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Daptomycin forms a stable complex with phosphatidylglycerol for selective uptake to bacterial membrane

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eLife Assessment

This **valuable** study describes the molecular mechanism of daptomycin insertion into bacterial membranes. The authors provide **solid** in vitro evidence for the early events of daptomycin interaction with phospholipid headgroups and stronger, specific interaction with phosphatidylglycerol. This work will be of interest to bacterial membrane biologists and biochemists working in the antimicrobial resistance field.

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Abstract

Daptomycin is a potent lipopeptide antibiotic used in the treatment of live-threatening Gram-positive infections, but the molecular mechanism of its interaction with bacterial membrane remains unclear. Here we show that this interaction is divided into two stages, of which the first is a fast and reversible binding of the drug to phospholipid membrane in milliseconds and the second is a slow and irreversible insertion into membrane in minutes, only in the presence of the bacteria-specific lipid phosphatidylglycerol, to a saturating point where the ratio of the drug to phosphatidylglycerol is 1:2. Fluorescence-based titration showed that the antibiotic simultaneously binds two molecules of phosphatidylglycerol with a nanomolar binding affinity in the presence of calcium ion. The resulting stable complex is easily formed in a test tube and readily isolated from the membrane of drug-treated bacterial cells, strongly supporting a unique drug uptake mechanism in which daptomycin forms a stable multi-component complex with calcium and phosphatidylglycerol. Revelation of this novel uptake mechanism provides fresh insights into the mode of action of daptomycin and paves the way to new strategies to attenuate resistance to the drug.

Introduction

Daptomycin (Dap) is a calcium-dependent lipopeptide antibiotic used to treat life-threatening Gram-positive infections.[1, 2] It exhibits potent bactericidal activity[3, 4] and was once used as a last-line-of-defence antibiotic against resistant pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci.[5] Resistance to Dap is mostly mild with moderate increases in the minimum inhibitory concentration.[6] However, recent years have witnessed an increasing number of failed treatment due to high-level Dap resistance of clinical pathogens, including *streptococci*,[7, 8] *Enterococcus faecium*,[9] and *Corynebacterium striatum*.[10] Currently, few daptomycin derivatives are available to attenuate the resistance.[11]

The effort to counter the increasing resistance is hampered by our insufficient understanding of the action mechanism of Dap, which has been suggested to involve inhibition peptidoglycan biosynthesis[12, 13] or membrane depolarization.[14, 15] Recent studies have provided supporting evidence for the inhibition of peptidoglycan biosynthesis either by disruption of membrane fluidity[16] or by the formation of a bactericidal complex between the drug and lipid II.[17] Another unresolved problem is how Dap recognizes Gram-positive bacteria and selectively accumulates in their membrane. Cumulative evidence has shown that this cell specificity is linked to phosphatidylglycerol (PG), a major phospholipid in most bacteria but rare in mammalian cells.[18] PG was first linked to susceptibility to Dap by the finding that the antibiotic accumulates in membrane microdomains rich in this lipid.[19] In addition, PG in pulmonary surfactants is able to neutralize Dap, rendering the drug ineffective against community-acquired, bacteria-caused pneumonia.[20, 21] More importantly, decreased membrane content of PG caused by reduction-of-function mutations in the PG synthase PgsA has been unambiguously shown to be primarily responsible for resistance to Dap, first in the model bacterium *Bacillus subtilis*[22] and then in clinical pathogens.[23] When PgsA is inactivated by a single loss-of-function mutation, Dap susceptibility is completely eliminated to result in a very high level of resistance.[24] Corroborated by *in vitro* liposome model studies,[24] these findings strongly support a critical role of PG in the recognition of bacterial cells by the antibiotic.

Previous studies have shown that Dap non-specifically binds membrane regardless of the lipid composition[18] and also interacts specifically with PG in membrane with conformational change.[25–27] However, the strength of this specific interaction and how it contributes to bacteria-specific uptake of the drug are not clear. In addition, these early investigations reached different conclusion on the stoichiometry of the Dap interaction with PG.[26, 27] In this study, we investigated the calcium-dependent interaction of Dap with model membranes and found that the mode of interaction is dependent on PG. In absence of PG Dap reversibly binds membrane in a fast process, whereas the drug undergoes a slow, irreversible insertion into membrane when PG is present. Further investigation showed that Dap forms a multi-component complex with calcium and two molecules of PG both *in vitro* and in drug-treated bacterial cells, thus revealing a unique mechanism for the selective uptake of Dap into bacterial membrane.

Results

Dependence of Dap uptake on phosphatidylglycerol

Interaction of Dap with membrane is known to increase the fluorescence of its kynurenine residue (Kyn-13, **Fig. 1**) with a 15 nm blue shift.[28] We used the kinetic of this fluorescent increase to study the interaction in micelles and found negligible fluorescence increase when the model membrane was comprised of 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) only (**Fig.**

2A). When 1, 2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol (DMPG) was added to the phospholipid micelles, the fluorescence increased and reached a plateau in about 10 min. Interestingly, the plateau level is limited by the DMPG content and in most cases is unable to reach a maximum level at which all Dap is absorbed into the model membrane. In addition, the initial rate of the fluorescence change sharply increases with the concentration of both DMPG and Ca^{2+} (Fig. S1). Similar kinetics of Kyn fluorescence was observed in the interaction of Dap with vesicles containing DMPG (Fig. S2), indicating that Dap interacts with PG-containing membrane in the same mode regardless of the model system.

Using PG-free micelles, no increase of fluorescence intensity was detected when the model membrane contains the same molar equivalent of other major lipid components in bacteria, including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), cardiolipin (CL), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), and FPP (a structural homolog of bactoprenol diphosphate) (**Fig. 2B**). Nevertheless, the steady-state Dap fluorescence also increases with the content of the lipids, particularly for the negatively charged POPS and CL. Noticeably, this enhanced background fluorescence is accompanied by the same blue shift observed in micelles containing PG (Fig. S3), suggesting that Dap also interacts with the PG-free membrane, but in a mode different from that for the PG-containing membrane. Corroboratively, the steady-state Dap fluorescence is linearly proportional to the DMPG content and reaches a maximum at a concentration about twice that of Dap, but it has a significantly different relationship with the content of POPS, CL, or POPA (**Fig. 2C**). PG-dependent increase of the steady-state fluorescence was also observed in giant unilamellar vesicles (GUVs).[29]

Two distinct modes of Dap interaction with membrane

In the interaction of Dap with micelles containing CL, the increase of fluorescence was found to be complete within 100 ms by stopped-flow kinetics (**Fig. 2D**), demonstrating that this mode of interaction occurs much faster than the slow accumulation of Dap in PG-containing membrane (over minutes, **Fig. 2A**). This fast process is believed to correspond to the Ca^{2+}-dependent recruitment of Dap, via diffusion, to the headgroup region of the phospholipid membrane, where the environment of the Kyn residue becomes more hydrophobic likely due to structural change to cause the Kyn fluorescence enhancement and blue shift. This binding to the membrane surface should be reversible and no Dap is irreversibly inserted into the hydrophobic acyl layer of membrane, since the Kyn fluorescence was not further increased afterwards. Indeed, when Ca^{2+} was sequestrated by EGTA, the bound Dap was completely released back into the bulk solution as indicated by decrease of the Kyn fluorescence to the same level of free Dap control without the lipids (**Fig. 3A**). In support of this reversibility, the dramatic conformational change identified for Dap bound to the CL micelles was completely reversed to be identical to free Dap after Ca^{2+} sequestration (**Fig. 3B**).

By contrast, Dap was inserted irreversibly into the DMPG-containing membrane, because the Kyn fluorescence continuously increased (**Fig. 2A**) until all Dap was exhausted when the DMPG content was high enough (**Fig. 2C**). This is supported by the substantial residual Dap fluorescence after EGTA sequestration of Ca^{2+} from the accumulated drug in DMPG-containing micelles (**Fig. 3A**). This residual fluorescence not only indicates that Dap remains bound to membrane after removal of the metal ion but also demonstrate that Dap is still associated with Ca^{2+} after insertion into membrane. The fluorescence decrease induced by Ca^{2+} sequestration suggests that Dap is buried deeper into membrane when bound by the metal ion. The irreversible insertion of Dap into DMPG-containing micelles is further supported by the conformation of Dap after Ca^{2+} sequestration, which remains significantly different from free Dap albeit with significant change (**Fig. 3B**). Interestingly, Dap shows a non-identical, similarly shaped circular dichroism spectrum in its reversible and irreversible binding with the CL and DMPG micelles (**Fig. 3B**), respectively, indicating a small conformational change in the insertion of the surface-bound Dap into the hydrophobic acyl layer of the membrane.

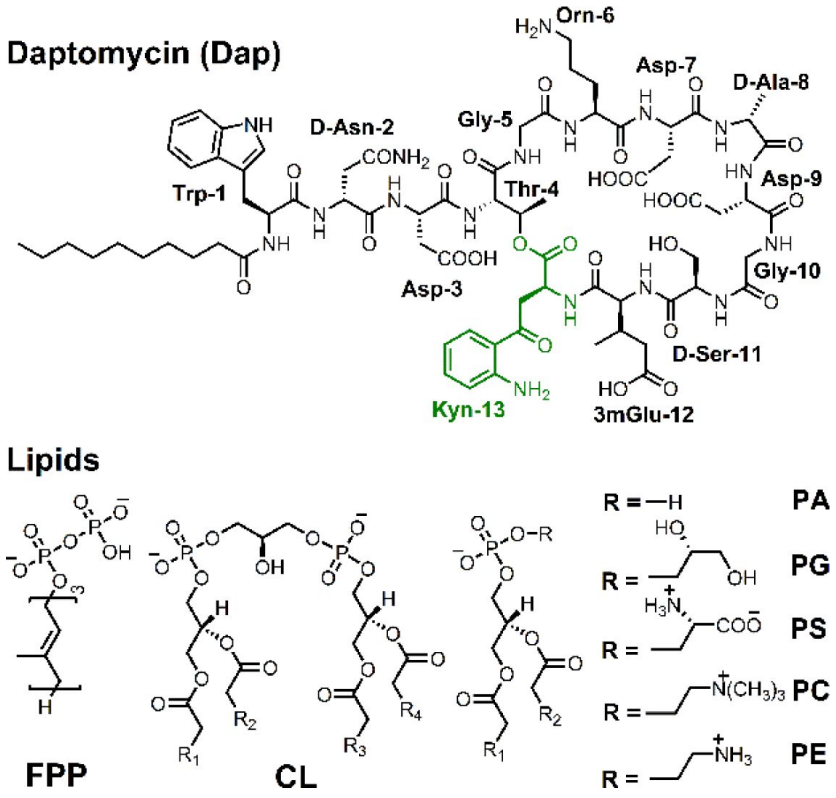

Figure 1.

Structure of daptomycin and lipids.

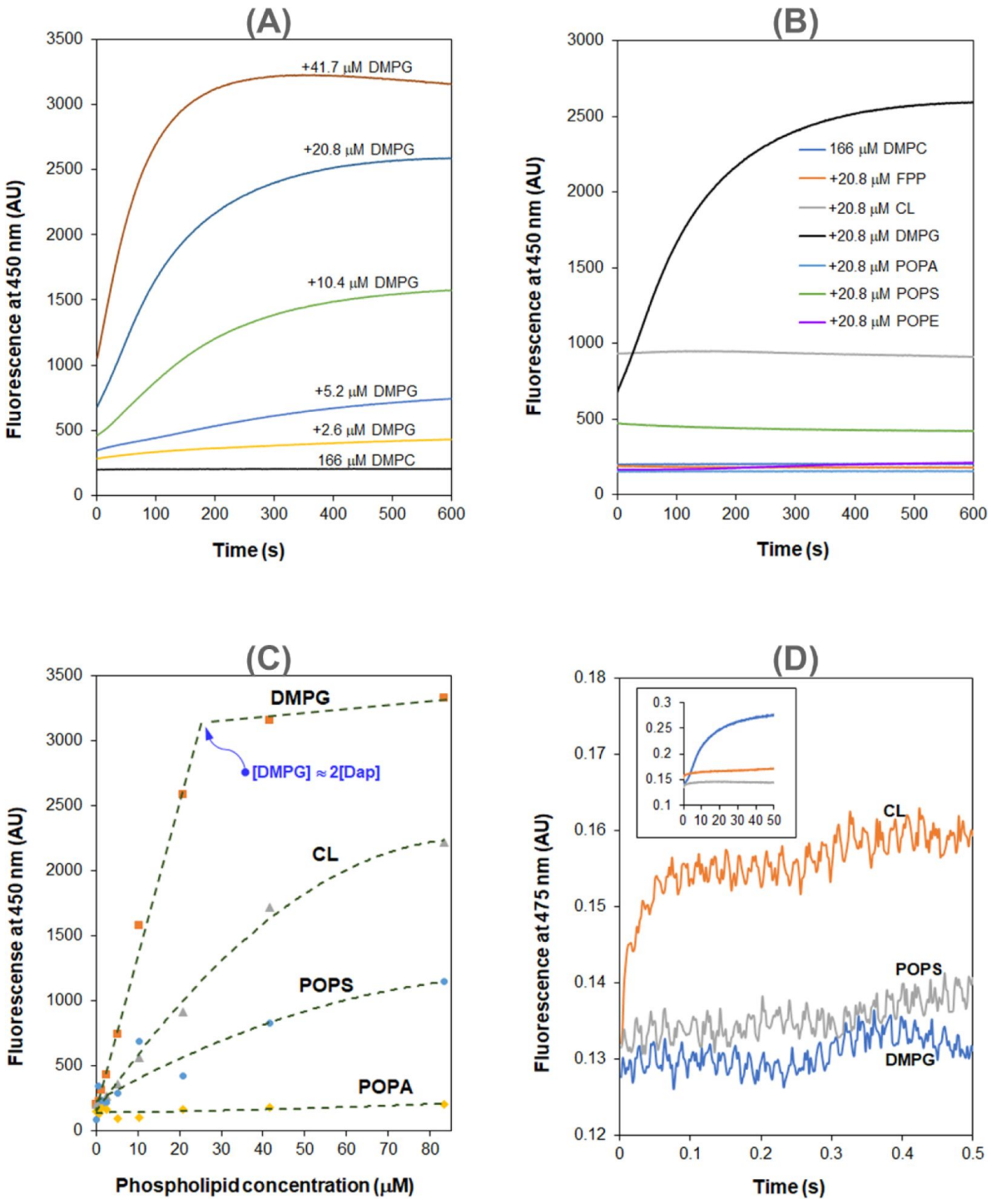

Figure 2.

Dap depends on PG in the interaction with membrane. (**A**) DMPG-dependent accumulation of Dap in phospholipid micelles. (**B**) Kinetics of the fluorescence of Dap in interaction with micelles containing different phospholipids. Micelles contained 20.8 μM various phospholipids (11.5%) and 167 μM DMPC. (**C**) Plot of the steady-state Kyn fluorescence *vs*. the content of different negative phospholipids in the micelles. (**D**) A fast Kyn fluorescence increase within 100 ms in the interaction of Dap with CL-containing micelles. The inset shows the Kyn fluorescence change over 50 s; micelles contained DMPG, POPS or CL at 20.8 μM. All the experiments in (**A**)-(**D**) were carried out in 20 mM HEPES (pH 7.57) containing 1.67 mM $CaCl_2$; Dap was always added at last at 15 μM. The micelles were prepared from DMPC at 167 μM alone or together with another lipid at a concentration or content indicated in the plots.

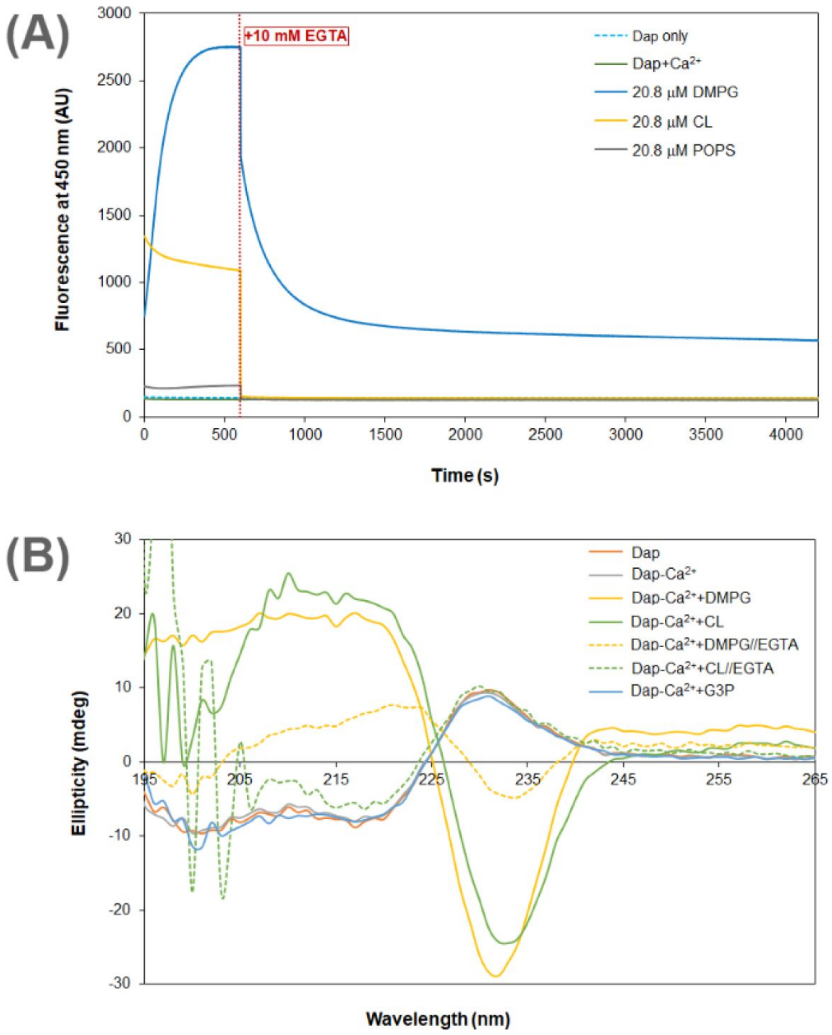

Figure 3.

Calcium dependence of the Dap structure and fluorescence in membrane. (**A**) Calcium sequestration decreases the Dap fluorescence in a way dependent on the phospholipid composition. The kinetic fluorescence measurement was performed under the same conditions as in **Figure 1(B)**. The red dotted line denoted the time point when 10 mM EGTA was added. The controls contained Dap at 15 μM Dap in the same buffer with or without calcium. (**B**) Circular dichroism spectra of Dap in different membrane environments. The buffer was 20 mM Tris.HCl, pH 7.57; Dap was 180 μM and $Ca(CH_3COO)_2$ was 1.0 mM; spectra were recorded 30 min after mixing with micelles contained pure DMPG or CL at 720 μM to maximize the amount of bound Dap; in Ca^{2+} sequestration experiments, spectra were recorded 30 min after adding 10.0 mM EGTA. The putative PG headgroup, *sn*-glycerol 3-phosphate (G3P), was 20 mM in attempt to detect its potential interaction with Dap.

Ca^{2+}-dependent interaction between Dap and DMPG

To assess whether the putative PG headgroup was responsible for the observed irreversible uptake of Dap, G3P was included in the kinetic measurement and found to slightly inhibit the drug uptake at millimolar levels (Fig. S4A). This interaction was not detectable by circular dichroism (**Fig. 3B**), isothermal titration calorimetry (ITC) or ligand-induced fluorescence change but was found to cause a small fluorescence-based thermal shift in the presence of 20 mM G3P (Fig. S4B). In the NMR titration of G3P into a Dap-Ca^{2+}solution at pH 5.40, the α-protons of Trp-1, D-Asn-2, Asp-9 and Kyn-13 were found to shift slightly in the fingerprint region (Fig. S4C). The α-proton signals were assigned by comparing the unique $N_{\alpha}H$-H_{α}-H_{β}-H_{γ} cross-peak patterns of the amino acid residues from Dap in TOCSY with those obtained for the drug at pH 5.0 in a previous study[30] (see Fig. S5 for the comparison and Table S1 for the assignments). These results show that Dap indeed binds G3P but the affinity is too low (in high millimolar range) to account for the DMPG-enabled membrane insertion.

Noticeably, Dap was saturated by DMPG at a ratio of 1: 2 in the steady-state fluorescence titration (**Fig. 2C**), indicating a binding interaction between the two in 1:2 stoichiometry that is consistent with the previous ITC titration.[26] To determine the binding affinity, Dap at 15 nM was titrated with sub-micromolar DMPG in the presence of Ca^{2+} and its Kyn fluorescence at 454 nm was found to increase sharply at low DMPG concentration and reach a saturating level at high concentration, whereas no fluorescence increase was observed for a control titration with POPS at an elevated Dap concentration of 1.5 μM (**Fig. 4**). The titration curve was best fitted with a model in which Dap binds two molecules of DMPG with a dissociation constant of $K_D = 7.2 \times 10^{-15}$ M^2. DMPG has a critical micelle concentration (CMC) higher than 1.0 μM (Fig. S6) and is homogeneously present in solution in the 0-1.0 μM concentration range under the given conditions. This titration result shows that Dap forms a high-affinity complex with two molecules of DMPG in the presence of calcium ion.

To obtain the complex, Dap (20 μM or higher) and DMPG were mixed at 1:2 molar ratio in the presence of $CaCl_2$ (1 mM or higher) and white precipitate was immediately formed, while mixing DMPG with $CaCl_2$ resulted in clear solution (Fig. S7). By comparison, precipitate was formed when POPS or POPA was mixed with $CalCl_2$ only, whereas no precipitate was observed when Dap was mixed with other lipids under the same conditions. This comparison suggested that the precipitate was formed from the specific calcium-dependent binding interaction between Dap and DMPG. The same precipitate was also formed by extracting the DMPG-containing micelles after the Dap uptake experiments (**Fig. 2A**) with chloroform, which was not observed in similar extraction of Dap-bound micelles without DMPG. This precipitate dissolved readily in aqueous solution containing 10 mM EGTA and was moderately soluble in a few organic solvents such as dimethyl sulfoxide (DMSO) and dichloromethane (DCM). In high performance liquid chromatography (HPLC), the dissolved complex was a pure single species in the elution profile (**Fig. 5A**).

Dap-PG complex isolated from drug-treated bacterial cells

To test whether Dap is complexed with PG in cells, *Bacillus subtilis subsp. Subtilis 168* was grown in Luria broth, treated with Dap at 0. 375 mg/mL and $CaCl_2$ at 6.8 mM, and lysed after washing. The cell membrane was isolated from the crude extract by ultracentrifugation and extracted with chloroform. After adding sodium dodecyl sulfate (SDS) to solubilize all precipitated proteins, white precipitate reminiscent of the Dap-DMPG complex was found as suspension in aqueous phase. After dissolved in DMSO, this precipitate was found to contain a pure Dap-containing complex with a retention time different from the Dap-DMPG complex in an HPLC analysis (**Fig. 5A**). In addition, it was fluorescent with a spectrum characteristic of membrane-bound Dap like the Dap-DMPG complex (**Fig. 5B**), strongly suggesting that the isolated precipitate was a similar phospholipid complex of Dap. In support of this, Dap was detected with a molecular ion at *m/z* =

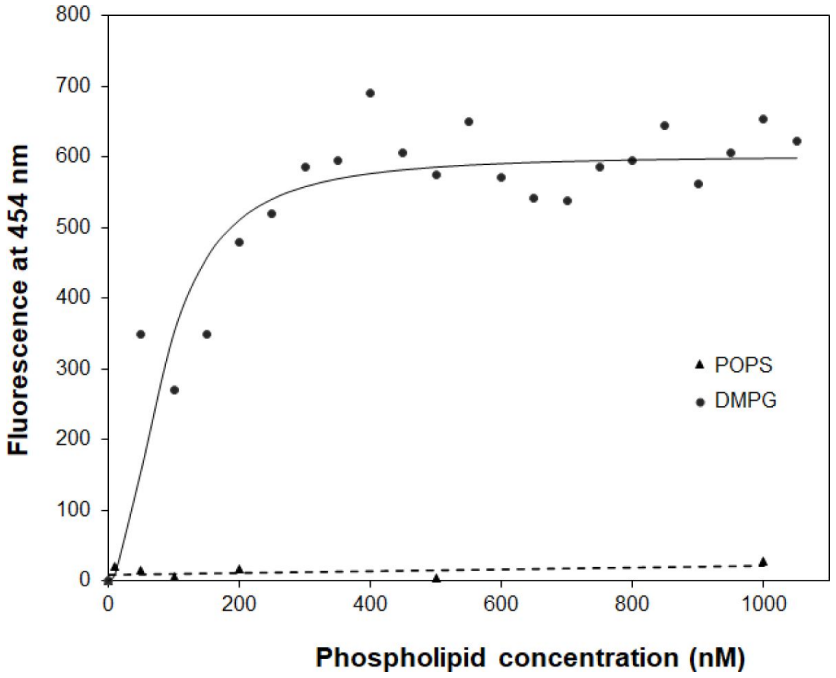

Figure 4.

The binding affinity of Dap for DMPG by titration. The titration with DMPG was performed in 20 mM HEPES buffer (pH 7.57) containing 15 nM Dap and 1.67 mM $CaCl_2$. The solid line is the fitting curve using the equation $F = F_{max} \times [DMPG]^2/(K_D + [DMPG]^2)$ where F = fluorescence at 454 nm, which is derived from a binding model in which a fluorescent {Dap·2DMPG} complex is dissociated into non-fluorescent Dap and two DMPG molecules under the condition [DMPG] >> [Dap]. The dashed line is the linear trend line for the control titration of 1.5 μM Dap with POPS in the same buffer containing 1.67 mM $CaCl_2$.

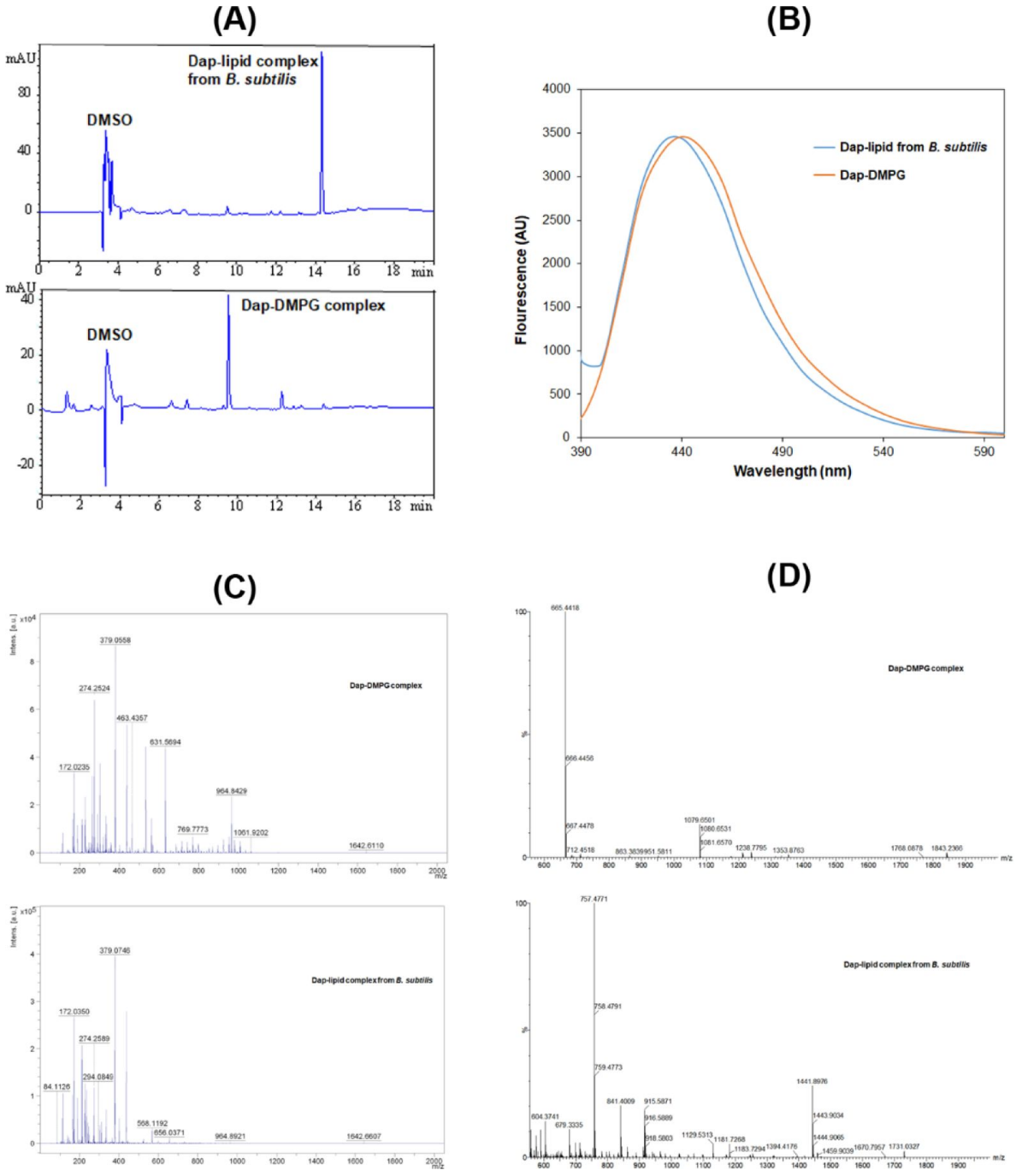

Figure 5.

Stable Dap-PG complexes formed *in vitro* and in *Bacillus subtilis*. (**A**) Reverse phase HPLC chromatogram of the complexes. Samples were dissolved in 10% DMSO and 90% water and filtered before injection into a Phenomenex Luna 5 µm C18 column (150 × 4.6 mm). The complexes were eluted by water containing 0.1% trifluoroacetic acid and a linear gradient of acetonitrile from 5-95% over 20 min and detected by ultraviolet absorbance at 254 nm. (**B**) Fluorescence spectra of the two complexes with excitation at 365 nm. The complexes were dissolved in DMSO and the fluorescence of the Dap-DMPG complex was scaled for easy comparison. (**C**) Comparative positive-ion MALDI-ToF mass spectrometry of the two complexes. All the peaks with *m/z* < 500 were from the matrix after comparison with a control. (**D**) Comparative negative-ion high-resolution ESIMS analysis of the two complexes.

1642.66 ([M+Na]$^+$, calcd. for $C_{17}H_{101}N_{17}O_{20}Na$: 1642.70) for both the cell-generated Dap-lipid complex and the Dap-DMPG complex in a comparative MALDI-ToF mass spectrometric analysis (**Fig. 5C**). However, the phospholipid was not detected by MALDI-ToF mass spectrometry. High-resolution electron spray ionization mass spectrometry (HRESIMS) in negative-ion mode turned out to be more sensitive for negative phospholipids, affording DMPG molecular ion at 665.4418 ([M-H]$^-$, calcd. for $C_{34}H_{66}O_{10}P$: 665.4394) for the Dap-DMPG complex (**Fig. 5D**). For the cell-generated complex, the phospholipid PG (32:0) was deduced from the main peak at *m/z* = 757.4771 ([M+Cl]$^-$, calcd. for $C_{38}H_{75}O_{10}PCl$: 757.4792) in the negative-ion HRESIMS as an adduct with the matrix chloride ion, which could readily be formed in electron-spray ionization.[31] PG (32:0) likely contains acyl chains derived from two most abundant *iso*-15:0 and *anteiso*-17:0 branched fatty acids in *Bacillus subtilis*,[32] and its presence in the isolated complex as the major lipid species provides strong support for the specific interaction between Dap and PG. Taken together, all these results consistently indicated that Dap indeed forms a stable complex with PG in bacterial cells, which closely resembles the *in vitro* Dap-DMPG complex.

Discussion

Daptomycin is well known to target bacterial membrane for its bactericidal effect, but the mechanism of its membrane uptake is poorly understood. By monitoring the kinetics of membrane-induced fluorescence, here we show that the calcium-dependent membrane uptake of Dap is divided into two stages. The first stage is reversible binding to the membrane surface, likely in the headgroup region, which happens very fast and reaches an equilibrium in milliseconds (**Fig. 2** and **Fig. 3**). The Dap binding capacity is dependent on the phospholipid, which is very low for neutral lipids PC and PE but is much higher for negative lipids, particularly cardiolipin (**Fig. 2**). After this reversible surface binding, Dap is slowly inserted into the membrane in minutes (**Fig. 2A** and **Fig. 3**) only in the presence of PG, culminating in the formation of a multi-component complex. This complex contains calcium and two PG molecules with nanomolar affinity (**Fig. 4**) and is easily formed *in vitro* and readily isolated from drug-treated bacterial cells (**Fig. 5**). This high-affinity interaction provides an impeccable rationale for the neutralization of Dap by the PG-rich pulmonary surfactants.[20, 21] Moreover, the complete dependence of the membrane insertion on PG also explains why Dap selectively attacks Gram-positive bacteria without affecting mammalian cells, because PG is a major phospholipid in bacterial membrane but is a minor component in mammalian membrane.

Using circular dichroism spectroscopy, Dap was structurally examined in every step of its interaction with membrane. In bulk aqueous solution, it takes a conformation with a characteristic positive peak at 230 nm and undergoes a large conformational change when bound to the headgroup region of membrane without PG, as evidenced from the dramatic change in circular dichroism (**Fig. 3B**) that is consistent with previous investigations.[25, 27] It may not bind Ca^{2+} in the bulk solution but requires the ion to bind the phospholipid headgroups, due to the observed release of the bound drug to the bulk solution with concomitant conformational reversal in the Ca^{2+} sequestration experiments (**Fig. 3**). In this surface-bound state, Dap is unable to insert into the acyl layer in membrane, suggesting that its decyl tail is folded back into the hydrophobic part of the cyclodepsipeptide ring. In addition, due to the specificity for negative phospholipids (**Fig. 2B** and 2C), this reversible binding of Dap likely involves both a non-specific Ca^{2+}-mediated ionic interaction and a specific interaction with the remaining part of the headgroups.

In the irreversible insertion into membrane, Dap undergoes a subtle conformational change from its surface-bound state to the inserted state, as seen from the small difference in their circular dichroism spectra (**Fig. 3B**). When Ca^{2+} is sequestrated, Dap undergoes another major conformational change from its inserted state to a new structure with circular dichroism features distinct from all other structures, supporting that the drug is still bound to Ca^{2+} in its membrane-inserted state and that its decyl tail is fully extended and firmly embedded in the hydrophobic acyl

layer of membrane. Besides binding Ca^{2+}, this inserted Dap should form a complex with PG in 1:2 ratio as indicated by titration of Dap with DMPG in both micromolar (**Fig. 2C**) and nanomolar range (**Fig. 4**), which was formed easily *in vitro* and readily isolated from drug-treated *Bacillus subtilis* (**Fig. 5**). This complex is structurally similar to the tripartite Dap-PG-undecaprenyl lipid complex proposed to be responsible for the bactericidal effect of the drug.[17] At present, it is not clear whether the Dap-Ca^{2+}-2PG complex contains one or more calcium ions.[33, 34] For simplicity of discussion, only one calcium ion is assumed to involve in the binding interaction to result in a quaternary Dap-Ca^{2+}-2PG complex.

Taking all the structural changes into consideration, we propose a mechanism for the Ca^{2+}-dependent uptake of Dap into bacterial membrane as shown in **Fig. 6**. In the reversible binding, Dap with a relatively random conformation is quickly bound to the headgroups of phospholipids likely through Ca^{2+}-dependent electrostatic interaction, accompanied by a large conformational change. With a hidden decyl tail, the surface-bound Dap is unable to insert into the membrane, even for the drug molecule bound by the PG headgroup. To enable membrane insertion, we propose that the bound Dap has to form a quaternary complex resembling the post-insertion Dap-Ca^{2+}-2PG complex, which is different from the latter in having a hidden rather than a fully extended decyl tail and is less stable. Subsequently, this pre-insertion complex undergoes structural change to expose the decyl tail of Dap and allows it to interact with the acyl chains of the two PG molecules to finish the insertion process, driven by the free energy to form the more stable post-insertion complex.

From this uptake mechanism, we can now understand how Dap recognizes bacteria and selectively accumulates in their membrane in a clinic setting. After absorption into the circulation, Dap is preferentially bound to the headgroup region of bacterial membrane due to the high content of PG and CL,[35] very quickly in milliseconds, although it is also bound to mammalian cell surface in a smaller amount per cell due to the very low content or absence of PG or CL in plasma membrane,[36] but in substantial total quantity due to the overwhelmingly larger amount of host cells. However, Dap absorbed on bacterial surface is continuously inserted into the acyl layer via formation of complex with PG in a time scale of minutes, whereas little irreversible insertion of Dap occurs on mammalian membrane due to the low content of PG while the bound Dap is continuously released to the circulation as the drug is depleted by the bacteria. The proposed requirement of the pre-insertion quaternary complex increases the threshold of PG content for the membrane insertion to happen and thus makes it less likely on the surface of mammalian cells that contain PG at a low level in the membrane. Consequently, most circulating drug is selectively inserted and accumulated in bacterial membrane within a short period of time where it exerts bactericidal effect via a mechanism still in debate.[12–17]

In the Dap uptake, PG plays the dual role of recruiting the drug to bacterial cell surface and enabling the irreversible insertion of the drug, of which both processes are critically affected in velocity and quantity by its membrane content. This PG dependence not only provides the basis for the reliance of Dap susceptibility on PG,[22] but also is fully consistent with the finding that a decrease in membrane PG content inevitably leads to resistance to Dap, no matter the decrease is due to the gain-of-function mutations of MprF in the domain responsible for lysinylation of PG,[37–40] or loss-of-function mutations of the PG synthase PgsA,[22, 41] or gain-of-function mutations of the cardiolipin synthase (Cls) to increase conversion of PG to CL.[42–45] Understandably, when the PG biosynthesis is abolished due to complete inactivation of the PgsA activity, the Dap susceptibility is completely lost to result in a very high level of resistance.[23, 24] However, a decreased PG content is found in only a fraction of resistant Enterococcus strains containing mutations in the LiaFSR operon and the PG metabolic enzymes GpdD and Cls in clinical isolates of Enterococci,[46, 47] suggesting that there exist PG-independent mechanisms to cause Dap resistance. In this connection, it is noteworthy that the PG content also appears not to be involved in the resistance caused by up-regulated expression of *dltABCD* operon that increases the production of teichoic acids and their D-alanylation.[48, 49]

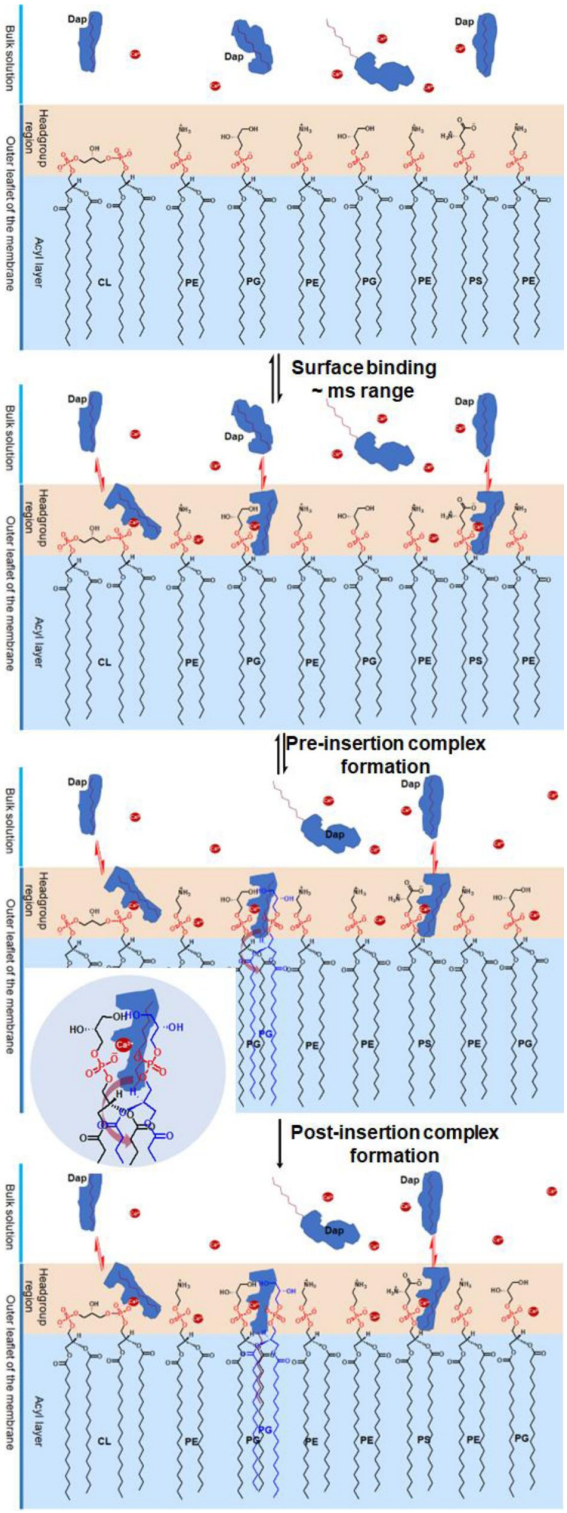

Figure 6.

Proposed mechanism for the two-phased uptake of Dap into bacterial membrane. In the first phase, Dap reversibly binds to negative phospholipids with a hidden tail in the headgroup region, where it combines with two PG molecules to form a pre-insertion complex. In the second phase, the hidden tail unfolds and irreversibly inserts into the membrane. The inset shows the headgroup of the pre-insertion complex with the broad arrow showing the direction for the unfolding of the hidden tail. The red dots denote Ca^{2+}.

In summary, we have shown that Dap is first reversibly bound to the membrane surface and then irreversibly inserted into the acyl layer by forming a stable complex with PG. Interestingly, the stable complex may exchange one PG molecule with an undecaprenyl lipid to form the previously proposed bactericidal Dap-PG-undecaprenyl lipid tripartite complex to play a critical role in the antibacterial activity of the antibiotic. In addition, this stable complex also forms the basis of a unique mechanism for the bacterial uptake of Dap, in which the negative phospholipid PG plays a pivotal role to affect both the susceptibility and resistance to Dap. Nevertheless, it remains unknown how the structural components of the drug affect nonspecific binding to the surface headgroup layer and specific binding with PG to influence the speed and dose of drug uptake. Elucidation of these structure-activity relationships may enable rational design of Dap analogues to counter the increasing drug resistance.

Materials and methods

Materials

Daptomycin (Dap) was purchased from MedChem Express. 1, 2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cardiolipin sodium salt (CL) from the bovine heart, farnesyl pyrophosphate ammonium salt (FPP), calcium chloride and calcium acetate were purchased from Sigma. The putative headgroups *sn*-glycerol-3-phosphate bis (cyclohexyl ammonium) salt (G3P) was also purchased from Sigma. Tris base was purchased from Fisher Bioreagents. 1, 2-Dimyristoyl-*sn*-glycero-3-phosphate sodium salt (DMPG) was purchased from Abcam. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] sodium salt (POPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate monosodium salt) (POPA) were purchased from Avanti Polar Lipids. All lipids or chemicals were used directly without further treatment.

Kinetics of the Kyn fluorescence change in micelles

Lipid stocks were prepared in 20 mM HEPES buffer with the pH adjusted to 7.57 at a concentration of 1.0 mM for DMPC and 0. 50 mM for other lipids including DMPG, POPA, POPE, POPS, CL, and FPP. In a typical kinetic measurement, a lipid micelle solution was prepared by mixing DMPC with or without another lipid in 20 mM HEPES (pH 7.57) and was sonicated for at least 4.5 h (Ultrasonic Cleaner, 37 kHz, 100%). After the micelle solution was added calcium chloride and Dap at the last, the kinetic of the Kyn fluorescence at 450 nm was immediately monitored in an Edinburgh FS-5 spectrofluorometer for 600 s with a time interval of 0.1 s and an excitation wavelength of 365 nm. The total volume of the solution was 300 µL (of which only 200 µL was added to the cuvette for reading) containing 167 µM DMPC with or without another lipid at a varied concentration, 1.67 mM $CaCl_2$, and 15.0 µM Dap in 20 mM HEPES (pH 7.57). After the kinetic measurement, an emission spectrum was recorded from 380 nm to 700 nm.

To study the effects of putative lipid headgroups on the fluorescence kinetics, their stocks were prepared at 50 mM with the pH adjusted to 7.57. The putative headgroup was then added at a pre-set concentration to the micelle solution for the kinetic measurements. In these measurements, final DMPC concentration was set at 80 µM and final DMPG concentration was set at 10 µM (or 11.5%), while all other components and the instrumental parameters remained unchanged.

In the calcium sequestration experiments, an EGTA stock was prepared at 42.85 mM and dissolved in HEPES by NaOH and adjusted to pH 7.57 by HCl. It was then added at a saturating concentration (10 mM) to the micelle solution at the end of the 600 s kinetic measurement and the fluorescence kinetics was monitored at 450 nm for another 10 min or a longer time (up to 70 min). The micelle solution contained 167 µM DMPC with or without another phospholipid, namely DMPG, or CL, or POPS at 20.8 µM (or 11.5%). All other conditions were the same as in other kinetic measurements. Similar experiments were also performed with a different combination of phospholipids such as 167 µM DMPC and 10.4 µM DMPG.

Kinetics of the Kyn fluorescence change in vesicles

Phospholipid vesicles were prepared in a varied ratio from a DMPC stock and the stock of another phospholipid, namely DMPG, CL or POPS, of which both were in chloroform at a concentration of 500 μM and 125 μM, respectively. After mixing, the solvent chloroform was evaporated by a Rotavap under reduced pressure at 37°C for 15 min, to form concentric rings at the bottom of the round bottom flask. The phospholipids were further dried under vacuum for 2 h and then hydrated overnight in 20 mM HEPES (pH 7.57). The hydrated phospholipid film was subjected to five freeze-thaw cycles, in each of which the vesicles were frozen in liquid nitrogen for 1 min, followed by 5 min thawing and 10 s vortex. Finally, the vesicle suspension in a 1 ml syringe was pushed through a 100 nm filter in an Avanti polar extruder with filter supports. Vesicles went through at least 10 passes and the extruder was warmed on a hot plate to ease the passing of vesicles. The vesicle suspension was diluted to the preset concentration of the phospholipid and used to interact with Dap for kinetic monitoring of the Kyn fluorescence similar to the measurements using the micelles.

Dap conformational change by circular dichroism spectroscopy

Circular dichroism (CD) was used to characterize the structure of Dap in its interaction with calcium, G3P and lipid micelles under various conditions using a Chirascan™ Circular Dichroism Spectrometer (Applied Photophysics). The samples were prepared to contain 0.30 mg/ml Dap from stocks adjusted to pH 7.57, to avoid pH change after mixing of different components. To reduce background, 20 mM Tris buffer (pH 7.57) was used instead of the HEPES buffer and calcium acetate was used at 1.0 mM to replace calcium chloride used in the fluorescence experiments. Each sample was scanned 10 times in a 1 mm Hellma Quartz cuvette from 180 to 270 nm with 1 nm bandwidth, 1 nm step size and 0.5 s per point (approximate 73 seconds). The CD spectrum was then averaged from the 10 scans using the Chirascan software after auto-subtraction with the background signal of a blank buffer.

For Dap interaction with model membranes, the micelle solution was prepared from lipid stocks adjusted to pH 7.57 to contain one or two lipids at preset concentrations, sonicated for 4 h, and then added 1.0 mM calcium acetate and 0.30 mg/mL Dap for incubation at room temperature for at least 15 min before the CD spectra were taken. To maximize the drug reversibly bound to the micelle surface, pure CL micelle solution was used at 720 μM (higher concentration led to precipitation) to bind Dap in the presence of 1 mM calcium acetate for CD spectroscopy. Similarly, pure DMPG micelles at 720 μM was used in the presence of 1 mM calcium acetate to ensure complete membrane insertion of Dap for structural characterization by CD spectroscopy. To characterize the conformation of membrane-bound Dap after calcium removal, EGTA in 20 mM HEPES at pH 7.57 was added at a saturating concentration of 10 mM to the solution of Dap bound to micelles of either pure CL or pure DMPG at 720 μM, incubated at room temperature for at least 30 min and then subjected to CD spectroscopy.

Interaction of Dap with G3P by NMR spectroscopy

For the NMR analysis, stocks of calcium chloride, G3P and Dap were prepared in 20 mM HEPES with 90% D_2O (v/v) and pH/pD adjusted to 5.40. They were used to prepare the samples (in 1000 μL,) that contained 1.0 mM Dap, 1 mM $CaCl_2$ and G3P at a varied concentration at 0 mM, 1.0 mM, 4.0 mM, 8.0 mM, 12.0 mM, 16.0 mM and 20.0 mM in the same deuterated buffer. Controls containing 1.0 mM Dap or 20 mM G3P were also prepared for the NMR experiments in the same buffer. The 1D ^{1}H-NMR spectrum and standard 2D spectra including TOCSY (spin lock system was 75 mS), COSY and HMBC were recorded for each sample on an 800 MHz Varian spectrometer at room temperature (23°C). All spectra were analyzed using Mestrenova and the cross-peaks in the

fingerprint region were assigned by comparing the $N_{\alpha}H$-H_{α}-H_{β}-H_{γ} correlation patterns of the amino acid residues of Dap in TOCSY with those obtained for the drug at pH 5.0 in a previous study.[30] The assignments are shown in Table S1 and the TOCSY comparison is shown in Fig. S5.

Critical micelle concentration (CMC) of DMPG and titration of Dap with DMPG in the nanomolar range

The CMC of DMPG was determined using a reported method.[50] Briefly, pyrene (~ 20 mg) was crushed to powder and added to 5.0 mL to obtain a 1.0 mM stock of DMPG in 20 mM HEPES buffer (pH 7.57). The mixture was sonicated for 4.5 h, filtered to remove undissolved pyrene, and then diluted in the appropriate medium (either pure water or 20 mM HEPES, pH 7.57) to contain DMPG at a varied concentration from 10 nM to 10 μM. All samples were added calcium chloride to a final concentration of 1.67 mM and their emission spectra from 340 nm to 600 nm were recorded on an FS-5 Edinburgh spectrofluorometer with an excitation wavelength at 330 nm and a bandwidth of 3 nm. The ratio of the fluorescence intensity at 473 nm and 484 nm was plotted against the DMPG concentration to determine CMC, as shown in Fig. S5.

For the fluorescence titration at low concentrations, each sample was prepared in 300 μL to contain 1.67 mM $CaCl_2$, 15 nM Dap and DMPG at a varied concentration ranging from 15 nM to 10 μM in an appropriate medium (pure water or 20 mM HEPES, pH 7.57). DMPG was added from a stock of DMPG at 5.0 μM in 20 mM HEPES (pH 7.57) while Dap was added from a stock at 150 nM. These samples were then recorded for their emission spectra in the range of 380 nm to 700 nm on an FS-5 Edinburgh spectrofluorometer with the excitation wavelength set at 365 nm. The bandwidth was set at 3 nm for both the excitation and emission. The emission value at 450 nm was then plotted against the DMPG concentration (as shown in **Figure 4A**). A control titration was performed similarly using 1.5 μM Dap against POPS in the concentration range of 0 – 1.0 μM.

Isolation of Dap-lipid complex from drug-treated *Bacillus subtilis*

Bacillus subtilis 168 was grown in 500 mL LB for 12 h at 37°C inoculated with 1 mL overnight LB culture from a single colony, harvested, resuspended in 10 mL fresh LB, and then added 0.375 mg/mL Dap and 0.75 mg/mL $CaCl_2$ for incubation at 37°C for another 2 h. The drug-treated cells were pelleted, washed once with 10 mL 0.9% NaCl solution, resuspended in 10 mL 1 mM NaCl and lysed by sonication (1s sonication, 2 second pause, Instrument: JY92-II) for 1 h over ice. The lysate was centrifuged to remove the cell debris and the supernatant was ultracentrifuged at 125,000 × g at 4°C for 1 h in Hitachi CP80WX Preparative Ultracentrifuge. After removing the supernatant, the pellet was suspended in 2 mL of 0.9% NaCl solution and extracted with 2 mL chloroform to observe a layer of white precipitate at the phase interface. At this point, 1 mL of 0.1 M SDS solution was added to the aqueous phase and the mixture was vortexed for 1 min. 3 mL of DMSO was then added to quench the bubbles and the aqueous phase appeared cloudy. The aqueous layer was separated and centrifuged at high speed to obtain a small amount of white pellet, which exhibits fluorescent emission typical of membrane-absorbed Dap at 365 nm excitation after suspension in water or being dissolved in DMSO. In a parallel control experiment starting from the same amount of cell culture treated with $CaCl_2$ but without Dap, the aqueous phase was clear without any precipitate after addition of SDS, indicating that SDS dissolved all protein precipitates formed from the chloroform extraction. Like the Dap-DMPG complex, the final precipitate or pellet obtained from the drug-treated cells was soluble in DMSO and partly soluble in CH_2Cl_2 and was subject to analysis by reverse-phase HPLC, MALDI-ToF mass spectrometry and high-resolution ESIMS.

Additional information

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Author contributions

Conceptualization: Z.G. Methodology: P. M., V. G. U., Y. L., Y. J., L. Z. Investigation: P. M., V. G. U., Y. L, L. Z. Supervision: Z. G. Writing—original draft: Z. G., P. M. Writing—review & editing: Z. G., P. M., V. G. U., Y. L., Y. J.

Competing interests

Authors declare that they have no competing interests.

Abbreviations

- CL: cardiolipin
- Dap: daptomycin
- Kyn: kynurine
- FPP: farnesyl pyrophosphate
- PC: phosphatidylcholine
- DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
- PG: phosphatidylglycerol
- DMPG: 1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol
- PA: phosphatidic acid
- POPA: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate
- PE: phosphatidylethanolamine
- POPE: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
- PS: phosphatidylserine
- POPS: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine
- G3P: *sn*-glycerol 3-phosphate
- oPS: *O*-phosphoserine
- oPC: *O*-phosphocholine
- oPE: phosphorylethanolamine or *O*-phosphoethanolamine

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Editors

Reviewer #3 (Public review):

Summary:

Machhua et al. in their work focused on unravelling the molecular mechanism of daptomycin binding and interaction with bacterial cell membranes. Daptomycin (Dap) is an acidic, cyclic lipopeptide composed of 13 amino acids, known for preferential binding to anionic lipids, particularly phosphatidylglycerol (PG), which are prevalent components in the membranes of Gram-positive bacteria. The process of binding and antimicrobial efficacy of Dap are significantly influenced by the ionic composition of the surrounding environment, especially the presence of Ca^{2+} ions. The authors underscore the presence of significant knowledge gaps in our understanding of daptomycin's mode of action. Several critical questions remain unanswered, including the basis for selective recognition and accumulation in membranes of Gram-positive strains, the specific role of Ca^{2+} ions in this process, and the mechanisms by which daptomycin binds to and inserts into the cell membrane.

Dap is intrinsically fluorescent due to its kynurenine residue (Kyn-13) and this property allows direct imaging of Dap binding to model cell membranes without the need of additional labeling. Taking advantage of this Dap autofluorescence, authors monitored the emission intensity of micelles, composed of varying DMPG content upon their exposure to Dap and compared it with the kinetics of fluorescence observed for zwitterionic DMPC and other negatively charged lipids such as cardiolipin (CA), POPA and POPS. The authors noted that the linear relationship between DMPG content and Dap fluorescence is strongly lipid-specific, as it was not observed for other anionic lipids. The manuscript sheds light on the specificity of Dap's interaction with CA and DMPG lipids. Through Ca^{2+} sequestration with EGTA, the authors demonstrated that the binding of Dap with CA is reversible, while its interaction with DMPG results in the irreversible insertion of Dap into the lipid membrane structure, caused by the significant conformational change of this lipopeptide. The formation of a stable DMPG-Dap complex was also verified in bacterial cells isolated from Gram-positive bacteria B. subtilis, where Dap exhibited a permanent binding to PG lipids.

Altogether, the authors endeavored to illuminate novel insights into the molecular basis of Dap binding, interaction, and the mechanism of insertion into bacterial cell membranes. Such understanding holds promise for the development of innovative strategies in combating drug resistance and the emerging of the so-called superbugs.

Strengths:

- The manuscript by Machhua et al. provides a comprehensive analysis of the Dap mechanism of binding and interaction with the membrane. It discusses various aspects of this, only apparently trivial interaction such as the importance of PG presence in the membrane, the impact of Ca2+ ions, and different mechanisms of Dap binding with other negatively charged lipids.
- The authors focused not only on model membranes (micelles) but also extended their research to bacterial cell membranes obtained from B. subtilis
- The research is not only a report of the experimental findings but tries to give potential hypotheses explaining the molecular mechanisms behind the observed results

Weaknesses:

- The authors overestimate their findings, stating that they propose a novel mechanism of Dap interaction with bacterial cell membranes. This research is the extension of the hypotheses that have already been reported.
- The literature study and overall discussion about the mechanism of action of Ca2+ ions or conformational changes of daptomycin could be improved.

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Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Summary:*
>
> *In this manuscript, the molecular mechanism of interaction of daptomycin (DAP) with bacterial membrane phospholipids has been explored by fluorescence and CD spectroscopy, mass spectrometry, and RP-HPLC. The mechanism of binding was found to be a two-step process. A fast reversible step of binding to the surface and a slow irreversible step of membrane insertion. Fluorescence-based titrations were performed and analysed to infer that daptomycin bound simultaneously two molecules of PG with nanomolar affinity in the presence of calcium. Conformational change but not membrane insertion was observed for DAP in the presence of cardiolipin and calcium.*
>
> *Strengths:*
>
> *The strength of the study is skillful execution of biophysical experiments, especially stoppedflow kinetics that capture the first surface binding event, and careful delineation of the stoichiometry.*
>
> *Weaknesses:*
>
> *The weakness of the study is that it does not add substantially to the previously known information and fails to provide additional molecular details. The current study provides incremental information on DAP-PG-calcium association but fails to capture the complex in mass spectrometry. The ITC and NMR studies with G3P are inconclusive. There are no structural models presented. Another aspect missing from the study is the reconciliation between PG in the monomer, micellar, and membrane forms.*

Besides the two-stage process, another important finding in the current work is the stable complex that plays a critical role in the drug uptake both *in vitro* and in *B. subtilis*. This complex has been shown to be a stable species in HPLC and its binding stoichiometry and

affinity have been quantitatively characterized. The complex may not be stable enough in gas phase to be detected in the MS analysis, which was designed to detect the phospholipid and Dap components, not the complex itself. The structural model of this complex is clearly proposed and presented in Figure 6.

The NMR and ITC studies have a very clear conclusion that Dap has a weak interaction with the PG headgroup alone, which is unable to account for the Dap-PG interaction observed in the fluorescence studies. Thus, the whole PG molecule has to be involved in the interaction, leading to the discovery of the stable complex.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) I appreciate and agree with the comment that there are stages of daptomycin insertion, and these might involve the formation of different complexes with different binding partners (e.g. pre-insertion vs quaternary vs bactericidal). However, it seems like lipid II is an apparent participant in daptomycin membrane dynamics (Grein et al. Nature Communications 2020). It's not clear why this was excluded from analysis by the authors, or what basis there is for the discussion statement that the quaternary complex can shift into the bactericidal complex by exchanging 1 PG for lipid II.*

We agree that lipid II and other isoprenyl lipids may be involved in the uptake and insertion of daptomycin into membrane according to the results of the *Nat. Comm.* paper. However, these isoprenyl lipids are very small components of the membrane in comparison to PG and their contribution to the drug uptake is thus expected to be much less significant. Nonetheless, we included farnesyl pyrophosphate (FPP) as an analog of bactoprenol pyrophosphate (C55PP), which was reported to have the same promoting effect as lipid II in the previous study, in our study but found no promoting effect in the fluorescence assay (Fig. 2B). In addition, no complex was formed when FPP replaced PG in our preparation and analysis of the drug-lipid complex. In consideration of these negative results and the expected small contribution, other isoprenyl lipids or their analogs were not included in the study.

The statement of forming the proposed bactericidal complex from the identified complex is a speculation that is possible only when lipid II has a higher affinity for Dap than a PG ligand. To avoid confusion, we deleted the sentence' in the revision.

> *(2) The detailed examination of daptomycin dynamics, particularly on the millisecond scale, in this paper is ideal for characterizing the effect of lipid II on daptomycin insertion. It would be helpful to either include lipid II in some analyses (micelle binding, fluorescence shifts, CD) or at least address why it was excluded from the scope of this work.*

As mentioned in the response to the first comment, we did not exclude isoprenyl lipids in our study but used some of their analogs in the fluorescence assay. Besides FPP mentioned above, we also tested geranyl pyrophosphate and geranyl monophosphate but obtained the same negative results. Lipid II was not directly used because it is one of the three isoprenyl lipids reported to have the same promoting effects in the *Nat. Comm.* paper and also because its preparation is not easy. Even if lipid II were different from other isoprenyl lipids in promoting membrane binding, its contribution is likely negligible at the reversible stage compared to the phospholipids because of its minuscule content in bacterial membrane. This is the main reason we did not use the isoprenyl lipids in the fast kinetic study (this stage only involves reversible binding, not insertion).

> *(3) Grein et al. 2020 saw that PG did not have a strong effect on daptomycin interaction with membranes. I believe this discrepancy is more likely due to the complex physical parameters of supported bilayers versus micelles/vesicles or some other methodological*

> *variable, but if the authors have more insight on this, it would be valuable commentary in the discussion.*

We totally agree that the discrepancy is likely due to the different conditions in the assays. It is hard to tell exactly what causes the difference. Thus, we did not attempt to comment on the cause of this difference in the discussion.

> *(4) Isolation of the daptomycin complex from B. subtilis cells clearly had different traces from the in vitro complex; is it possible that lipid II is present in the B. subtilis complex? If not, a time-course extraction could be useful to support the model that different complexes have different activities. Isolates from early-stage incubation with daptomycin may lack lipid II but isolates from longer incubations may have lipid II present as the complex shifts from insertion to bactericidal.*

From the day we isolated the complex from *B. subtilis*, we have been looking for evidence for the previously proposed lipid complexes containing lipid II or other isoprenyl lipids but have not been successful. We did not see any sign of lipid II or other isoprenyl lipids in the MALDI or ESI mass spectroscopic data. The minute peaks in the HPLC traces are not the expected complexes in separate LC-MS analysis. However, this does not mean that such complexes are not present in the isolated PG-containing complex because: (1) the amount of such complexes may be too small to be detected due to the low content of the isoprenyl lipids; (2) the isoprenyl lipids, particularly lipid II, are not easily ionizable due to their size and unique structure for detection in mass spectrometry.

We don't think the drug treatment time is the reason for the failure in detecting lipid II or other isoprenyl lipids. In our reported experiment, the cells were treated with a very high dose of Dap for 2 hours before extraction. In a separate experiment done recently, we treated *B. subtilis* at 1/3 of the used dose under the same condition and found all treated cells were dead after 1 hour in a titration assay, consistent with the results from reported time-killing assays in the literature. From this result, the proposed bactericidal lipid-containing complex should have been formed in the treated cells used in our extraction and isolated along with the PG-containing complex. It was not detected likely due to the reasons discussed above. To avoid the interference of the PG-containing complex, a large amount of bacterial cells might have to be treated at a low dose to isolate enough amount of the lipid II-containing complex for identification. However, isolation or identification of the lipid II-containing complex is outside the scope of the current investigation and is therefore not pursued.

> *(5) Part of the daptomycin mechanism of interacting with bacterial membranes involves the flipping of daptomycin from one leaflet to another. There was some mentioned work on the consistency of results between micelles and vesicles, but the dynamics or existence of a flipping complex in the bilayer system wasn't addressed at all in this paper.*

The current investigation makes no attempt to solve all problems in the daptomycin mode of action and is limited to the uptake of the drug, up to the point when Dap is inserted into the membrane. Within this scope, flipping of the complex is not yet involved and is thus irrelevant to the study. How the complex is flipped and used to kill the bacteria is what should be investigated next.

> *(6) The authors mention data with phosphatidylethanolamine in the text, but I could not find the data in the main or supplemental figures. I recommend including it in at least one of the figures.*

It is much appreciated that this error is identified. The POPE data was lost when the graphic (Fig. 2B) was assembled in Adobe to create Figure 2. We re-draw the graphic and reassemble

the figure to solve this problem. Fig. 2B has also been modified to use micromolar for the concentration of the lipids.

> *(7) Readability point: I'd suggest some consistency in the concentrations mentioned. Making the concentrations either all molar-based or all percentage-based would make comparison across figures easier.*

As suggested, we have changed the % into micromolar concentrations in Fig. 2B and also in Fig. 3A.

> *(8) The model figure is quite difficult to interpret, particularly the final stage of the tail unfolding. I recommend the authors use a zoomed-in inset for this stage, or at least simplify the diagram by removing the non-participating lipid structures. The figure legend for the model figure should also have a brief description of the events and what the arrows mean, particularly the POPS□ PG arrow in the final panel of the figure. I am assuming here the authors are implying that daptomycin can transiently interact with one lipid species and move to another, but the arrow here suggests that daptomycin is moving through the lipid headgroup space.*

We really appreciate the suggestions. As suggested, we put an inset to show the preinsertion complex more clearly. In addition, we have removed the green arrows originally intended to show the re-organization/movement of the phospholipids. Moreover, the legend is changed to 'Proposed mechanism for the two-phased uptake of Dap into bacterial membrane. In the first phase, Dap reversibly binds to negative phospholipids with a hidden tail in the headgroup region, where it combines with two PG molecules to form a pre-insertion complex. In the second phase, the hidden tail unfolds and irreversibly inserts into the membrane. The inset shows the headgroup of the pre-insertion complex with the broad arrow showing the direction for the unfolding of the hidden tail. The red dots denote Ca2+.'

> *(9) The authors listed the Kd for daptomycin and 2 PG as 7.2 x 10-15 M2. Is this correct? This is an affinity in the femtomolar range.*

Please note that this Kd is for the simultaneous binding of two PG molecules, not for the binding of a single ligand that we usually refer to. Assuming that each PG contributes equally to this interaction, the binding affinity for each ligand is then the squared root of 7.2 x 10-15 M2, which equals to 8.5 x 10-8 M. This is equivalent to a nanomolar affinity for PG and is a reasonably high affinity.

> ***Reviewer #3 (Recommendations For The Authors):***
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> *(1) The authors reported an increase in daptomycin intensity with the increasing amount of negatively charged DMPG. A similar observation has been reported for GUVs, however, the authors did not refer to this paper in their manuscript: E. Krok, M. Stephan, R. Dimova, L. Piatkowski, Tunable biomimetic bacterial membranes from binary and ternary lipid mixtures and their application in antimicrobial testing, Biochim. Biophys. Acta - Biomembr. 1865 (2023) [1]. This paper is also consistent with the authors' observation that there is negligible fluorescence detected for the membranes composed of PC lipids upon exposure to the Dap treatment.*

As suggested, this paper is cited as ref. 29 in the revision by adding the following sentence at the end of the section 'Dependence of Dap uptake on phosphatidylglycerol.': 'PG-dependent increase of the steady-state fluorescence was also observed in giant unilamellar vesicles (GUVs).29'. The numbering is changed accordingly for the remaining references.

> *(2) Please include the plot of the steady-state Kyn fluorescence vs the content of POPA (Figure 2C shows traces for DMPG, CL, and POPS). Both POPA and POPS lipids are negatively charged, however, POPS seems to interact with Dap, while POPA does not. In my opinion, this observation is really interesting and might deserve a more thorough discussion. The authors might want to describe what could be the mechanism behind this lipid-specific mode of binding.*

As suggested, a plot is now added for POPA in Fig. 2C, which is basically a flat line without significant increase for the Kyn fluorescence. Indeed, the different effect of the negative phospholipids is very interesting, indicating that the reversible binding of Dap to the lipid surface is dependent not only on the Ca2+-mediated ionic interaction but also the structure of the headgroup. In other words, Dap recognizes the phospholipids at the surface binding stage. Considering this headgroup specificity, the last sentence in the second paragraph in "Discussion' is changed from 'In addition, due to the low lipid specificity, this reversible binding likely involves Ca2+-mediated ionic interaction between Dap and the phosphoryl moiety of the headgroups.' to 'In addition, due to the specificity for negative phospholipids (Fig. 2B and 2C), this reversible binding of Dap likely involves both a nonspecific Ca2+-mediated ionic interaction and a specific interaction with the remaining part of the headgroups.'

> *(3) The authors write that they propose a novel mechanism for the Ca2+-dependent insertion of Dap to the bacterial membrane, however, they rather ignored the already published findings and hypotheses regarding this process. In fact the role of Ca2+, as well as the proposed conformational changes of Dap, which allow its deeper insertion into the membrane are well known:*
>
> *The role of Ca2+ ions in the mechanism of binding is actually three-fold: (i) neutralization of daptomycin charge [2], (iii) creating the connection between lipids and daptomycin and (iii) inducing two daptomycin conformational changes. It should be noted that the interactions between calcium ions and daptomycin are 2-3 orders of magnitude stronger than between daptomycin and PG lipids [3,4]. Thus, upon the addition of CaCl2 to the solution, the divalent cations of calcium bind preferentially to the daptomycin, rather than to the negatively charged PG lipids, which results in the decrease of daptomycin net negative charge but also leads to its first conformational change [4]. Upon binding between calcium ions and two aspartate residues, the area of the hydrophobic surface increases, which allows the daptomycin to interact with the negatively charged membrane. In the next step, Ca2+ acts as a bridge connecting daptomycin with the anionic lipids. This event leads to the second conformational change, which enables deeper insertion of daptomycin into the lipid membrane and enables its fluorescence [4]. The overall mechanism has a sequential character, where the binding of daptomycin-Ca2+ complex to the negatively charged PG (or CA) occurs at the end.*
>
> *The authors should focus on emphasizing the novelty of their manuscript, keeping in mind the already published paper.*

We agree with the comments on the three general roles of calcium ion in the Dap interaction with membrane. The current investigation does not ignore the previous findings, which involve many more works than mentioned above, but takes these findings as common knowledge. Actually, the role of calcium ion is not the focus of current work. Instead, the current work focuses on how the drug is taken up and inserted into the membrane in the presence of the ion and how its structure changes in this process. With the known roles of calcium ion in mind, we propose an uptake mechanism (Fig. 6) that shows no conflict with the common knowledge.

We would like to point out that the 'deeper insertion into the membrane' in the comment is different from the membrane insertion referred to in our manuscript. This 'deeper insertion' still remains in the reversible stage of binding to the membrane surface because all negative phospholipids can do this (causing a conformational change and fluorescence increase, as quantified in Fig.2C) but now we know that only PG can enable irreversible membrane insertion because of our work. In addition, the comment that calcium binding to daptomycin causes first conformational change is not supported by our finding that no conformational change is found for Dap in the presence of calcium in a lipid-free environment (Fig. 3B). One important aspect of novelty and contribution of our work is to clear up some of these ambiguities in the literature. Another contribution of our work is to demonstrate the formation of a stable complex between Dap and PG with a defined stoichiometry and its crucial role in the drug uptake.

> *(4) One paragraph in the section "Ca2+- dependent interaction between Dap and DMPG" is devoted to a discussion of the formation of precipitate upon extraction of DMPG-containing micelles, exposed to Dap in the calcium-rich environment. Contrary, in the absence of Dap, no precipitate was detected. The authors did not provide any visual proof for their statement. Please include proper photographs in the supplementary information.*

The precipitate formed upon extraction of the DMPG-containing micelles was too little to be visually identifiable but could be collected by centrifugation and detected by fluorescence or HPLC after dissolving in DMSO. For visualization, we show below the precipitate formed using higher amount of Dap and DMPG. The Dap-DMPG-Ca2+ complex (left tube) was formed by mixing 1 mM Dap, 2 mM DMPG and 1 mM Ca2+ and the control (right tube) was a mixture of 2 mM DMPG and 1 mM Ca2+. This is now added as Fig. S7 in the supplementary information (the index is modified accordingly) and cited in the main text.

> *(5) The authors wrote that it is not clear how many calcium ions are bound to Dap-2PG complex (page 11, Discussion section). There are already reports discussing this issue. I recommend citing the paper discussing that exactly two Ca2+ ions bind to a single Dap molecule: R. Taylor, K. Butt, B. Scott, T. Zhang, J.K. Muraih, E. Mintzer, S. Taylor, M. Palmer, Two successive calcium-dependent transitions mediate membrane binding and oligomerization of daptomycin and the related antibiotic A54145, Biochim. Biophys. Acta - Biomembr. 1858, (2016) 1999-2005 [5]*

We were aware of the cited work that shows binding of two Ca2+ but also noted that there are more works showing one Ca2+ in the binding, such as the paper in [Ho, S. W., Jung, D., Calhoun, J. R., Lear, J. D., Okon, M., Scott, W. R. P., Hancock, R. E. W., & Straus, S. K. (2008), Effect of divalent cations on the structure of the antibiotic daptomycin. *European Biophysics Journal*, *37*(4), 421–433.]. That was the reason we said 'it is not clear how many calcium ions are bound to Dap-2PG complex'. Now, both papers are cited (as Ref. #33, 34) to support this statement.

> *(6) The authors wrote two contradictory statements:*
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> *- PG cannot be found in mammalian cell membranes:*
>
> *"Moreover, the complete dependence of the membrane insertion on PG also explains why Dap selectively attacks Gram-positive bacteria without affecting mammalian cells, because PG is present only in bacterial membrane but not in mammalian membrane. " (Page 10, Discussion section, last sentence of the first paragraph)*

> *"However, Dap absorbed on bacterial surface is continuously inserted into the acyl layer via formation of complex with PG in a time scale of minutes, whereas no irreversible insertion of Dap occurs on mammalian membrane due to the absence of PG while the bound Dap is continuously released to the circulation as the drug is depleted by the bacteria." (Page 13, Discussion section)*
>
> *- PG in trace amounts is present in mammalian membranes:*
>
> *"The proposed requirement of the pre-insertion quaternary complex increases the threshold of PG content for the membrane insertion to happen and thus makes it impossible on the surface of mammalian cells even if their plasma membrane contains a trace amount of PG." (Page 13, Discussion section).*
>
> *In fact, phosphatidylglycerol comprises 1-2 mol% of the mammalian cell membranes. Please, correct this information, which in this form is misleading to the readers.*

We appreciate the comments about the PG content in mammalian cells. Changes are made as listed below:

(1) p10, the sentence is changed to 'Moreover, the complete dependence of the membrane insertion on PG also explains why Dap selectively attacks Gram-positive bacteria without affecting mammalian cells, because PG is a major phospholipid in bacterial membrane but is a minor component in mammalian membrane.'

(2) p13, the sentence is changed to 'However, Dap absorbed on bacterial surface is continuously inserted into the acyl layer via formation of complex with PG in a time scale of minutes, whereas little irreversible insertion of Dap occurs on mammalian membrane due to the low content of PG while the bound Dap is continuously released to the circulation as the drug is depleted by the bacteria.'

(3) p13, another sentence is modified to 'The proposed requirement of the pre-insertion quaternary complex increases the threshold of PG content for the membrane insertion to happen and thus makes it less likely on the surface of mammalian cells that contain PG at a low level in the membrane.'

> *(7) Please include information that Dap is effective only against Gram-positive bacteria and does not show antimicrobial properties against Gram-negative strains. The authors focused on emphasizing that Dap does not affect mammalian membranes, most likely due to the low PG content, however even membranes of Gram-negative bacteria are not susceptible to the Dap, despite the relatively high content of negatively charged PG in the inner membrane (e.g. inner cell membrane of E. coli has ~20% PG).*

The requested information is already included in 'Introduction'. In this part, Dap is introduced to be only active against Gram-positive bacteria, implicating that it is not active against Gram-negative bacteria. The reason Dap is inactive against *E. coli* or other Gramnegative bacteria is because the outer membrane prevents the antibiotic from accessing the PG in the inner membrane to cause any harm. When the outer membrane is removed, Dap will also attack the plasma membrane of Gram-negative bacteria.

Literature cited in the comments:

(1) E. Krok, M. Stephan, R. Dimova, L. Piatkowski, Tunable biomimetic bacterial membranes from binary and ternary lipid mixtures and their application in antimicrobial testing, Biochim. Biophys. Acta - Biomembr. 1865 (2023). https://doi.org/10.1101/2023.02.12.528174.

(2) S.W. Ho, D. Jung, J.R. Calhoun, J.D. Lear, M. Okon, W.R.P. Scott, R.E.W. Hancock, S.K. Straus, Effect of divalent cations on the structure of the antibiotic daptomycin, Eur. Biophys. J. 37 (2008) 421-433. https://doi.org/10.1007/S00249-007-0227-2/METRICS.

(3) A. Pokorny, P.F. Almeida, The Antibiotic Peptide Daptomycin Functions by Reorganizing the Membrane, J. Membr. Biol. 254 (2021) 97-108. https://doi.org/10.1007/s00232-02100175-0.

(4) L. Robbel, M.A. Marahiel, Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery, J. Biol. Chem. 285 (2010) 2750127508. https://doi.org/10.1074/JBC.R110.128181.

(5) R. Taylor, K. Butt, B. Scott, T. Zhang, J.K. Muraih, E. Mintzer, S. Taylor, M. Palmer, Two successive calcium-dependent transitions mediate membrane binding and oligomerization of daptomycin and the related antibiotic A54145, Biochim. Biophys. Acta - Biomembr. 1858 (2016) 1999-2005. https://doi.org/10.1016/J.BBAMEM.2016.05.020.

https://doi.org/10.7554/eLife.93267.2.sa0