Daptomycin – A New Twist To An Old Tale

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The lipopeptide antibiotic daptomycin, naturally produced by a nonribosomal peptide synthetase (NRPS) in the soil actinomycete *Streptococcus roseosporus*, was discovered by scientists at Eli Lilly in the early 1980s. It is now used as a front-line antibiotic for severe or deep-seated infections caused by Gram-positive bacteria, including multiple drug resistant (MDR) organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) and penicillin-resistant *Streptococcus pneumoniae*. Despite the importance of daptomycin in the current weaponry to fight bacterial pathogens, the history of its development and introduction to the clinic is tortuous and its mode of action has proven elusive until recently.

DISCOVERY AND CLINICAL DEVELOPMENT

The potential of daptomycin was realised early on by Eli Lilly, who conducted Phase I and II trials during the late 1980s in patients suffering from complicated skin and skin-structure infection (SSSI). However, due to manifestation of severe musculoskeletal adverse side effects in several healthy volunteers in the Phase I trial, Eli Lilly discontinued further development of daptomycin. Cubist Pharmaceuticals subsequently took up clinical development of daptomycin following an in-licensing agreement with Eli Lilly in 1997 (for the fascinating background to this see Eisenstein et al, 2010). Scientists at Cubist believed that the excellent therapeutic prospects of daptomycin warranted efforts to overcome the toxicity problems. Very soon after in-licensing it became apparent that there existed a pressing need to fight the emerging and increasing threat of MRSA and VRE. Cubist therefore decided to develop an intravenous (i.v.) formulation of daptomycin for the treatment of serious Gram-positive infections including complicated SSSI and bacteremia with suspected infective endocarditis. Carefully-designed pre-clinical studies yielded the surprising result that a once daily i.v. dose of daptomycin was optimal and resulted in less side effects than a twice daily fractionated treatment regimen. Thence, clinical trials with daptomycin were designed based on a once-daily treatment regimen. FDA approval for daptomycin for injection ('Cubicin') was obtained in 2003, for the treatment of complicated SSSI caused by specific Gram-positive bacteria and in 2006, for the treatment of *S.aureus* bacteremia, including right-sided infective endocarditis; approval by the European regulatory agency for the same indications was obtained in 2006.

Since its launch in 2003, daptomycin has proven to be the most successful i.v. antibiotic in terms of sales revenues in US dollars.

CHEMICAL STRUCTURE AND SEQUENCE

Daptomycin is a 13-residue anionic lipopeptide cyclized through an ester linkage between the side-chain hydroxyl of T4 and the backbone carboxyl group of the C-terminal kynurenine (Kyn13) residue. As a result, the daptomycin primary structure is a 10-residue ring with a three-residue tail (Figure 1). Daptomycin contains a number of unusual amino acids including ornithine (O), (2S,3R)-3 methylglutamic acid (mE), and kynurenine (Kyn) [3-(2-aminobenzoyl)-L-alanine], as well as three D-amino acids, and has the sequence ^{1}WDNDTGODDADGDSmEKyn^{13}. A defining characteristic of the lipopeptide family of antibiotics, along with ring and tail structure, is a lipid N-cap on residue 1. In daptomycin, the N-terminal W residue is capped by a decanoyl aliphatic chain (Rotondi and Gierasch, 2005).

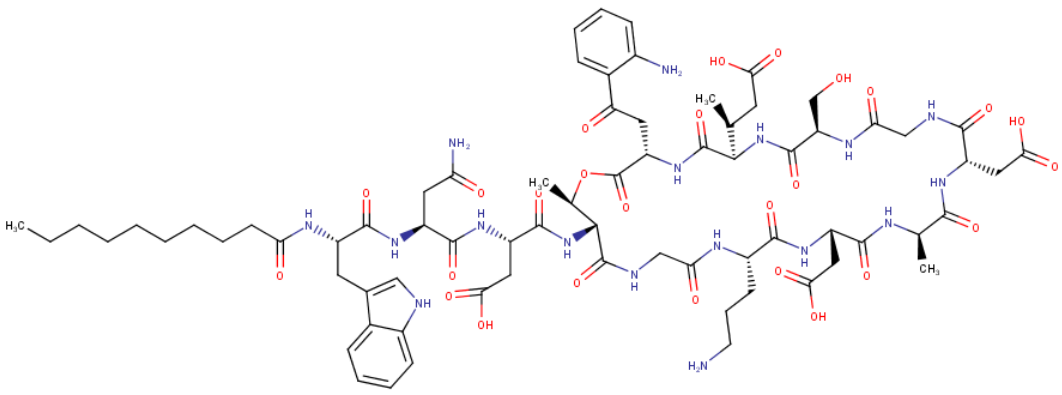

Figure 1. Chemical structure of daptomycin - note the large peptide part and short acyl tail (Depicted using MarvinSketch [ChemAxon Ltd.]).

MECHANISM OF ACTION

Scientists have been puzzling over the mechanism of action of daptomycin since its discovery. It was clear that daptomycin acted on the cell envelope of susceptible bacteria. Initial research at Eli Lilly suggested that daptomycin interfered with peptidoglycan synthesis (Allen et al, 1987). Another early study demonstrated that daptomycin inhibited the synthesis of lipoteichoic acid (Canepori et al, 1990); however, other researchers failed to reproduce these results for *S.aureus* and *Enterococcus faecalis*.

Yet another hypothesis was that daptomycin caused loss of membrane potential and thus general disruption of cell function. Loss of membrane potential following formation of pores in the bacterial cell membrane is a mechanism observed for other antimicrobial peptides, such as nisin (Ageitos et al, 2016).

In 2003, researchers at Cubist, having shown a correlation between loss of membrane potential and bactericidal effect of daptomycin, proposed a multistep model for its mechanism of action. They proposed that following Ca^{2+}-dependent insertion into the bacterial cytoplasmic membrane, daptomycin forms oligomers, resulting in subsequent disruption of membrane integrity, release of intracellular ions and eventually cell death (Silverman et al, 2003). The slow depletion of membrane potential observed with daptomycin compared to the almost immediate loss of membrane potential seen with other antimicrobial peptides which cause pore formation, suggested a novel mechanism for daptomycin.

Over the years, a variety of experiments gave rise to different models for the mechanism of action of daptomycin including, i) blockage of cell wall synthesis; ii) bacterial membrane pore formation; iii) generation of altered membrane curvature resulting in an aberrant recruitment of essential cell-envelope proteins.

In the meantime a number of NMR-determined structures became available for daptomycin, providing structural insights into its mode of action, although the results from different groups were not entirely compatible. One study of daptomycin in aqueous solution, in the absence of calcium, revealed that the cyclic portion of the peptide adopts a bent hairpin conformation with two transannular hydrogen bonds and a type II beta-turn centered at residues ^{7}DDADG^{10} (Rotondi & Gierasch, 2005). Two of the four negatively charged residues in daptomycin are in the beta-turn, and the flanking residues are predominantly polar. The other end of the hairpin, bridged by the side-chain– backbone ester linkage of T4 and Kyn13, forms a hydrophobic cluster composed of the side chains of W1, Kyn13, and the lipid N-cap (Figure 2). The amphipathic nature of daptomycin predicted that the hydrophobic end of the molecule could bury itself in the membrane bilayer. The structural studies led to the proposal that daptomycin inserts its lipid tail between fatty acyl chains of the membrane bilayer and association with Ca^{2+} reduces excess negative charge, allowing oligomerization (Jung et al, 2004). The increased affinity of the Ca^{2+}-daptomycin complex for negatively charged phospholipids, including phosphotidylglycerol, found in abundance in bacterial cell membranes, corroborates this mechanism.

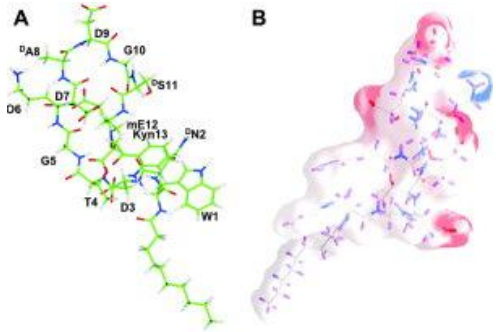

Figure 2. A. All-atom structure of the best structure of daptomycin. B. Surface of the structure in A colored by electrostatic potential. The clustering of charge in the beta-turn end of the hairpin is in contrast to the uncharged hydrophobic tail end of daptomycin (Rotondi and Gierasch, 2005).

The study by Müller et al (2016), reported recently in PNAS, finally brings clarification to daptomycin's mode of action. In this thorough and systematic study using the model organism *Bacillus subtilis*, the effects of daptomycin on cell wall synthesis, membrane potential, potassium efflux, membrane pore formation and curvature were revisited.

The inhibitory effect of daptomycin on cell wall synthesis, previously reported for *S.aureus* and *Enterococcus faecium*, was demonstrated for *B.subtilis* and further scrutinised by comparing the proteome stress response of daptomycin-treated cells with a proteome reference library containing the unique stress profiles of B. subtilis treated with more than 60 different antibiotics. Five proteins showed twofold or higher upregulation (marker proteins) in daptomycin treated bacteria, three of which are connected to membrane-associated lipid biosynthesis. On the other hand, proteins normally upregulated following treatment with other cell wall synthesis inhibiting or membrane-disrupting antibiotics were not found in the daptomycin proteome stress profiles. Neither did the proteome profile of daptomycin match those of pore-forming antimicrobials such as nisin. The proteome profile of daptomycin was unique – suggesting a novel mode of action.

The question of daptomycin-induced potassium efflux was examined using inductively coupled plasma optical emission spectroscopy (ICP-OES) to measure intracellular element concentration. No reduction in the the levels of K^+ or any other element could be demonstrated following application of daptomycin, although leakage of K^+ was seen with the specific potassium ionophore valinomycin.

The fluorescence potentiometric probe DiSC3 was used to follow dissipation of membrane potential. Only the highest concentration of daptomycin caused gradual dissipation of membrane potential, complete after 30 minutes, in stark contrast to the immediate drop in potential brought about by the membrane-disrupting peptide gramicidin. Uptake of the large fluorescent dye propidium iodide, an indicator of pore formation, was not observed after daptomycin treatment.

Localization of GFP-tagged proteins monitored by fluorescence light microscopy revealed a gradual delocalization of proteins involved in cell division and cell shape determination following daptomycin exposure. Furthermore, localization of the transmembrane ATPase complex used as an indicator of abnormal cell shape owing to its uniform membrane distribution, revealed no perturbance in the normal distribution of this protein in the presence of daptomycin. On the basis of this observation it was concluded that previous reports of daptomycin-induced membrane curvature could have been due to artefacts caused by GFP dimerisation.

The stunning finding of this work was that in the presence of certain fluorescent membrane dyes, daptomycin brought about the formation of large fluorescent membrane patches. Using a lipid-mimicking dye which localizes to fluid lipid regions, it was demonstrated that these fluorescent patches were enriched in fluid lipids and that daptomycin caused clustering and redistribution of fluid lipids in the membrane, an effect barely perceptible when bacteria were tested with other antibiotics targeting the cell envelope. Using the membrane fluidity-sensitive dye laurdan, it was shown that within two minutes of exposure to daptomycin a redistribution of regions of increased fluidity (RIFs) had occurred, resulting in overall increase in membrane rigidity.

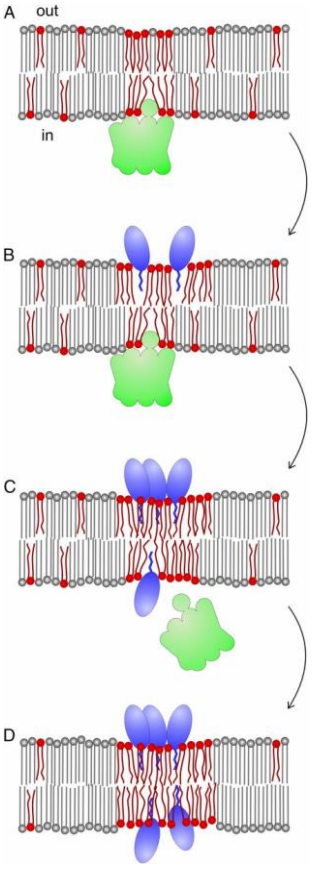

Figure 3. Model of daptomycin interaction with fluid lipid domains. (A) Peripheral membrane proteins involved in cell wall (e.g., MurG) and lipid (e.g., PlsX) synthesis localize to RIFs indicated by a high concentration of fluid lipids (red). "Out" and "in" represent the cell wall side and the cytoplasmic side, respectively. (B) Daptomycin,, owing to its bulky nature inserts into the bacterial membrane with preference for regions of high fluid lipid content, such as RIFs. The lipids are forced apart creating bilayer distortions and attracting fluid lipids to the sites of daptomycin insertion to compensate for gaps in the lipid bilayer. (C) Ca^{2+} ions stimulate daptomycin oligomerization, exacerbating this effect. This attraction occurs first in the outer membrane leaflet and then causes a similar redistribution of lipids in the inner leaflet, facilitating the flipping of daptomycin molecules through the bilayer to the inner leaflet. Consequently, peripheral membrane proteins are displaced from RIFs. (D) Daptomycin blocks access to fluid lipids in the inner leaflet, which are required for the association of these proteins, and clusters them into inflexible domains, resulting in the withdrawal of fluid lipids from the bulk and overall increased membrane rigidity (Müller et al, 2016).

One question remained: how does daptomycin affect cell wall synthesis. The authors answered this question by first making the astute conjecture that in binding to fluid lipids and reducing their disorder, daptomycin might interfere with the attachment of peripheral membrane proteins to the membrane. Indeed one peripheral membrane protein, the N-acetylglucosamine transferase, MurG, responsible for the last synthesis step of the peptidoglycan precursor, lipid II, became immediately dissociated from the membrane less than two minutes after addition of daptomycin. Another peripheral membrane protein, PlsX, involved in phospholipid synthesis showed similar rapid dissociation from the cell membrane upon daptomycin exposure.

Müller and co-workers concluded that daptomycin does not cause formation of membrane pores or rapid K^+ efflux, neither does it cause membrane curvature. Daptomycin acts on the cell membrane by bringing about a clustering of fluid lipids which in turn increases membrane rigidity and causes delocalization of peripheral membrane proteins MurG and PlsX, which are involved in peptidoglycan and phospholipid synthesis. As a result of these observations, a revised daptomycin working model was proposed (Figure 3). According to this model, daptomycin, upon reaching the bacterial cell membrane, inserts its short lipid tail (C_{10}) between the fatty acyl chains of phospholipid molecules, its large peptide ring structure causing gross disturbance to their regular packing. Ca^{2+}-dependent oligomerization further distorts the membrane bilayer. Owing to its bulky structure, daptomycin will be accommodated preferably in regions of increased fluidity, RIFs. Oligomerization exacerbates this effect. Daptomycin has even been shown to flip from one membrane leaflet to the other. Thus daptomycin has a dramatic effect on the fluid lipid order/disorder balance and biophysical properties of the bacterial cell membrane and this is the basis of its bactericidal effect, a mode of antimicrobial action described for the first time by Müller and colleagues.

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