substantial (50% in 60 min) release from POPG:POPE liposomes, which was (i) concentration-dependent (0–600 nM; [microbiologically meaningful concentrations]); (ii) enhanced by an increase in POPG:POPE ratio, and decreased when replacing POPG by DPPG. AFM of CL:POPE supported bilayers showed that oritavancin (84 nM) caused a remodeling of the lipid domains combined with a redistribution of the drug and degradation of the borders. In all the above studies, vancomycin was without a significant effect at 5.5 μM. Electrostatic interactions, together with lipid curvature, lipid polymorphism as well of fluidity play a critical role for the permeabilization of lipid bilayer and changes in lipid organization induced by oritavancin.

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## 1. Introduction

Biological membranes act as semi-permeable barriers to allow the inside environment of cells or organelles to differ from that outside medium, and mediate cell communications and interactions. In Gram-positive bacteria, the pericellular membrane is surrounded by a thick cell wall of peptidoglycan that confers mechanical resistance to external osmotic and other physical stress. Both the pericellular membrane and the cell wall of peptidoglycan are essential for cell survival, and, therefore, are privileged targets in antibacterial chemotherapy. Antibiotics that act on the membrane of Gram-positive organisms include daptomycin and nisin. Daptomycin is a lipopeptide approved for clinical use which, after complexation with Ca<sup>2+</sup>, forms an oligomeric assembly exerting a detergent-like effect towards bilayers with a high content in negatively-charged headgroups, such as those found in bacteria, causing leakage of cytosolic contents [1]. Nisin kills bacteria by forming a pore

complex together with lipid II [2]. Antibiotics acting on the formation of the bacterial cell wall include the β-lactams, which act primarily by inhibition of the transpeptidases (or penicillin-binding proteins and impair the reticulation of the peptidoglycan [3] and the glycopeptides (vancomycin, teicoplanin) that act by binding to the D-Ala–D-Ala sequence of the pentapeptide of lipid II, thereby preventing the transpeptidation and transglycosylation reactions in the late stages of peptidoglycan synthesis [4]. Both approaches, however, have their limitations, with the most striking examples of the emergence of resistance of a major medical importance being shown by *Staphylococcus aureus*. This pathogen has indeed acquired mechanisms of resistance to β-lactams (under the form of the so-called methicillin-resistant organisms that produce a transpeptidase [PBP 2a] with decreased affinity for β-lactams [5], to glycopeptides (with the emergence of the so-called glycopeptide-intermediate *S. aureus* characterized by a thickened cell wall, and also of fully resistant organisms in which the terminal D-Ala is replaced by Ser or a lactate [6], and to daptomycin (by alteration of the biosynthetic pathway of phosphatidylglycerol and/or by acquisition of a thickened cell wall (see [7] for review).

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Chemical modification of the glycopeptides, however, has provided a new class of derivatives called collectively lipoglycopeptides which show improved activity against such multi-resistant strains (see [8] for review). Among them, oritavancin (LY333328) is the most active against vancomycin-resistant organisms [7]. Compared to vancomycin, oritavancin possesses a 4-(4-chlorophenyl)-phenylmethyl side chain [9,10], synthetically added to the natural product precursor, chloroeremomycin, which differs from vancomycin by the presence of a 4-epi-vancosamine sugar (Fig. 1). Oritavancin, as well as another lipoglycopeptide, telavancin, is considerably more rapidly bactericidal than vancomycin, suggesting a novel mode of action that could involve membrane destabilization [11–13]. This mechanism ought to be specific of bacteria since both compounds are devoid of marked toxicities towards eukaryotic cells and have been successfully brought to clinical applications in humans [7,11].

Because interactions of lipoglycopeptides in general, and of oritavancin in particular, with model membranes is still largely unexplored, we attempted to further characterize them using a multidisciplinary approach but taking care of using conditions that mimicked those under which the drug exerts its antibacterial effects. As maintenance of a permeability barrier is the central property of biological membranes, we first compared the ability of oritavancin and vancomycin to permeabilize lipid bilayers. The results were correlated with real-time observations of the interactions of both molecules with lipids at the nanoscale level using Atomic Force Microscopy (AFM) [14]. Since the bacterial membrane is rich in acidic phospholipids [15], we used liposomes and supported bilayers made of cardiolipin (CL) or phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). PG is the predominant phospholipid in the membranes of *S. aureus* [16], and cardiolipin is also found at high levels in these bacteria [17] as well several other Gram-positive organisms [15,18]. While being more abundant in Gram-negative bacteria, PE is nevertheless present in *S. aureus* [19,20].

## 2. Materials and methods

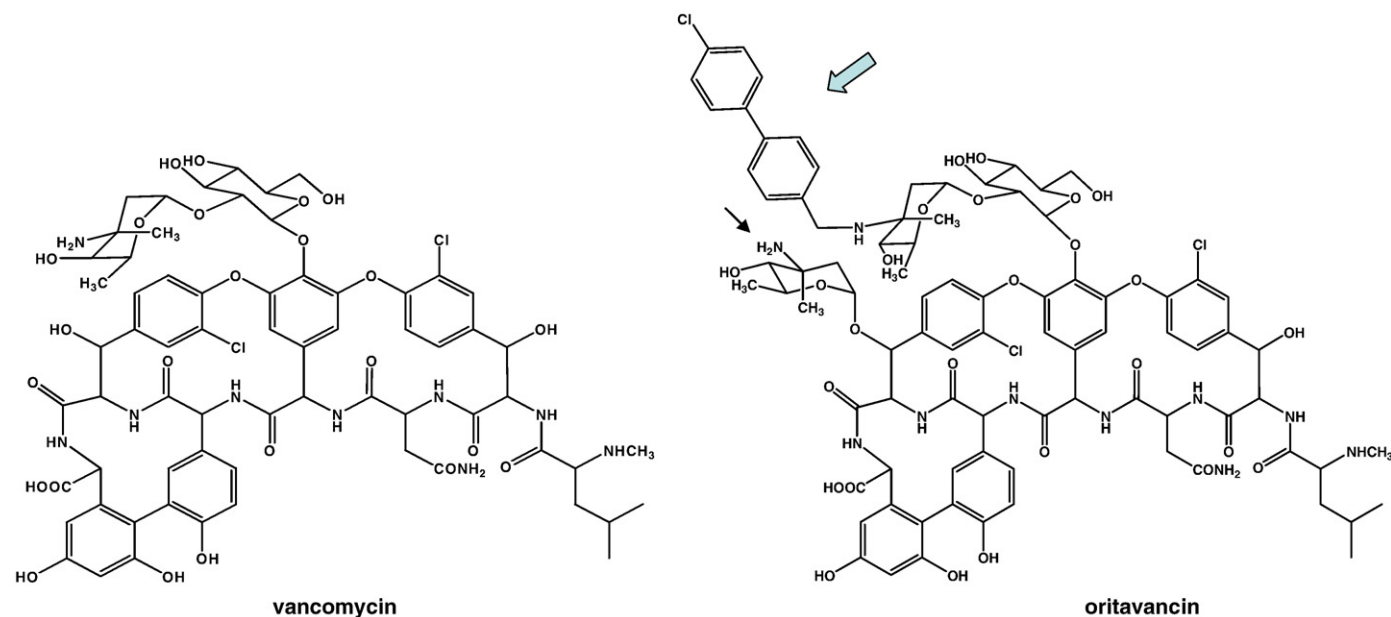
### 2.1. Materials

Beef heart cardiolipin (CL; Disodium Salt; purity >99%), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmi-

toyl-2-palmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol-amine (POPE) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Calcein was purchased from Sigma-Aldrich (St. Louis, MO) and was purified as described previously [21]. Briefly, calcein was dissolved in 6 N NaOH and subjected to size-exclusion chromatography through a Sephadex<sup>®</sup> LH-20 column. The final concentration of calcein solution in 20 mM Tris-HCl was 73 mM with an osmolarity of 434 mOsm/kg (measured by the freezing point technique, using a model 3C2 Advanced Cryomatic Osmometer [Advanced Instruments, Needham Heights, MA]). Oritavancin diphosphate powder was supplied by Targanta Therapeutics, Cambridge, MA, as microbiological standard for *in vitro* investigations. It was dissolved in water containing 0.002% polysorbate-80 (vol:vol). Vancomycin was obtained from GlaxoSmithKline s.a., Genval, Belgium as the product registered for parenteral use in humans and complying with the provisions of the European Pharmacopeia.

### 2.2. Large unilamellar vesicle preparation

Lipids were dissolved in CHCl<sub>3</sub>:CH<sub>3</sub>OH (2:1, v/v) and mixed to obtain the desired composition in a round flask. The solvent was evaporated using a Rotavapor system (model R-210, Buchi Labortechnik AG, Flawil, Switzerland). Dried films were maintained under reduced pressure overnight and thereafter hydrated with 20 mM Tris-HCl, 150 mM NaCl, 20 mM CaCl<sub>2</sub> 2H<sub>2</sub>O, pH 7.4 for AFM studies and with the purified calcein for permeability experiments. Large Unilamellar Vesicles (LUV) were obtained after 5 cycles of freeze/thawing and 10 cycles of extrusion in a 10 mL Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada) under a nitrogen pressure of 10 bars through two polycarbonate filters of 100 nm or 400 nm pore size for permeability and AFM studies, respectively (Nucleopore, Costar Corporation, Badhoevedorp, The Netherlands). Non-entrapped calcein was removed using minicolumn centrifugation [21]. The size and polydispersity of liposome suspensions (Table 1) were monitored by quasi-elastic light scattering with a Zetasizer Nano SZ (Malvern Instruments, Worcestershire, UK). Lipid concentration on the liposomal suspensions was measured by phosphorous quantification [22].



**Fig. 1.** Structural formulae of vancomycin and oritavancin. Key differences are (i) the chloro-biphenyl methylene moiety (block arrow; absent in vancomycin) that confers a more hydrophobic character to the molecule, and (ii) the amino group present in the additional epi-vancosamine moiety (thin arrow) that contributes to its amphipathic character. The calculated log P and log D (at pH 7) of vancomycin are –1.44 and –4.70, and those of oritavancin 4.10 and –3.43.

**Table 1**

Diameter and polydispersity index of liposomes used for permeability studies (measured by quasi-elastic light scattering).

| Composition              | Diameter (nm) | Polydispersity index |
|--------------------------|---------------|----------------------|
| POPC:POPE (4:8, mol:mol) | 114.1         | 0.172                |
| POPG:POPE (4:8, mol:mol) | 109.9         | 0.110                |
| CL:POPE (2:8, mol:mol)   | 127.6         | 0.145                |
| DPPG:POPE (4:8, mol:mol) | 103.1         | 0.079                |

### 2.3. Calcein release

The liposome total phospholipid concentration was adjusted to a final concentration of 5  $\mu\text{M}$  with isoosmotic buffer (20 mM Tris–HCl, 200 mM NaCl, pH 7.4). After overnight storage at 4 °C, liposomes were exposed to the drug under study at 37 °C at the desired concentration and for the suitable time with continuous stirring and protection from light. Leakage of entrapped calcein was monitored by the increase in fluorescence of the samples, resulting from the dilution and release of self-quenching of this tracer [23]. All fluorescence determinations were performed on an LS 55 fluorescence spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, UK) using  $\lambda_{\text{exc}}$  and  $\lambda_{\text{em}}$  of 476 nm and 512 nm, respectively and slits fixed at 3 nm. The percentage of calcein released under the influence of drug was defined as  $[(F_t - F_{\text{contr}}) / (F_{\text{tot}} - F_{\text{contr}})] \times 100$ , where  $F_t$  is the fluorescence signal measured at time  $t$  in the presence of drug,  $F_{\text{contr}}$  is the fluorescence signal measured at the same time in the absence of drug, and  $F_{\text{tot}}$  is the total fluorescence signal obtained after complete disruption of liposomes by Triton X-100 at a final concentration of 2% (checked by quasi-elastic light spectroscopy) [21].

### 2.4. Atomic force microscopy

Before each experiment, the contact mode cell was extensively washed with ethanol and water. Mica squares (0.25 cm<sup>2</sup>) were glued onto a steel disc, cleaned carefully with water before use and cleaved to obtain a flat and uniform surface. Immediately, an aliquot of 50  $\mu\text{L}$  of vesicles (CL:POPE [2:8] or POPE only; adjusted to 250  $\mu\text{M}$  in 20 mM Tris–HCl, 150 mM NaCl, 20 mM CaCl<sub>2</sub> 2H<sub>2</sub>O, pH 7.4), was deposited on the mica surface and incubated for 1 h at room temperature. The sample was thereafter washed with buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) to eliminate non-adsorbed vesicles. AFM contact mode images in liquid were obtained using a Nanoscope IV Multimode AFM (Veeco Metrology Group, Santa Barbara, CA) with triangular Si<sub>3</sub>N<sub>4</sub> cantilevers (Microlevers, Veeco Metrology Group, Santa Barbara, CA.) with a nominal spring constant of 0.01 N m<sup>−1</sup>. The instrument was equipped with a “J” scanner (120  $\mu\text{m}$ ). To minimize the applied force on the sample the set point was continuously adjusted during imaging. Images were acquired at 90° scan angle with a scan rate of 2 Hz. All images were processed using the Veeco software.

## 3. Results

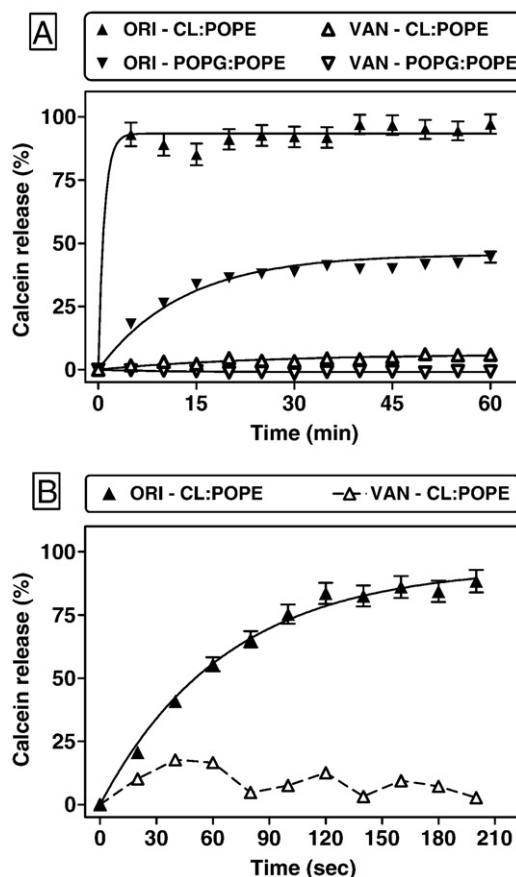
### 3.1. Membrane permeability

The effect of oritavancin and vancomycin at a fixed concentration (600 nM) on the permeability of liposomes made of CL:POPE (2:8) or POPG:POPE (4:8) (so as to obtain the same amount of negative charges) is shown in Fig. 2A. Oritavancin caused a release of calcein that was almost immediate (less than 5 min) and complete when added to CL:POPE liposomes. The release of calcein was slower but eventually reached about 50% within 60 min when oritavancin was added to POPG:POPE liposomes (maintaining oritavancin in contact with these liposomes for up to 8 h caused about 60% calcein release). Vancomycin was almost without effect on both CL:POPE and POPG:POPE liposomes. To obtain more information about the true rate of release of calcein from CL:POPE liposomes upon exposure to

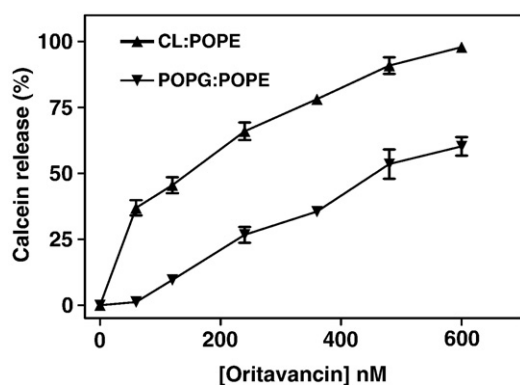
oritavancin, experiments were repeated using shorter periods of contact (0–200 s). Results presented in Fig. 2B show that 50% calcein release was obtained within about 50 s. Vancomycin was without constant effect under these conditions.

In a second series of experiments, we examined the effect of oritavancin concentration on the permeabilization of CL:POPE and POPG:POPE vesicles. The concentration range chosen (0–600 nM) was meant to attain the values reported as causing bacterial death of *S. aureus* *in vitro* (0.12–1 mg/L [66–550 nM], depending upon assay conditions [24,25]. The results, as seen after 8 h of contact (to ensure maximal effects) are presented in Fig. 3. Oritavancin caused a concentration-dependent release of calcein that reached a value close to 98% at 600 nM when tested against CL:POPE liposomes, and 60% for POPG:POPE liposomes. Vancomycin was without significant effect over the same range of concentrations (data not shown).

The experiments reported so far did not address the question as to whether the permeabilization effect seen with oritavancin was related to a bilayer charge per se or to hydrophobic interactions between this lipoglycopeptide and membranes. We, therefore, used POPG:POPE and POPC:POPE vesicles and examined the effect of changing the ratio between POPG and POPE and between POPC and POPE on the ability of oritavancin to cause calcein release. The results presented in Fig. 4 show that an increase of the POPG to POPE ratio from 0.5 to 0.75 was associated with an increase of calcein release, above which only a



**Fig. 2.** Release of calcein from liposomes (5  $\mu\text{M}$  phospholipids) made of CL:POPE (2:8 molar ratio; upright triangles) or POPG:POPE (4:8 molar ratio; upside down triangles) upon exposure at 37 °C to 600 nM oritavancin (ORI; filled symbols) or vancomycin (VAN; open symbols). (A) 0–60 min exposure time; (B) 0–200 s exposure time (CL:POPE liposomes only). The ordinate shows the percentage of calcein released compared to what was observed after addition of 2% Triton X-100. Each value is the mean of three independent experimental determinations  $\pm$  SEM (when not visible, the bars are smaller than the symbols). For oritavancin, the graph shows the functions obtained by fitting a one phase exponential association function to the data by non-linear regression.



**Fig. 3.** Release of calcein from liposomes (5  $\mu$ M phospholipids) made of CL:POPE (2:8 molar ratio; upright triangles) or POPG:POPE (4:8 molar ratio; upside down triangles) upon exposure at 37 °C and for 8 h to increasing concentrations of oritavancin (0–600 nM; 0–1.08 mg/L). The ordinate shows the percentage of calcein released compared to what was observed after addition of 2% Triton X-100. Each value is the mean of three independent experimental determinations  $\pm$  SEM (when not visible, the bars are smaller than the symbols).

more modest increase was seen. Conversely, the extent of release of calcein, which was substantial when using liposomes with a low POPC:POPE ratio (0.5) was decreased when the POPC:POPE ratio was increased to 0.75 or more. The influence of the nature of the fatty acid chain was also investigated by replacing POPG by DPPG in liposomes while leaving the ratio PG:PE identical (4:8). This caused a drastic reduction of the permeabilization effect of oritavancin that dropped from 60% to 24% (measured after 8 h of contact with 600 nM oritavancin).

### 3.2. Atomic force microscopy

To go further in the study of the interaction of oritavancin and lipid bilayers and especially to characterize at the nanoscale, the effect of this antibiotic on membrane organization, we prepared supported lipid bilayers made of POPE only, or of CL:POPE (2:8). The latter composition has been selected in view to the larger permeabilizing effect induced by oritavancin on CL:POPE (2:8) as compared to POPG:POPE (4:8). Supported lipid bilayers made of POPG:POPE were already studied by AFM for some of us [26] showing no lipid separation between POPE and POPG. Supported bilayers of POPE and CL:POPE were prepared by fusion of unilamellar vesicles on mica, and their organization was imaged by AFM. Preliminary experiments assessed the stability of POPE and CL:POPE bilayers during consecutive scanning in buffer. Successive images of the same bilayer location were recorded but this did not cause any significant change of the surface morphology, indicating that the bilayer was stable in these conditions for at least 3 h.

#### 3.2.1. POPE bilayers

Fig. 5A shows a typical topographic image of a POPE bilayer supported on mica recorded in buffer solution where the darkest region is the mica surface while the brightest region is the surface of the supported planar bilayer. The step height with respect to the mica surface was 5.24 nm ( $n=50$ ), with a roughness ( $R_a$ ) mean value of 0.07 nm ( $n=25$ ). Fig. 5B shows a typical image of the same bilayer after 1 h exposure to vancomycin (5.5  $\mu$ M [8 mg/L]), a concentration resulting in a bactericidal effect towards *S. aureus* *in vitro* [27,28]. The drug induced an erosion in the unprotected edges, border and holes, of the supported planar bilayer, but did not substantially change its thickness, the step height being 4.90 nm ( $n=50$ ) with a roughness of 0.12 nm ( $n=25$ ). When the experiment was performed in the presence of oritavancin (84 nM [0.15 mg/L], a concentration at which oritavancin becomes bactericidal *in vitro* [25], the drug induced an erosion of the bilayer together with a decrease of the height down to

4.00 nm (Fig. 5D) vs. 4.80 nm [ $n=50$ ] for control (Fig. 5C) without change in roughness (0.09 nm [ $n=25$ ]). These results suggest, therefore, that vancomycin had no major effect on the supported planar bilayer of pure POPE whereas oritavancin induced a slight erosion and thinning of the bilayer (Table 2).

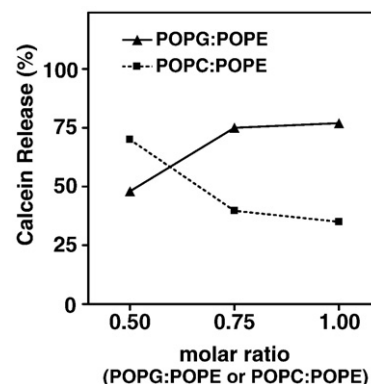
#### 3.2.2. CL:POPE (2:8) bilayers

In control supported bilayers, two laterally segregated domains were observed. The step height between the uncovered mica, and the underlying domain was 4.55 nm ( $n=50$ ) with a  $R_a$  value of 0.17 nm ( $n=25$ ) (Fig. 6A). Several smaller domains were seen which were 1.03 nm ( $n=50$ ) taller than the underlying domain with a mean  $R_a$  value of 0.09 nm ( $n=25$ ). These domains were clearly seen in the cross section supported below as a protruding planar structure on the more extended domain.

After 1 h of contact with vancomycin (5.5  $\mu$ M [8 mg/L]), the frontiers of the more extended lipid domain were eroded (Fig. 6B). From section analysis, the more extended domain presented a height of 3.79 nm ( $n=50$ ) from the mica surface with a mean  $R_a$  value of 0.15 nm ( $n=25$ ). In parallel, reorganisation and/or disappearance of several small lipid domains was observed with solubilisation and afterwards spreading of the material on the mica surface. Moreover, new domains appeared which were 0.75 nm higher than the underlying domain with a mean  $R_a$  value of 0.07 nm ( $n=25$ ). When the supported bilayers were exposed to oritavancin to a final concentration of 84 nM (0.15 mg/L) for 1 h, a completely different effect was seen compared to vancomycin. While the control supported bilayers (Fig. 6C) showed two segregated domains of 4.57 nm and 5.26 nm heights and 0.08 nm and 0.14 nm roughness, respectively, oritavancin (Fig. 6D) caused a remodelling of the lipid domain combined with a redistribution of the drug as well as a degradation of the unprotected borders of the supported bilayer. Thus, oritavancin induced a decrease of the height from 4.57 nm to 4.10 nm ( $n=50$ ) with a roughness of 0.15 nm. This underlying domain was covered by two other domains. The first one was located at 0.48 nm ( $n=50$ ) with a roughness of 0.13 nm whereas the second one, more heterogeneous, ranged from 0.51 to 1.40 nm ( $n=25$ ).

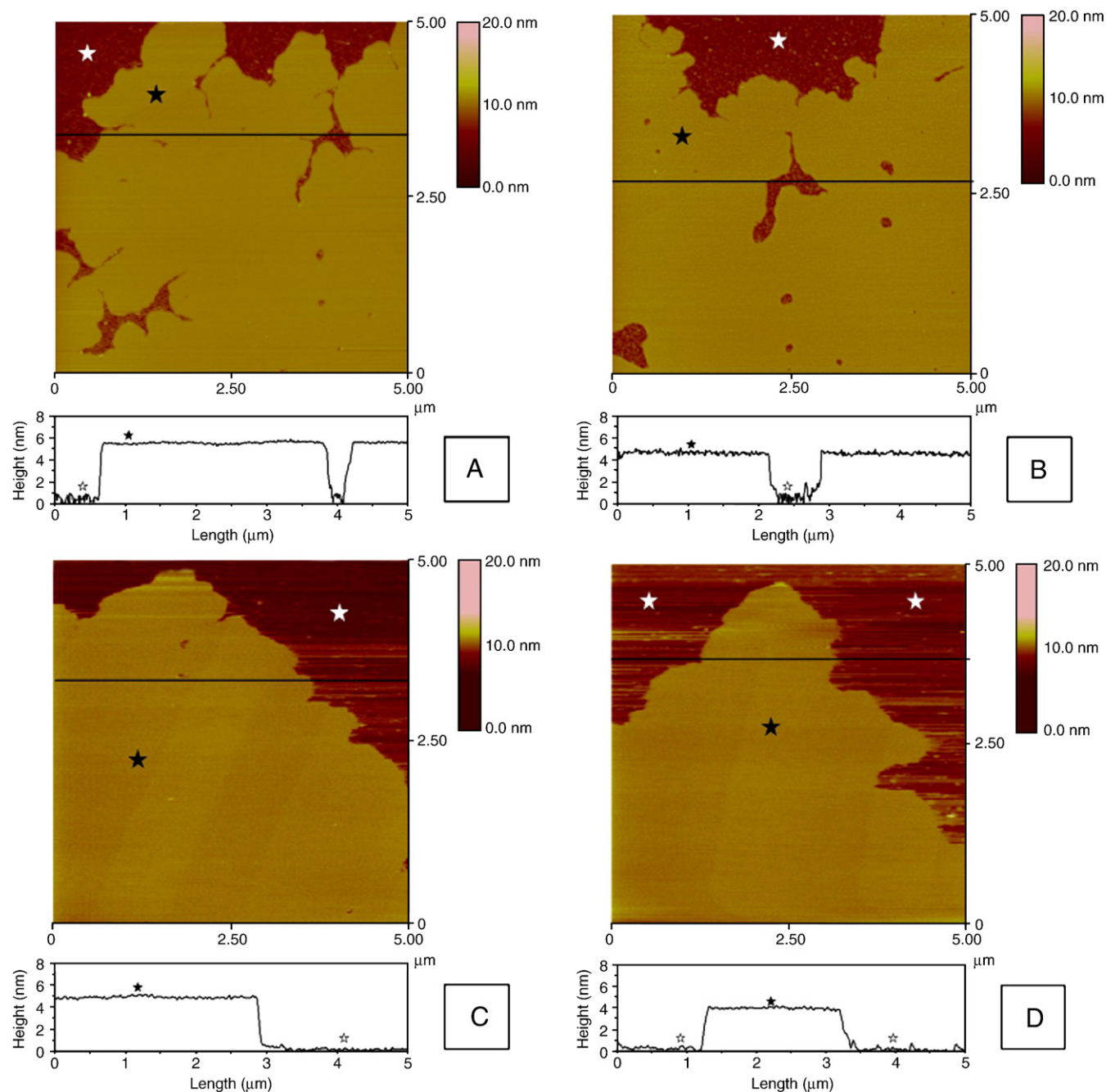
## 4. Discussion

The bacterial envelope has long been recognized as a critical target for antibiotics, with  $\beta$ -lactams and glycopeptides—both directed against the peptidoglycan—being the most widely used today in clinical practice to fight Gram-positive bacteria and *S. aureus* in particular. In contrast, antibiotics acting on the lipid membrane part of



**Fig. 4.** Release of calcein from liposomes (5  $\mu$ M phospholipids) made of POPG:POPE (triangles) or POPC:POPE (squares) of variable composition (see abscissa) upon exposure at 37 °C and for 8 h to 600 nM (1.08 mg/L) oritavancin. The ordinate shows the percentage of calcein released compared to what was observed after addition of 2% Triton X-100. Each value is the mean of three independent experimental determinations  $\pm$  SEM (when not visible, the bars are smaller than the symbols).





**Fig. 5.** Nanoscale membrane activity of vancomycin (top) or oritavancin (bottom) on POPE supported bilayer. AFM height images (5 μm × 5 μm; z-scale: 20 nm) of POPE supported planar bilayer recorded prior (0 min) (A; C) or after exposure for 1 h to vancomycin (5.5 μM [8 mg/L]) (B) or oritavancin (84 nM [0.15 mg/L]) (D). Vertical cross-sections were taken along the position indicated by the continuous line. Upper domain and mica are indicated by black and white stars, respectively.

the bacterial membrane have for long remained largely unused as human medicines, mainly because of lack of specificity resulting in a globally less favorable toxicological profile. The emergence of widespread resistance to β-lactams and the slow but inescapable loss of potency of glycopeptides has, however, directed again the attention to membrane-acting antibiotics, as these may potentially offer novel modes of action coupled with powerful antibacterial activities [7]. Among these, lipoglycopeptides attracted increasing attention since these have now been successfully developed clinically [29,30] and are being considered for approval for the treatment of multi-resistant Gram-positive organisms.

For lipoglycopeptides, differences in activities relative to vancomycin do not relate to the aglycone part of these molecules, i.e. in the D-Ala–D-Ala binding sites, since these are similar with affinities

essentially identical among all of them [31]. However, the presence of a lipophilic tail confers the ability to lipoglycopeptides to interact with membranes. This has been shown for telavancin, a drug with an overall structure and antibacterial properties similar to those of oritavancin, for which 25% only of the antibiotic is recovered in the peptidoglycan while 75% remains associated the protoplast upon separation of these two constituents of the bacterial envelope (vs. >90% bound to the peptidoglycan and <10% to the protoplasts for vancomycin) [12]. The novel mode of action proposed involves dissipation of membrane potential and membrane permeabilization [13], and has been related to loss of membrane integrity favored by interaction of the drug with lipid II (the molecule that carries the D-Ala–D-Ala terminated pentapeptide from the cytosolic to the extracellular face of the bacterial membrane). Similarly, ongoing studies

**Table 2**

Height and roughness values in supported phospholipid bilayers of POPE (top) and CL:POPE (2:8; mol:mol) (bottom).

|             |        | Height $\pm$ SD (nm)        | Roughness (nm)  |                 |                       |              |      |
|-------------|--------|-----------------------------|-----------------|-----------------|-----------------------|--------------|------|
| Vancomycin  | Before | 5.24 $\pm$ 0.19             | 0.07            |                 |                       |              |      |
|             | After  | 4.90 $\pm$ 0.20             | 0.12            |                 |                       |              |      |
| Oritavancin | Before | 4.80 $\pm$ 0.20             | 0.09            |                 |                       |              |      |
|             | After  | 4.00 $\pm$ 0.15             | 0.09            |                 |                       |              |      |
|             |        | Domain height $\pm$ SD (nm) |                 |                 | Domain roughness (nm) |              |      |
|             |        | Low                         | Intermediate    | High            | Low                   | Intermediate | High |
| Vancomycin  | Before | –                           | 4.55 $\pm$ 0.20 | 5.58 $\pm$ 0.16 | –                     | 0.17         | 0.09 |
|             | After  | –                           | 3.79 $\pm$ 0.30 | 4.54 $\pm$ 0.11 | –                     | 0.15         | 0.07 |
| Oritavancin | Before | –                           | 4.57 $\pm$ 0.15 | 5.26 $\pm$ 0.20 | –                     | 0.08         | 0.14 |
|             | After  | 4.10 $\pm$ 0.30             | 4.58 $\pm$ 0.07 | 4.61–5.50       | 0.15                  | 0.13         | –    |

have now described the depolarization of membranes of vancomycin non-susceptible *S. aureus* following incubation with oritavancin (G.A. McKay, S. Beaulieu, T.R. Parr Jr, G. Moeck, in Program and Abstracts of the 48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington DC (2008) abstract no. C1-4182; [13]).

In the present work, and using calcein as a validated probe for the study of lipid membrane permeabilization [21,23], and AFM to investigate the effect of the lipopeptide at the nanoscale, we showed that oritavancin induced membrane permeabilization together with changes in the lipid membrane organization in domains. It is, however, important to remember that the two models used, liposomes and supported lipid bilayers, are different, notably regarding the curvature of the bilayer and the ability of the both leaflets of lipids to be in contact with aqueous phases. The effects induced by oritavancin are observed in the absence of any binding to a D-Ala–D-Ala motif, indicating that alteration of membrane integrity is probably not due to the aglycone part of the molecule, since its is common to that of vancomycin, which exerts no effect in our models.

The mechanisms responsible for membrane permeabilization are complex, but four main mechanisms have been proposed, namely (i) the barrel-stave channel, i.e. a bundle of membrane-spanning helices aligned with the polar side chains oriented towards the center [32,33] (ii) the carpet-like mechanism where a monolayer of surface-lying peptides covers the membrane surface [34], (iii) the toroidal model [35,36], and (iv) the detergent-like peptide model [37]. Our study does not provide direct evidence for either of these models, which should be the subject of future investigations. Especially the ability of oritavancin to insert in the outer monolayer of the bilayer, leading to an asymmetric increase in lateral pressure that tends to bend the bilayer and induce a transient disruption of the membrane [37] has to be examined. Moreover, the formation of rich clusters in the membrane and destabilization of the lipid membrane by formation of mixed micelles, could be determined.

If the permeabilization clearly depends on drug structure, the lipid composition, and therefore the physical properties of membranes, can also play a critical role for permeabilization. This is clearly reflected by the ranking found for the ability of oritavancin to permeabilize lipid bilayers (CL:POPE > POPC:POPE > POPG:POPE > DPPG:POPE). Four parameters could be critical in this respect.

First, one feature that distinguishes the membrane of prokaryotic organisms from that of eukaryotic organisms is that only the former harbor negatively-charged lipids in the outer leaflet of the plasma membrane. So a large proportion of negatively-charged lipids, especially POPG, is generally considered critical for the selectivity of action of antibacterial peptides and lipopeptides [1]. Recently, however, this point has been questioned. Some amphipathic  $\alpha$ -helical peptide analogues derived from *Helicobacter pylori* [38] had strong antibacterial activity despite weak binding to phosphatidylglycerol. Similarly, homologous antimicrobial oligomers mimicking host defense peptides are not sensitive to charge [39]. Regarding oritavancin, clearly, the presence of negatively-charged lipids is not

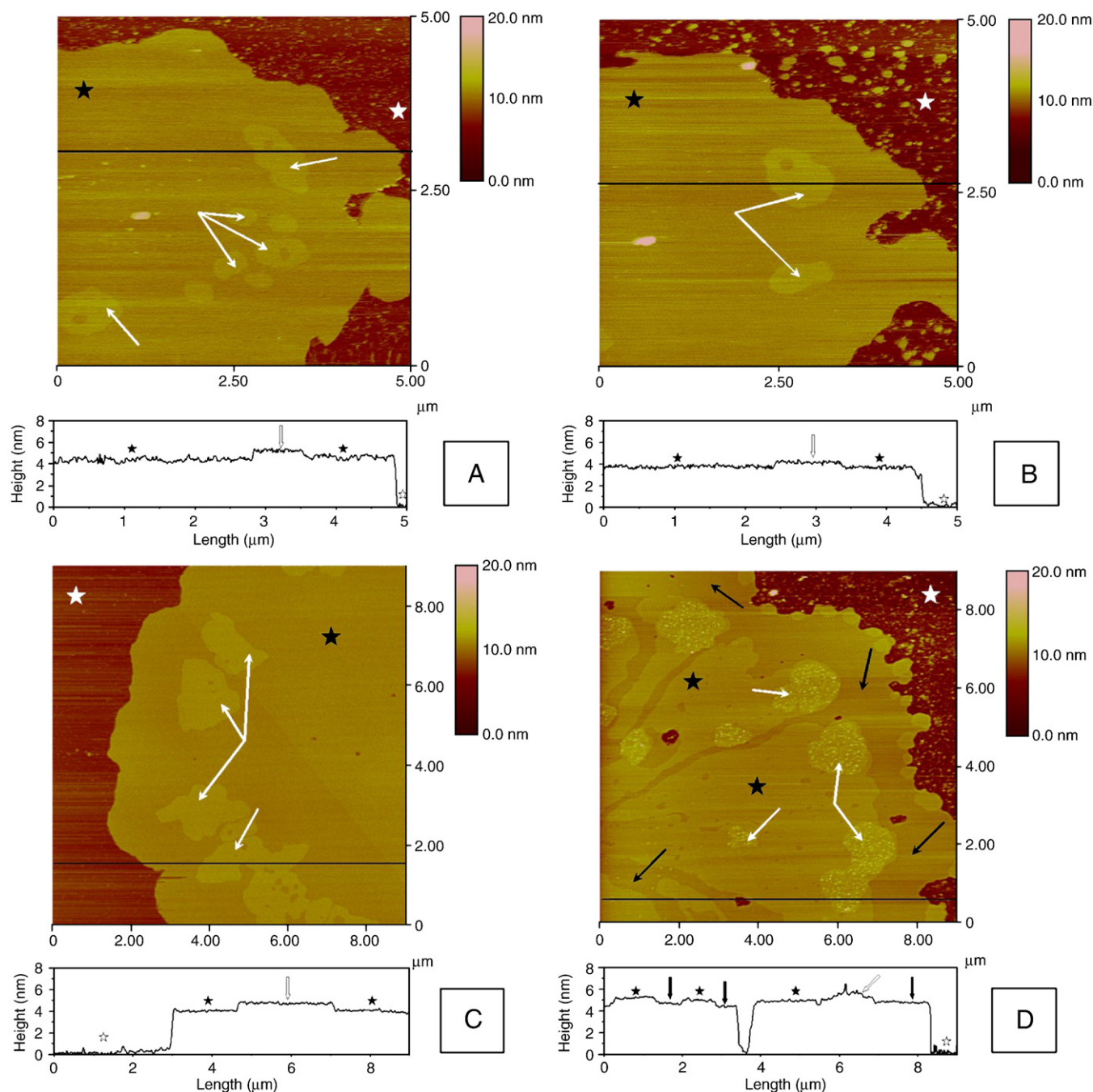
the only parameter required to induce the maximal membrane permeabilization since a higher permeabilizing effect has been observed on bilayers of POPC:POPE as compared to POPG:POPE. This is in agreement with other studies performed on plantaricin A, a 26-residue peptide pheromone, for which leakage of calcein from SOPC:POPG vesicles is less extensive than from SOPC vesicles [40]. Similarly, pardaxin, a membrane-lysing peptide originally isolated from the fish *Pardachirus marmoratus*, also shows a lower ability to disrupt POPG:POPE as compared to homogenous POPC bilayers [41].

Second, besides electrostatic interactions, the ability of phospholipids to form H bonds could also be a major parameter governing their interaction with glycopeptides. Indeed, POPG:POPE bilayers show a high propensity to interact through hydrogen bonds with phosphate and carbonyl oxygen atoms, as well as a much denser packing of chain atoms in the near-the-interface regions of the hydrophobic core, as compared to POPC bilayers [42]. Moreover, POPG is less hydrated and shows a low probability of gauche conformation in the beginning of the  $\beta$ -chain as compared to POPC [43]. This may contribute to disturb packing of atoms in the near-the-interface regions of the POPC bilayer core, creating defects and explaining why oritavancin showed greater permeabilizing effect towards POPC:POPE (4:8) than POPG:POPE (4:8).

Third, the propensity of CL and PE to induce negative curvature and adopt hexagonal phase [44,45] may also contribute to favor the permeabilization induced by oritavancin. At a molecular level, the polar headgroup of PE is characterized by a smaller diameter than the hydrocarbon chain region in the fluid phase. This results in a molecular shape of a truncated cone and an intrinsic propensity of PE bilayers to form surfaces with a negative curvature, leading to the formation of non-lamellar phases. Formation of hexagonal phase can be related to high compressibility in fluid phase [46], to an ability to easily adapt to highly irregular surfaces [47], and to provide the lateral force for an adequate stress profile [46,48]. This could thus explain why, when the POPE content is increased in liposomes containing POPC:POPE, an enhancement of calcein release is observed. In CL:POPE liposomes, the rapid and important effect of oritavancin on membrane permeability could result from a local reduction in electrostatic repulsion between cardiolipin molecules, thus imposing negative curvature strain on the membrane. In comparison to CL:POPE, the lower permeabilizing effect of oritavancin on POPG:POPE liposomes, could be related to the fact that PG preferentially adopts a cylindrical molecular shape and forms flat bilayer, even at high temperatures.

Fourth, the unsaturated nature of the bacterial phospholipid confers fluidity to the membrane and possibly augments the insertion of exogenous compounds like antimicrobial peptides [49] or the glycopeptides themselves [50]. The higher release of calcein from liposomes of POPG:POPE: as compared to DPPG:POPE could result from this.

AFM, a now well-established technique for imaging supported lipid bilayers at nanometer resolution [14] has also provided us with useful information on the changes brought by oritavancin on bilayer



**Fig. 6.** Nanoscale membrane activity of vancomycin (top) or oritavancin (bottom) on CL:POPE (2:8) supported bilayer. AFM height images (5 μm × 5 μm; z-scale: 20 nm) of CL:POPE supported planar bilayer recorded prior (0 min) (A; C) or after exposure for 1 h to vancomycin (5.5 μM [8 mg/L]) (B) or oritavancin (84 nM [0.15 mg/L]) (D). Vertical cross-sections were taken along the position indicated by the continuous line. Upper domain and mica are indicated by black and white stars, respectively. White arrows showed domains enriched in cardiolipin.

organization. Previous studies performed by mixing CL and POPE at the air–water interface, and comparing the values of the excess energy of mixing, showed that the composition chosen (CL:POPE at 2:8 molar ratio) was the most stable [51]. In addition to the lateral phase separation due to the differences in molecular structure, POPE and CL can simultaneously undergo a thermal transition at the same time [52], explaining the occurrence of the three domains in a binary system observed by AFM. Since increasing the amount of cardiolipin resulted in an increase of the area occupied by the upper domains, it has been assumed that the lower domain is mainly formed by POPE whereas the upper domain is mainly formed by cardiolipin [52].

We show here that oritavancin did not modify the height of a supported bilayer of POPE, but decreased the step height between the

mica surface and the surface of the more extended domain in the CL:POPE bilayer. In a supported bilayer of pure POPE, the PE headgroups can form hydrogen bonds between adjacent molecules [53] conferring rigidity to the surface, while in a CL:POPE mixed supported bilayer, cardiolipin molecules can disturb the formation of the hydrogen bonds network [51]. Thus, oritavancin could interact with CL, which tends to form non-lamellar phases, promoting a negative curvature in the region of the domain and destroying its integrity (detergent-like peptide model) [37].

Our studies were performed under conditions that are pertinent to the antibacterial activity of oritavancin, in that we (i) used bilayers that, like bacterial membranes, are rich in both a negatively-charged lipid like phosphatidylglycerol and/or cardiolipin and phosphatidy-



lethanolamine [16,17,19,20]; (ii) performed all observations at concentrations of oritavancin that are similar to those at which bactericidal effects can be demonstrated *in vitro* towards both vancomycin-susceptible and vancomycin-resistant *S. aureus* or other Gram-positive organisms [24,54,55]. These ranges of concentrations are also those reached in serum during conventional administration of the drug to humans [55]. All together, the data, therefore, strongly suggest that membrane effects are responsible for the increased potency of oritavancin as compared to vancomycin (and its activity against vancomycin-resistant organisms). Moreover, recent studies have demonstrated that oritavancin induced rapid bactericidal activity against both exponential phase and stationary-phase inocula of *S. aureus* in relation with membrane depolarization and membrane permeabilization [13]. It remains, however, that membrane-destabilizing effects of oritavancin, if critical for its antibacterial activity, must be largely specific to prokaryotic cells since cellular experiments with eukaryotic cells failed to markedly affect the cell viability (assessed by measuring the release of a cytosolic enzyme, the lactate dehydrogenase) of macrophages incubated with concentrations of up to 25 mg/L, i.e. 200-fold the minimal inhibitory concentration observed *in vitro* for the least susceptible bacteria [25]. The explanation probably lies in the difference in membrane composition and more specifically, in the lack of significant amounts of POPG or CL in the pericellular membrane of eukaryotic cells.

An important aspect underlined by our studies is that only weak alterations in membrane permeability or bilayer morphology were seen with large concentrations of vancomycin. These cause killing of Gram-positive organisms *in vitro*, but through a slow-acting mechanism unrelated to direct membrane destabilization but, most likely, to the activation of autolytic enzyme systems [56] through signal transduction (via a death signal peptide) common to vancomycin and  $\beta$ -lactams [57,58].

Other hypotheses, however, could be suggested. If oritavancin and vancomycin are comparable inhibitors of transglycosylation, oritavancin is also a potent inhibitor of transpeptidation [59]. Moreover, in addition to the binding to the primary site D-Ala–D-Ala, sequestration of lipid II and inhibition of transglycosylation, the hydrophobic biphenyl moiety and components of the aglycone structure of oritavancin could form a secondary binding site for pentaglycyl segments [59,60] due to the biphenyl group that contributes to an enlargement of the drug hydrophobic surface area [61]. Also, the fact that all effects can be seen at very low concentrations is of direct importance. Indeed, oritavancin has been shown to dimerize easily, and this, together with its membrane anchoring properties, has been proposed as an important determinant in its superior activity to vancomycin by favoring its interaction with the D-Ala–D-Ala motif [62]. Dimerization is not only critical for peptide recognition but also for membrane destabilization as it has been shown for antibacterials, such as cathelicidin [63] or magainin [64]. Oligomerization could enhance the kinetics and thermodynamic stabilities of the complex formed [65] and affect the rate of association/dissociation to/from lipids [66]. Lastly, a hydrophobic effect could also be important but with some degree of specificity, as no beneficial effect is observed when an aliphatic chain was introduced to the E-ring of vancomycin [67].

In conclusion, our work has advanced the characterization of the interaction between oritavancin, a novel lipoglycopeptide endowed with potent antimicrobial activity, and phospholipid bilayers. Our observations indicate that lipid composition is a critical parameter in this context with a clear role played by cardiolipin. This effect is probably related to changes in the local physico-chemical properties of the membranes, such as the surface charge, ability to form H bonds, curvature, and fluidity. Future biological and pharmaceutical research may help in developing and expanding these observations on what may constitute essential parts of antimicrobial armamentarium in the future.

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