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On the quest for the elusive mechanism of action of daptomycin: Binding, fusion, and oligomerization

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ABSTRACT

Daptomycin, sold under the trade name CUBICIN, is the first lipopeptide antibiotic to be approved for use against Gram-positive organisms, including a number of highly resistant species. Over the last few decades, a number of studies have tried to pinpoint the mechanism of action of daptomycin. These proposed modes of action often have points in common (e.g. the requirement for Ca^{2+} and lipid membranes containing a high proportion of phosphatidylglycerol (PG) headgroups), but also points of divergence (e.g. oligomerization in solution and in membranes, membrane perturbation vs. inhibition of cell envelope synthesis). In this study, we investigate how concentration effects may have an impact on the interpretation of the biophysical data used to support a given mechanism of action. Results obtained from small angle neutron scattering (SANS) experiments and molecular dynamics (MD) simulations show that daptomycin oligomerizes at high concentrations (both with and without Ca^{2+}) in solution, but that this oligomer readily falls apart. Photon correlation spectroscopy (PCS) experiments demonstrate that daptomycin causes fusion more readily in DMPC/PG membranes than in POPC/PG, suggesting that the latter may be a better model system. Finally, fluorescence and Förster resonance energy transfer (FRET) experiments reveal that daptomycin binds strongly to the lipid membrane and that oligomerization occurs in a concentration-dependent manner. The combined experiments provide an improved framework for more general and rigorous biophysical studies toward understanding the elusive mechanism of action of daptomycin. This article is part of a Special Issue entitled: Biophysics in Canada, edited by Lewis Kay, John Baenziger, Albert Berghuis and Peter Tieleman.

1. Introduction

Daptomycin is a lipopeptide antibiotic composed of 13 amino acid residues (Fig. 1), approved in 2003 for use against Gram-positive organisms, including a number of highly resistant species [1–3]. Among the amino acid residues are three D-amino acids (D-asparagine, D-alanine, and D-serine) and three uncommon amino acids, the latter including ornithine, (2*S*,3*R*)-3-methyl-glutamic acid, and kynurenine. The N-terminus of daptomycin is acylated with a *n*-decanoyl fatty acid chain [4]. Daptomycin is one of the few available antibiotics that are effective against many resistant bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) [5], vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-resistant *Staphylococcus aureus* (VRSA), and vancomycin-resistant *Enterococci* (VRE) [6]. Its antimicrobial activity is entirely dependent on the presence of calcium ions (Ca^{2+}) and lipids with negatively charged headgroups (e.g., phosphatidylglycerol (PG), as found in bacteria) [1–3,7–9]. Despite the widespread use of daptomycin in hospital and clinical settings, the complete mechanism of its action is still not fully understood and is hence the subject of recent investigations [3,10–13].

Over the last few decades, several biophysical and bacterial cell studies have helped to piece together key steps in the mode of action of daptomycin. Early clues pointed to daptomycin's ability to affect peptidoglycan biosynthesis [14,15], but this was then refuted [16], only for blockage of cell wall synthesis to gain in importance again recently [3]. Likewise, it was suggested that daptomycin functions by membrane depolarization [7] or pore formation [17], but evidence to the contrary has also been published [3,18–21]. Finally, other studies have shown that daptomycin causes membrane deformation, leading to aberrant recruitment of cell-wall proteins such as DivIVA [22]. But again, other evidence [3] suggests otherwise. As recently discussed [3], this diversity of explanations regarding the mode of action of daptomycin may

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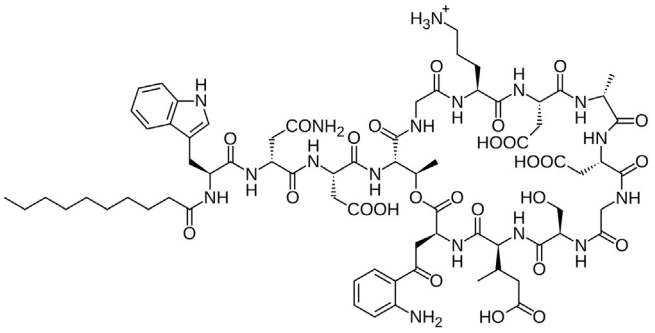

Fig. 1. Chemical structure of daptomycin, drawn at acidic pH. At neutral pH, the 3 Asp and 1 mGlu have negatively charged side-chains, resulting in a net −3 charge for daptomycin.

be linked to the variety of methods used to elucidate the mechanism. As with all scientific endeavours, every experiment has led to new models being proposed and a deeper understanding of how this lipopeptide antibiotic might function. However, one key element, which also plays a part, particularly in biophysical studies, is daptomycin's concentration-dependent properties—namely its ability to form aggregates in solution and in membranes, and under particular conditions, to cause membrane fusion [23].

The propensity for daptomycin to form aggregates in solution was first discovered when groups tried to solve its structure by NMR [24–26]. Indeed, Ball et al. [24] and Rotondi and Gierasch [26] found sample conditions where line-broadening in the ^{1}H NMR spectra was evident. Hence, Rotondi and Gierasch [26] carefully optimized sample preparation conditions to produce an aggregate-free sample through the slow addition of daptomycin to a well-degassed solution of 10 mM sodium phosphate buffer, pH 5.3 [26]. As detailed in the recent review by Taylor and Palmer [2], it is the ionization state of daptomycin that plays a role, with samples prepared at neutral pH [25,27] showing no or minimal aggregation, if the daptomycin concentration is at millimolar concentrations or lower. Upon addition of Ca^{2+}, Ball et al. [24] and Jung et al. [25] both demonstrated that line-broadening occurs, suggesting aggregation of the sample. Nevertheless, Jung et al. (and not Straus et al., as stated by Taylor and Palmer [2]) determined the structure of a Ca^{2+}-bound form of daptomycin, assuming that all long-range Nuclear Overhauser Effect distance restraints (NOEs) found were intramolecular. It was Buncókzi et al. [28] who then first suggested that some of these NOEs might be intermolecular. Ho et al. [29] then re-analyzed the Ca^{2+}-bound structure of daptomycin and also investigated the aggregate formed in detail. Using analytical ultracentrifugation [29], they[1] found that daptomycin forms aggregates consisting of 14–16 daptomycin molecules in the presence of one equivalent of Ca^{2+}. This finding led to the suggestion that daptomycin forms a micellar structure and that this aggregated form may be important for the mode of action of daptomycin [1,24]. This view was further supported by fluorescence measurements of daptomycin (at concentration of 60 μM and above, in the presence of 1 mM Ca^{2+}) [27]. However, other fluorescence measurements by Muraih et al. [12] with daptomycin concentrations of 5–10 μM (i.e. close to the minimal inhibitory concentration (MIC) of 0.02–5 μM) and calcium suggest that solution-phase oligomerization is not a key step in the mechanism of action of daptomycin.

As mentioned above, daptomycin has been found to cause membrane fusion under certain conditions [23]. Since a peptidoglycan layer surrounds Gram-positive bacteria, the primary targets of daptomycin, membrane fusion is unlikely to be important in the mechanism of action of daptomycin. It could, however, have an impact on the interpretation of biophysical data used to elucidate the mode of action of daptomycin (Fig. 2). For instance, if one wants to determine how daptomycin binds (steps 1–2) and oligomerizes (steps 3–4) in the membrane (Fig. 2A), then fusion (Fig. 2B, shown for the hemifusion step here only) could introduce anomalies or lead to erroneous interpretation of data. Previous work by Jung et al. [23] showed that fusion occurs only when a combination of daptomycin, Ca^{2+} and a phosphatidylglycerol-containing model membrane is used. Interestingly, this phenomenon occurs for low concentrations of daptomycin that are close to its MIC (i.e. 8 μg/mL or 5 μM). Recently, a number of fluorescence studies [10–12] proposed that daptomycin forms well-defined membrane-bound oligomers consisting of 6 or 7 daptomycin molecules, with Ca^{2+} binding occurring in two steps [10]. These biophysical studies used conditions similar to those in Jung et al. where fusion occurred [23], leading us to question whether the choice of conditions has an impact on the resulting model.

In this work, we have examined daptomycin's concentration-dependent properties in solution and in model membranes. To assess the potential importance of solution-phase micellar structures of daptomycin, we have used a unique combined approach of small angle neutron scattering (SANS) and molecular dynamics (MD) simulation in order to probe the size and shape of the oligomer and the packing of the daptomycin molecules within it. Unrestrained MD simulations of the oligomer provide further insight into the evolution of the micellar structure under conditions where it is diluted. Aggregation was also tested in solution in the presence of 150 mM NaCl using ^{1}H NMR spectroscopy. We have also systematically determined conditions under which fusion occurs using photon correlation spectroscopy (PCS), and whether or not the fusion state persisted after dilution, for a number of model membrane systems: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG) (50%/50%), 1:1 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG) (50%/50%), POPC/POPG/cardiolipin (CL) (40%/50%/10%), and finally POPC alone (100%). Fluorescence experiments were conducted to quantify the binding of daptomycin to DMPC/PG and POPC/POPG membranes under conditions where fusion does not occur. Finally, FRET experiments were conducted using daptomycin and NBD-daptomycin, again under conditions where there is no fusion, to investigate if daptomycin forms oligomers in a concentration-dependent manner. Taken together, these experiments provide an improved framework for more general and rigorous biophysical studies toward understanding the elusive mechanism of action of daptomycin, and are discussed in this context.

2. Materials and methods

2.1. Materials

Daptomycin was a generous gift from Cubist Pharmaceuticals (Lexington, MA). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG), 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (CL) were from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. 4-Chloro-7-nitrobenzofurazan (NBD-Cl), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and acetonitrile were from Sigma-Aldrich (St. Louis, MO). Sodium borate buffer (0.5 M, pH 8.0) was obtained from Alfa Aesar (Ward Hill, MA). Acetic acid, ammonium acetate, and ethylenediaminetetraacetic acid (EDTA) were from Fisher Scientific (Fair Lawn, NJ).

2.2. Preparation of NDB-daptomycin

Daptomycin was labeled with NBD at the ornithine side-chain (6th residue, Fig. 1), as reported by Muraih et al. [12]. Daptomycin

[1] It should be noted that in Taylor and Palmer, the ultracentrifugation data is referenced as being reported in Jung et al. [23], but it was reported in Ho et al. [29].