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Mechanistic studies on the effect of membrane lipid acyl chain composition on daptomycin pore formation

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Abstract

Daptomycin is a lipopeptide antibiotic that binds and permeabilizes the cell membranes of Gram-positive bacteria. Membrane permeabilization requires both calcium and phosphatidylglycerol (PG) in the target membrane, and it correlates with the formation of an oligomer that likely comprises eight subunits, which are evenly distributed between the two membrane leaflets. In both bacterial cells and model membranes, changes in the fatty acyl composition of the membrane phospholipids can prevent permeabilization. We here used liposomes to study the effect of phospholipids containing oleoyl and other fatty acyl residues on daptomycin activity, and made the following observations: 1) Oleic acid residues inhibited permeabilization when part not only of PG, but also of other phospholipids (PC or cardiolipin). 2) When included in an otherwise daptomycin-susceptible lipid mixture, even 10% of dioleoyl lipid (DOPC) can strongly inhibit permeabilization. 3) The inhibitory effect of fatty acyl residues appears to correlate more with their chain length than with unsaturation. 4) Under all conditions tested, permeabilization coincided with octamer formation, whereas tetramers were observed on membranes that were not permeabilized. Overall, our findings further support the notion that the octamer is indeed the functional transmembrane pore, and that fatty acyl residues may prevent pore formation by preventing the alignment of tetramers across the two membrane leaflets.

Keywords: calcium-dependent lipopeptide antibiotics, phosphatidylglycerol, cardiolipin, membrane fluidity, membrane permeabilization

1. Introduction

The lipopeptide antibiotic daptomycin is used clinically to treat infections with Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecium*. It

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binds to calcium and to phosphatidylglycerol in the cytoplasmic membrane [1]. It then oligomerizes [2, 3] and permeabilizes the membrane. In certain model liposomes, oligomerization is accompanied by the formation of cation-selective pores [4]. Our current working model postulates that the oligomeric pore comprises eight daptomycin molecules overall and consists of two tetramers, which reside in the inner and the outer membrane leaflet, respectively [5].

The existence of such pores may also account for various experimental observations on Gram-positive bacteria, namely, the depolarization of the cell membrane [6–8] with concomitant disruption of amino acid uptake [6, 9] and the release of K^+ from the cells [8, 9]; in contrast, the membrane remains impervious to the negatively charged permeability probe calcein [10]. A recent study [11], however, did not detect direct permeabilization of *Bacillus subtilis* cells for K^+, whereas delayed, partial membrane depolarization was still observed. This study also documented the dissociation of biosynthetic enzymes from the cell membrane, as well as changes in membrane lipid dynamics, to which the authors ascribed the observed membrane depolarization.

While oligomerization of daptomycin has been implicated in its bactericidal activity on *Bacillus subtilis* [3], the observations reported in [11] suggest that the bactericidal effect may not arise from direct pore formation. It has recently emerged that, both in model membranes [12] and in bacterial cells [13, 14], the fatty acyl composition of the membrane phospholipids can affect daptomycin activity. Model liposomes composed of dimyristoyl-phosphatidylcholine (DMPC) and -phosphatidylglycerol (DMPG) are readily permeabilized by daptomycin [4]; in these lipids, both fatty acyl chains are 14 carbon atoms long and contain no double bonds (14:0/14:0; for lipid structures, see Figure S1). In contrast, liposomes that contain palmitoyl-oleoyl lipids (POPC/POPG; 16:0/18:1) or dioleoyl lipids (DOPC/DOPG; 18:1/18:1) are not permeabilized [12]. We here examine in more detail the effect of fatty acyl composition of PC/PG model membranes on daptomycin pore formation, as well as on other aspects of its activity.

2. Materials and Methods

For brevity, methods are described here only in outline, and the pertinent references are cited. Where indicated, additional detail is given in the Supplementary Materials.

2.1. Antibacterial Activity Assay

Daptomycin and its semisynthetic derivatives were tested against *Bacillus subtilis* ATCC 1046 by broth microdilution as described in [12]. Further detail is given in the Supplementary Materials.

2.2. Liposome preparation

All the lipids used in this study were acquired from Avanti Polar Lipids (Alabaster, Alabama). Lipids include: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3phospho-(1'-rac-glycerol) (sodium salt; DMPG),

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt; DOPG), 1',3'-bis[1,2dimyristoyl-sn-glycero-3-phospho]-sn-glycerol (sodium salt; TMCL), 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (sodium salt; TOCL), 1,2-dipentadecanoyl-sn-glycero-3phosphocholine (DPDPC), and 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine (DMPC-Δ9-Cis). The lipids were dissolved into chloroform and methanol (3:1) and were used without further purification. The structures of all lipids used in this study are shown in the Supplementary Materials (Figure S1).

Large unilamellar vesicles (LUVs) were prepared using the polycarbonate membrane extrusion method [15] as detailed before [2]. Further detail is given in the Supplementary Materials.

2.3. Kynurenine fluorescence

The intrinsic fluorescence of daptomycin's kynurenine residue, which increases upon membrane binding [16], was measured using excitation and emission wavelengths of 365 and 445 nm, respectively. These measurements (as well as all other fluorescence measurements) were carried out in a QuantaMaster 4 steady-state spectrofluorimeter (PTI, London, ON).

2.4. Membrane Permeabilization Assay

This assay was carried out as described before in [12]. The results shown are the average of 6 independent experiments each. Assays were run at a temperature higher than the phase transition (see Results for specific temperatures). Further detail is given in the Supplementary Materials.

2.5. Daptomycin subunit stoichiometry

These experiments were carried out essentially as described in [17]. Further detail is given in the Supplementary Materials. The results shown are the averages and standard deviations of eight independent experiments.

2.6. Daptomycin bilayer translocation

The distribution of daptomycin between the outer and the inner membrane leaflets of liposomes was measured using dithionite quenching [18] of NBD-daptomycin essentially as described in [12]. The results shown are the averages and standard deviations of three independent experiments. Further detail is given in the Supplementary Materials.

2.7. Fluorescence assay of membrane fluidity

The phase transition temperatures of liposomes containing tetra-myristoyl-cardiolipin (TMCL) or di-pentadecanoyl-phosphatidylcholine (DPDPC) were determined using the diphenylhexatriene polarization assay [19]. 1,6-Diphenyl-1,3,5-hexatriene (DPH; Millipore-Sigma) dissolved in methanol was added to liposomes in a 1 mL cuvette to a final concentration of 10 μM, followed by incubation for 10 minutes. For fluorescence anisotropy measurements, DPH was excited at 350 nm and emission measured at 428 nm.

2.8. Synthesis of NBD-daptomycin and of C_{14}-daptomycin

These were synthesized essentially according to previously reported methods [2, 20]. Further detail is given in the Supplementary Materials.

3. Results

3.1. Inhibition of daptomycin by oleoyl residues is independent of the phospholipid head group

Previous studies have shown that PG in the target membrane mediates high-affinity binding, conformational change, and oligomerization of daptomycin [2, 21]. Moreover, membrane levels of PG are strongly correlated with bacterial susceptibility to daptomycin [1, 13, 22–25]. In contrast, PC alone permits low-affinity binding, but it fails to induce the subsequent stages of activity [2, 21]. Since daptomycin activity thus depends on PG, the question arises whether oleoyl residues inhibit permeabilization only if they are part of this specifically required phospholipid, or also if they are part of an inert "bulk" lipid such as PC.

In the experiments shown in Figure 1, the activity of daptomycin was measured on membranes composed of DMPC and DMPG, as well as of various combinations of these with dioleoyl lipids as indicated. This assay measures the effect of daptomycin indirectly: when daptomycin permits the influx of sodium into the liposomes, protons are released in exchange by the protonophore CCCP. The accompanying change of pH is then detected by an increase in the fluorescence of the pH-sensitive fluorophore pyranine entrapped inside the liposomes [4, 26].

Figure 1A shows that daptomycin readily permeabilizes DMPC/DMPG membranes, which agrees with previous findings [4]. In contrast, membranes in which either DMPC or DMPG is replaced with the corresponding dioleoyl lipid are not permeabilized (Figure 1B,C). Thus, oleoyl acyl chains inhibit daptomycin even if they are not covalently associated with the PG head group. This indicates that, while the PG head group is specifically required for daptomycin binding, other lipids (PC in this case) are part of the microenvironment that controls the activity of membrane-bound daptomycin. Furthermore, even at a molar fraction as low as 10%, DOPC still suppresses permeabilization; the inhibition only wanes once DOPC is reduced to 5% (Figure 1D,E). Thus, the inhibitory action of DOPC is remarkably strong.

3.2. Mechanistic aspects of the inhibition of permeabilization by dioleoyl lipids

In our previous study [12], we showed that DOPC/DOPG membranes bind daptomycin at lower calcium concentrations, and thus more avidly, than DMPC/DMPG membranes; the greater bulk of the oleoyl chains may distend the head group layer, which may help accommodate daptomycin in the membrane (cf. [5]). Therefore, a conceivable explanation for the high inhibitory potency of dioleoyl lipids in mixed membranes with dimyristoyl lipids would be that they create high-affinity binding sites which sequester daptomycin and prevent it from interacting with DMPC/DMPG. However, as Figure 2 shows, neither DOPC/DMPG nor DMPC/DOPG membranes exceed the daptomycin affinity of DMPC/DMPG membranes. Therefore, daptomycin does not bind preferentially to dioleoyl lipids in mixtures with dimyristoyl lipids.

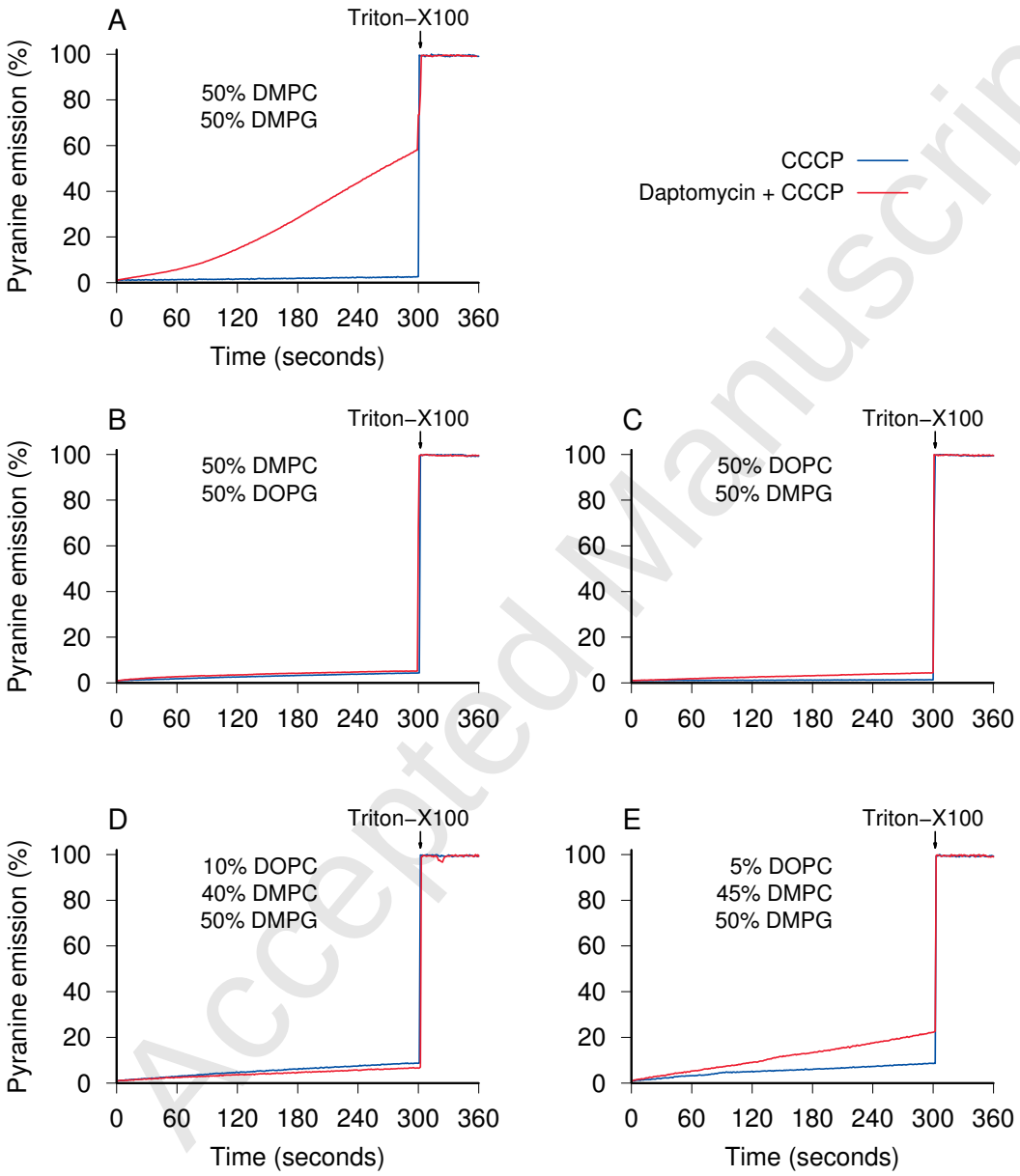

Figure 1: Permeabilization of LUVs composed of DOPC or DMPC and DOPG or DMPG in various proportions, as indicated. LUVs were loaded with the pH-sensitive fluorescent dye pyranine at pH 6, diluted into a buffer with pH 8, and exposed to the proton ionophore CCCP (5 nM) and to daptomycin (2 μM). An increase in fluorescence indicates membrane permeabilization for both protons and sodium ions. Solubilization with Triton-X100 abolishes the pH gradient and provides a reference value for 100% permeabilization [4, 12, 26].

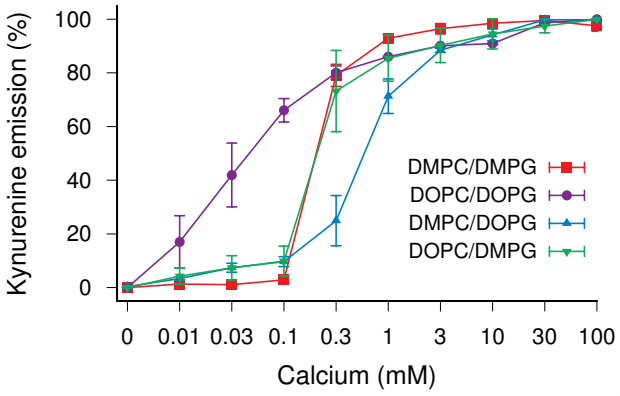

Figure 2: Calcium-dependent binding of daptomycin to liposomes composed of equimolar mixtures of DMPC or DOPC with DMPG or DOPG as indicated. Membrane binding is detected by the concomitant increase in the intrinsic fluorescence of daptomycin's kynurenine residue [16]. Error bars represent standard deviations from four independent experiments.

With pure DOPC/DOPG membranes, we had previously found that daptomycin formed tetramers rather than octamers, and moreover that these tetramers were unevenly distributed between the two membrane leaflets, such that approximately two thirds remain in the outer leaflet [12]. Since our working model assumes that the pore is octameric, the observed inhibition of octamer formation by dioleoyl lipids accounts for the lack of pore formation. On the other hand, it seemed possible that at a mere 10% of dioleoyl lipids, octamers might still form, which would however fail to permeabilize the membrane; such non-functional oligomers have been described with some pore-forming protein toxins on non-susceptible cell types [27, 28].

The subunit stoichiometry of daptomycin oligomers was determined by a previously reported FRET method [17], whereas the distribution of daptomycin across leaflets was measured using dithionite quenching of NBD-labeled daptomycin [4, 12]. Somewhat to our surprise, we found that indeed both the subunit stoichiometry and the distribution across leaflets was closely similar between pure DOPC/DOPG membranes and those that contained a mere 10% DOPC combined with 40% DMPC and 50% DMPG (Figure 3). In contrast, on pure DMPC/DMPG membranes, the oligomer subunit stoichiometry was close to eight—slightly lower, in fact, as it usually is; possible reasons have been discussed before [5, 17]—and the distribution of daptomycin across leaflets was close to even.

As reported previously [12], the uneven distribution of daptomycin between leaflets of membranes containing dioleoyl lipids did not change after prolonged incubation and thus likely reflects the equilibrium. The asymmetry might be explained by the lack of calcium inside these liposomes, which would destabilize oligomers bound to the inner leaflet. In contrast, the pores on DMPC/DMPG liposomes would most likely admit calcium to the interior, which would stabilize oligomers bound to the inner leaflet. (This has not been directly demonstrated, since at the concentration required for the permeant ion in our permeability assay [100 mM], Ca^{++} causes the liposomes to aggregate; but two other divalent cations have been shown to cross the membrane

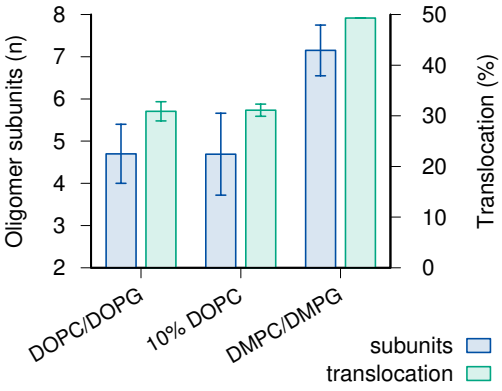

Figure 3: Oligomer subunit stoichiometry and translocation to the inner membrane leaflet of daptomycin on PC/PG liposomes differing in acyl chain composition. Liposomes labeled with "10% DOPC" consisted of DMPC (40%) and DMPG (50%) otherwise. Data for pure DMPC/DMPG membranes were adapted from [5]. Error bars indicate standard deviations of 3 or more experiments.

[4]). However, adding the calcium ionophore ionomycin to DOPC/DOPG liposomes—either alone, or together with CCCP to render the influx of calcium electroneutral by permitting an efflux of protons—did not increase the extent of daptomycin translocation (Figure S6). We conclude that the asymmetric distribution of daptomycin across membranes that contain dioleoyl lipids is not caused by a lack of calcium on the inside. This question will be revisited in the discussion.

3.3. Revisiting the effect of cardiolipin on daptomycin activity

We previously reported that addition of cardiolipin to DMPC/DMPG membranes at a molar fraction of 10 or 20% inhibits daptomycin pore formation, as well as its translocation to the inner membrane leaflet; and we ascribed this inhibitory effect to cardiolipin's head group [5]. However, the particular species of cardiolipin used in our earlier study was TOCL, which means that the membranes contained an amount of oleoyl groups that has now turned out to be inhibitory apparently regardless of the lipid head group. This raises the possibility that the fatty acyl chains of TOCL, rather than its head group, were really causing the inhibition observed earlier. To decide this question, we prepared liposomes that contained TMCL (10 or 20%) in combination with DMPG (50%) and DMPC (to 100%); that is, the membranes contained some cardiolipin head groups but only myristoyl residues.

In preliminary experiments, we observed that TMCL dose-dependently increased the gel-to-liquid transition temperature of the membranes (Figure 4A), and furthermore that 20% TMCL prevented the stable encapsulation of pyranine, our fluorescence probe for membrane permeabilization. All further experiments were therefore conducted with 10% TMCL and at 30°C, at which temperature the membranes are in the fluid state and do not leak pyranine.

Unlike membranes consisting of DMPC and DMPG only (cf. Figure 1A), membranes containing TMCL are only weakly permeabilized by 2 μM daptomycin (Figure 4C). However, when these liposomes were treated with 10 μM daptomycin, they were permeabilized to a greater degree than another sample that contained an equal amount

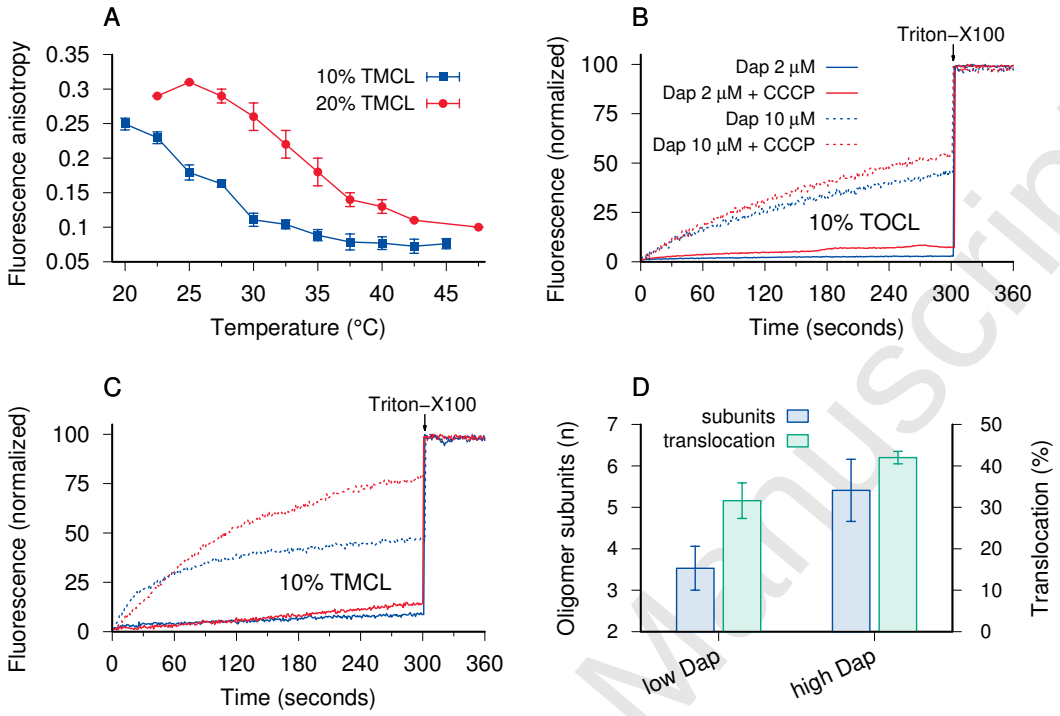

Figure 4: Characterization of daptomycin activity on liposomes containing TMCL (A, C, D) or TOCL (B) together with DMPG (50%) and DMPC (to 100%). **A**: Fluorescence polarization of diphenylhexatriene (DPH). The polarization drops as the membrane transitions from the gel to the fluid state [29]. **B**: Pyranine permeabilization assay on liposomes containing 10% TOCL by 2 and 10 μM daptomycin. **C**: Pyranine permeabilization assay on liposomes containing 10% TMCL by 2 and 10 μM daptomycin. Traces are colored and patterned as in A. **D**: Oligomer subunit stoichiometry and translocation to inner membrane leaflet of daptomycin on membranes containing 10% TMCL. 'Low' daptomycin concentrations were 2 and 3 μM daptomycin for stoichiometry and translocation, while 'high' one were 10 μM and 8 μM, respectively.

of TOCL instead of TMCL (Figure 4B). Therefore, in keeping with our previous report [5], the cardiolipin head group on its own indeed has an inhibitory effect, yet the oleoyl residues of TOCL endow it with greater inhibitory potency than TMCL.

As an aside, with both TMCL and TOCL, daptomycin at 10 μM caused a significant rise in pyranine fluorescence intensity even without the protonophore CCCP. As discussed earlier [4, 26], an efflux of protons is required to maintain electroneutrality as sodium enters the liposomes. It thus appears that, at sufficiently high concentrations, daptomycin can cause a significant efflux of protons by itself; we have previously observed a similar effect with a dimeric derivative of daptomycin even at lower concentrations [20].

Finally, we also measured daptomycin oligomer subunit stoichiometry as well as translocation to the inner membrane leaflet on TMCL-containing liposomes, at different daptomycin concentrations. We observed that both the number of subunits and the extent of translocation to the inner leaflet increased as the daptomycin concentration was raised from non-permeabilizing to permeabilizing levels (Figure 4D). This

appears consistent with the earlier conclusion that permeabilization correlates with the formation of the membrane-spanning octamer.

3.4. Is inhibition caused by acyl chain length or unsaturation?

The acyl chains in DOPC/DOPG and POPC/POPG, which form membranes that are impervious to daptomycin permeabilization, differ from those in susceptible model membranes (DMPC/DMPG) with respect to both chain length and degree of unsaturation. This raises the question if either or both of these traits are responsible for the inhibition. To answer it, we prepared liposomes that contained 10% of a PC species that differed from DMPC (di-C14:0-PC) either in length only (DPDPC; di-C15:0-PC) or in saturation only (DMPC-Δ9-Cis; di-C14:1-PC). Figure 5 shows the permeabilization of these two liposome species. Both showed some leakage even when either or both of daptomycin and CCCP were absent, yet it is apparent that di-C14:1-PC is more permissive toward daptomycin-mediated permeabilization than is di-C15:0-PC. This suggests that acyl chain length has a greater effect on permeabilization than unsaturation.

The reader may note that most of the unbranched fatty acyl residues found in bacterial membranes have even numbers of carbons. We nevertheless chose DPDPC (di-C15:0-PC) for this experiment rather than dipalmitoyl-PC (di-C16:0-PC) or distearoyl-PC (di-C18:0-PC) in order to minimize effects such as decreased membrane fluidity and lateral phase segregation, which become more likely with increasing difference in acyl chain length. Figure S8 shows that mixed membranes with up to 20% of di-C15:0-PC indeed remain in the fluid phase at the temperature of the permeabilization assay (30°C).

3.5. Does the length of the daptomycin acyl chain affect the lipid permeability threshold?

Previous experiments with a pyrene-acyl-labeled daptomycin analogue suggest that daptomycin's N-terminally attached fatty acyl chain participates in the transmembrane interaction that joins two tetramers in opposed membrane leaflets into an octamer [30]. Considering that oleoyl residues disrupt the alignment of tetramers between leaflets, and that this inhibition appears to correlate with fatty acyl chain length, the question arises if inhibition can be countered by extending the length of daptomycin's fatty acyl residue. Thus, we semisynthetically prepared a daptomycin derivative in which the N-terminal decanoyl residue was replaced by myristoyl residue (C_{14}-daptomcyin). The MIC of this derivative on *B. subtilis* closely resembled that of native daptomycin (0.5 μg/ml and 0.75 μg/ml, respectively). It was tested on a series of membranes containing dimyristoyl-, dioleoyl-, or palmitoyl-oleoyl lipids. In all cases, the activity C_{14}-daptomycin closely resembled that of native daptomycin (see Figure S7). Therefore, at least with this derivative, we obtained no evidence that the length of daptomycin's fatty acyl residue controls its susceptibility to inhibition by membrane lipids.

4. Discussion

In this study, we have shown that oleoyl and other fatty acyl residues in phospholipids can potently inhibit the formation of daptomycin pores in otherwise susceptible

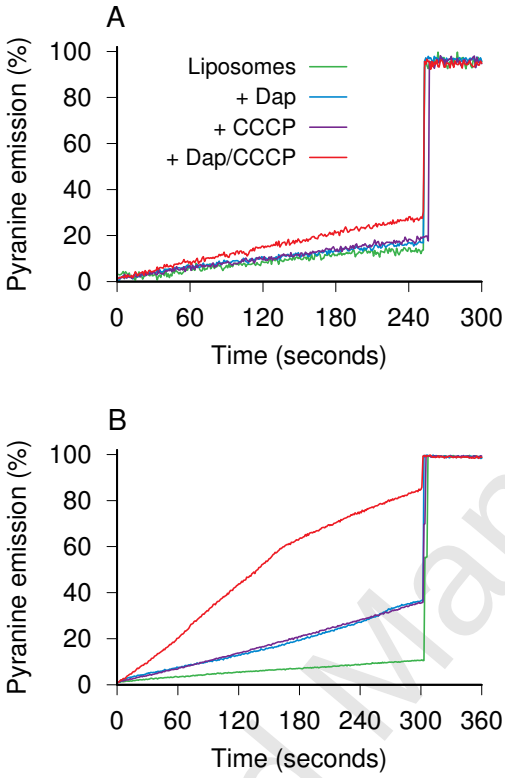

Figure 5: Permeabilization of liposomes that contain 10% DPDPC (A) or DMPC-Δ9-Cis (B), respectively, in combination with 40% DMPC and 50% DMPG. Daptomycin (Dap), where present, was used at 2 μM. Curves in B are colored as in A. Each curve represents the average of triplicate experiments.

membranes. This inhibitory effect appears to correlate with the chain length rather than with degree of unsaturation of the fatty acyl residue in question, and furthermore to not be restricted by the head group of the phospholipid. On the other hand, the reported observations with TMCL show that the cardiolipin head group can also inhibit permeabilization, which confirms our previous conclusion [5]. Furthermore, the inhibitory effects of both acyl chains and head groups are manifest at remarkably low molar fractions of the lipids in question.

Bacterial membranes represent fairly complex mixtures of lipids, with respect to both acyl chains and head groups, and lateral lipid segregation and domain formation will further diversify the membrane composition. In light of this, one might expect bacterial species to differ in their susceptibility to cation-selective membrane permeabilization by daptomycin [8, 9, 11], and furthermore that even minor shifts in membrane lipid composition may significantly alter daptomycin susceptibility [13, 14]. The latter two studies found that an increase in the fraction of longer acyl chains in the membrane lipids of *Staphylococcus aureus* correlates with daptomycin resistance, which agrees with our observations in model membranes. It must be noted, however, that membrane lipids of *S. aureus* and also other Gram-positives contain a large fraction of

branched fatty acyl chains, whose effect on daptomycin activity remains to be elucidated.

One further comment is in order regarding the relevance of this study to bacterial cells. Firstly, the neutral component of our model membranes was phosphatidylcholine; while this lipid is not a major component of bacterial cell membranes, we made this choice in order to keep the experiments consistent and comparable with previous studies by others [21, 31–33] and ourselves [4, 5, 12].

Our study further corroborates the hypothesis that cation-selective permeabilization, which is observed on DMPC/DMPG model membranes [4] and at least some bacteria [8, 9], is caused by a daptomycin octamer that spans both membrane leaflets [5]. Complete conversion of membrane-bound daptomycin to this octameric form implies an even distribution between both leaflets, which is indeed observed on the susceptible (DMPC/DMPG) model membranes. On the other hand, if they do not connect, then the tetramers should remain free to partition independently between leaflets; and whenever tetramers predominate, they exhibit a preference for the outer leaflet. This preference is not driven by calcium; it may be due to the difference in curvature strain between the two leaflets. We note that the (considerably larger) curvature strain in the division septum of *Bacillus subtilis* cells has previously been implicated in the preferential binding of daptomycin to this structure [34]. In any case, our findings do confirm that daptomycin can translocate to the inner leaflet of lipid membranes even if it fails to then permeabilize them, which suggests that bactericidal mechanisms which involve the interaction of daptomycin with proteins at the inner surface of such membranes [11] are feasible in principle.

While the interpretation given above is based on the assumption that the daptomycin oligomer itself is the functional transmembrane pore [5, 8], we cannot rule out that the observed inhibitory effects of oleoyl and other fatty acyl residues might be accounted for by the lipid phase segregation model of membrane permeabilization [11, 35]. This possibility will be addressed in a future experimental study.

5. Acknowledgments

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6. Bibliography

[1] R. H. Baltz, Daptomycin: mechanisms of action and resistance, and biosynthetic engineering, Curr Opin Chem Biol 13 (2009) 144–151. doi:10.1016/j.cbpa.2009.02.031.

[2] J. K. Muraih, A. Pearson, J. Silverman, M. Palmer, Oligomerization of daptomycin on membranes, Biochim Biophys Acta 1808 (2011) 1154–1160. doi:10.1016/j.bbamem.2011.01.001.

[3] T. Zhang, J. K. Muraih, E. Mintzer, N. Tishbi, C. Desert, J. Silverman, S. Taylor, M. Palmer, Mutual inhibition through hybrid oligomer formation of daptomycin and the semisynthetic lipopeptide antibiotic CB-182,462, Biochim Biophys Acta 1828 (2013) 302–308. doi:10.1016/j.bbamem.2012.10.008.

[4] T. Zhang, J. Muraih, B. MacCormick, J. Silverman, M. Palmer, Daptomycin forms cation- and size-selective pores in model membranes, Biochim Biophys Acta 1838 (2014) 2425–2430. doi:10.1016/j.bbamem.2014.05.014.

[5] T. Zhang, J. K. Muraih, N. Tishbi, J. Herskowitz, R. L. Victor, J. Silverman, S. Uwumarenogie, S. D. Taylor, M. Palmer, E. Mintzer, Cardiolipin prevents membrane translocation and permeabilization by daptomycin, J Biol Chem 289 (2014) 11584–11591. doi:10.1074/jbc.M114.554444.

[6] N. E. Allen, W. E. J. Alborn, J. N. J. Hobbs, Inhibition of membrane potential-dependent amino acid transport by daptomycin, Antimicrob Agents Chemother 35 (1991) 2639–2642. doi:10.1128/AAC.35.12.2639.

[7] W. E. J. Alborn, N. E. Allen, D. A. Preston, Daptomycin disrupts membrane potential in growing *Staphylococcus aureus*, Antimicrob Agents Chemother 35 (1991) 2282–2287. doi:10.1128/AAC.35.11.2282.

[8] J. A. Silverman, N. G. Perlmutter, H. M. Shapiro, Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*, Antimicrob Agents Chemother 47 (2003) 2538–2544. doi:10.1128/AAC.47.8.2538-2544.2003.

[9] N. E. Allen, J. N. Hobbs, W. E. J. Alborn, Inhibition of peptidoglycan biosynthesis in Gram-positive bacteria by LY146032, Antimicrob Agents Chemother 31 (1987) 1093–1099. doi:10.1128/AAC.31.7.1093.

[10] N. Cotroneo, R. Harris, N. Perlmutter, T. Beveridge, J. A. Silverman, Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*, Antimicrob Agents Chemother 52 (2008) 2223–2225. doi:10.1128/AAC.01410-07.

[11] A. Müller, M. Wenzel, H. Strahl, F. Grein, T. N. V. Saaki, B. Kohl, T. Siersma, J. E. Bandow, H.-G. Sahl, T. Schneider, L. W. Hamoen, Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains, Proc Natl Acad Sci U S A 113 (2016) E7077–E7086. doi:10.1073/pnas.1611173113.

[12] R. Taylor, D. Beriashvili, S. Taylor, M. Palmer, Daptomycin pore formation is restricted by lipid acyl chain composition, ACS Infectious Diseases 3 (2017) 797–801. doi:10.1021/acsinfecdis.7b00138.

[13] K. M. Hines, A. Waalkes, K. Penewit, E. A. Holmes, S. J. Salipante, B. J. Werth, L. Xu, Characterization of the mechanisms of daptomycin resistance among Gram-positive bacterial pathogens by multidimensional lipidomics, mSphere 2 (2017) e00492–17. doi:10.1128/mSphere.00492-17.

[14] R. Boudjemaa, C. Cabriel, F. Dubois-Brissonnet, N. Bourg, G. Dupuis, A. Gruss, S. Lévêque-Fort, R. Briandet, M.-P. Fontaine-Aupart, K. Steenkeste, Failure of daptomycin to kill *Staphylococcus aureus*: impact of bacterial membrane fatty acid composition, Antimicrob. Agents Chemother. 62 (2018) e00023–18. doi:10.1128/AAC.00023-18.

[15] L. D. Mayer, M. J. Hope, P. R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim Biophys Acta 858 (1986) 161–168. doi:10.1016/0005-2736(86)90302-0.

[16] J. H. Lakey, M. Ptak, Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes, Biochemistry 27 (1988) 4639–4645. doi:10.1021/bi00413a009.

[17] J. K. Muraih, M. Palmer, Estimation of the subunit stoichiometry of the membrane-associated daptomycin oligomer by FRET, Biochim Biophys Acta 1818 (2012) 1642–1647. doi:10.1016/j.bbamem.2012.02.019.

[18] J. C. McIntyre, R. G. Sleight, Fluorescence assay for phospholipid membrane asymmetry, Biochemistry 30 (1991) 11819–11827. doi:10.1021/bi00115a012.

[19] M. Shinitzky, Y. Barenholz, Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate, J. Biol. Chem. 249 (1974) 2652–7.

[20] R. M. Taylor, B. Scott, S. Taylor, M. Palmer, An acyl-linked dimer of daptomycin is strongly inhibited by the bacterial cell wall, ACS Infect Dis 3 (2017) 462–466. doi:10.1021/acsinfecdis.7b00019.

[21] D. Jung, A. Rozek, M. Okon, R. E. W. Hancock, Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin, Chem Biol 11 (2004) 949–957. doi:10.1016/j.chembiol.2004.04.020.

[22] A. S. Bayer, T. Schneider, H.-G. Sahl, Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall, Ann N Y Acad Sci 1277 (2013) 139–158. doi:10.1111/j.1749-6632.2012.06819.x.

[23] A.-B. Hachmann, E. Sevim, A. Gaballa, D. L. Popham, H. Antelmann, J. D. Helmann, Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*, Antimicrob Agents Chemother 55 (2011) 4326–4337. doi:10.1128/AAC.01819-10.

[24] N. N. Mishra, S.-J. Yang, A. Sawa, A. Rubio, C. C. Nast, M. R. Yeaman, A. S. Bayer, Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*, Antimicrob Agents Chemother 53 (2009) 2312–2318. doi:10.1128/AAC.01682-08.

[25] N. N. Mishra, A. S. Bayer, Correlation of cell membrane lipid profiles with daptomycin resistance in methicillin-resistant *Staphylococcus aureus*, Antimicrob Agents Chemother 57 (2013) 1082–1085. doi:10.1128/AAC.02182-12.

[26] N. R. Clement, J. M. Gould, Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles, Biochemistry 20 (1981) 1534–1538. doi:10.1021/bi00509a019.

[27] I. Walev, M. Palmer, A. Valeva, U. Weller, S. Bhakdi, Binding, oligomerization, and pore formation by streptolysin O in erythrocytes and fibroblast membranes: detection of nonlytic polymers, Infect Immun 63 (1995) 1188–1194.

[28] A. Valeva, I. Walev, M. Pinkernell, B. Walker, H. Bayley, M. Palmer, S. Bhakdi, Transmembrane β-barrel of staphylococcal α-toxin forms in sensitive but not in resistant cells, Proc Natl Acad Sci U S A 94 (1997) 11607–11611. doi:10.1073/pnas.94.21.11607.

[29] B. R. Lentz, Y. Barenholz, T. E. Thompson, Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes, Biochemistry 15 (1976) 4521–4528. doi:10.1021/bi00665a029.

[30] T. Zhang, S. D. Taylor, M. Palmer, J. Duhamel, Membrane binding and oligomerization of the lipopeptide a54145 studied by pyrene fluorescence, Biophys J 111 (2016) 1267–1277. doi:10.1016/j.bpj.2016.07.018.

[31] D. Jung, J. P. Powers, S. K. Straus, R. E. W. Hancock, Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes, Chem Phys Lipids 154 (2008) 120–128. doi:10.1016/j.chemphyslip.2008.04.004.

[32] J. Zhang, K. Scoten, S. K. Straus, Daptomycin leakage is selective, ACS infectious diseases 2 (2016) 682–687. doi:10.1021/acsinfecdis.6b00152.

[33] Y.-F. Chen, T.-L. Sun, Y. Sun, H. W. Huang, Interaction of daptomycin with lipid bilayers: a lipid extracting effect, Biochemistry 53 (2014) 5384–5392. doi:10.1021/bi500779g.

[34] J. Pogliano, N. Pogliano, J. A. Silverman, Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins, J Bacteriol 194 (2012) 4494–4504. doi:10.1128/JB.00011-12.

[35] M.-T. Lee, P.-Y. Yang, N. E. Charron, M.-H. Hsieh, Y.-Y. Chang, H. W. Huang, Comparison of the effects of daptomycin on bacterial and model membranes, Biochemistry (in press). doi:10.1021/acs.biochem.8b00818.

- Daptomycin forms cation-selective, octameric pores in membranes containing PG
- Pore formation is inhibited by phospholipids with acyl chains longer than 14 carbons
- Inhibition occurs even if the acyl chains are part of the “bulk” lipid, not of PG
- Inhibition is observed with low percentages (10%) of inhibitory phospholipid
- When inhibitory lipids are present, daptomycin forms tetramers rather than octamers

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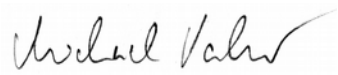
27 August 2018

Re: Conflict of interest statement for manuscript entitled "Mechanistic studies on the effect of membrane lipid acyl chain composition on daptomycin pore formation", by Beriashvili, Taylor, Kralt, Abu Mazen, Taylor, and Palmer

On behalf of all authors, I state that we have no conflicts of interest to declare.

Best regards,



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