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Activity of daptomycin on biofilms produced on a plastic support by *Staphylococcus* spp.

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Abstract

The aim of this study was to assess whether the novel lipopeptide daptomycin might be capable of disrupting or inhibiting the synthesis of biofilms produced by staphylococci. Fourteen recently isolated slime-producing methicillin-susceptible (MET-S) and methicillin-resistant (MET-R) strains (three MET-S *Staphylococcus aureus*, three MET-R *S. aureus*, three MET-S *Staphylococcus epidermidis,* three MET-R *S. epidermidis* and two vancomycin-intermediate *S. aureus* (VISA)) were tested. Slime formation on polystyrene plates was quantified spectrophotometrically. Daptomycin (2–64 mg/L) inhibited slime synthesis by ≥80% in MET-S strains, by 60–80% in MET-R *S. aureus* and by 70–95% in MET-R *S. epidermidis*. At 64 mg/L, biofilm synthesis decreased by 80% in the VISA isolates. Daptomycin also disrupted pre-formed biofilm: >50% breakdown of initial biofilm (5 h) was observed in all strains. Disruption of mature biofilms (48 h), in terms of percentage, was more variable depending on the strain, ranging from ca. 20% in a MET-R *S. epidermidis* strain to almost 70% in two MET-S strains (one *S. aureus* and one *S. epidermidis*). Daptomycin at concentrations achievable during therapy promoted a statistically significant inhibition of slime synthesis (preventing biofilm building) and induced slime disruption (disaggregating its structure) both in initial and mature biofilms on a plastic support in all staphylococcal strains studied.

Keywords: Slime; Staphylococci; Lipopeptide

1. Introduction

The use of surgically implanted devices has increased in almost all fields of medicine as a result of their beneficial effect on quality of life and, in some circumstances, on patient survival rates. However, they can be associated with a variety of complications, the worst being the risk of bacterial and fungal infections. Development of device-related infections begins with colonisation of the foreign material, followed by a complex physiological metamorphosis by the microorganisms resulting in biofilm formation [1–4]. Biofilm represents a structured community of bacterial cells embedded in a self-produced polymeric matrix adherent to the artificial surface. In this peculiar form, bacteria display several altered phenotypic properties. These changes, in conjunction with the protective layer provided by the biofilm, render the success of antimicrobial therapy, even if prolonged, uncertain. In fact, antibiotic treatment frequently fails to eradicate these infections despite the use of drugs with proven in vitro activity [5–7]. The host defence mechanisms also appear to be unable to eliminate pathogens from an implanted device. Surgical removal of implanted devices is often required, with all the underlying serious costs for the patient [2,8]. Whilst a variety of microorganisms may be involved as pathogens, staphylococci are responsible for the majority of medical device-related infections. The most important feature of the pathogenic process is their ability to adhere to plastic materials and to promote formation of a biofilm [3,8–10].

It may be safely assumed that compounds capable of inhibiting the synthesis of slime and of disrupting the biofilms formed by pathogens commonly involved in natural or device-associated infections (*Staphylococcus aureus* and *Staphylococcus epidermidis* among the Gram-positive species) may offer distinct advantages over molecules not endowed with these abilities. In this regard, it must be underlined that telavancin, a new molecule belonging to

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the chemical class of lipoglycopeptides, was recently shown to be more effective than the available glycopeptides, vancomycin and teicoplanin, and was the most active agent against staphylococcal biofilms, including those formed by vancomycin-intermediate *S. aureus* (VISA) strains [11]. Telavancin has a dual mechanism of action that involves not only the inhibition of cell wall synthesis but also the disruption of bacterial cell membrane barrier functions [12]. Exposure to telavancin resulted in increased membrane permeability, leakage of intracellular ATP and K^+ ions, and rapid dissipation of the cell membrane potential [13]. This ability of telavancin to produce membrane depolarisation presents a parallelism with the mode of action of daptomycin [14]. On the other hand, daptomycin has also demonstrated superior activity against biofilms synthesised by *Enterococcus faecium* isolates resistant to vancomycin compared with minocycline, quinupristin/dalfopristin and linezolid [15]. The aim of this study was to assess the in vitro activity of the novel lipopeptide daptomycin against staphylococcal biofilms using an in vitro model already successfully applied to other experimental conditions [16].

2. Materials and methods

2.1. Microorganisms

In this study, three strains each of methicillin-susceptible (MET-S) and methicillin-resistant (MET-R) *S. aureus* and *S. epidermidis* recently isolated from clinical specimens, identified according to standard procedures [17] and shown to be capable of slime production by the Congo red agar plate test described by Freeman et al. [18], were tested. Two VISA strains, previously described by our group [19], were also included.

2.2. Drug

Preparation of sterile stock solutions of daptomycin (Chiron Pharmaceuticals, Milan, Italy) was performed according to the manufacturer's instructions. The concentrations of antibiotic used in these experiments corresponded to plasma concentrations achievable in humans [20].

2.3. Susceptibility tests

Minimal inhibitory concentrations (MICs) of daptomycin were determined following the microdilution procedure suggested by the Clinical and Laboratory Standards Institute [21] using cation-adjusted Mueller–Hinton (MH) broth (Biolife, Milan, Italy) supplemented with 50 mg/L calcium as the test medium.

2.4. Biofilm production

The presence and extent of biofilm formation was quantified spectrophotometrically using a method based on that reported by Cramton et al. [22] and as previously reported by this group [16,23]. To produce biofilms, 25 μL of stationary growth phase bacterial cultures were added aseptically to a well of a 96-well, polystyrene, tissue culture plate (Corning, Milan, Italy) containing 175 μL of cation-adjusted MH broth supplemented with calcium (50 mg/L). Biofilms were obtained (at 35 °C) after 5–6 h (initial biofilm) or 48 h (mature biofilm), with the growth medium discarded and replaced every 12 h.

To evaluate the effect of daptomycin on biofilm synthesis and slime disruption, the same procedures were followed with the exception that the drug was added to the growth medium. Each well was washed three times with phosphate-buffered saline (PBS) under aseptic conditions to eliminate unbound bacteria and the required concentration of daptomycin in growth medium was added. After 24 h exposure, drug solutions were discarded and each well was washed three times with PBS. Adherent microorganisms were fixed with Bouin's solution and stained with crystal violet. Excess stain was rinsed off with running tap water and the plates were dried. Adherent bacterial films were quantified spectrophotometrically by determining the optical density at 570 nm ($OD_{570\ nm}$). To calculate the percentage of biofilm formed, the $OD_{570\ nm}$ value obtained for each strain without drug was used as the control. The percentage of biofilm formed in the presence of different concentrations of drug was calculated employing the ratio between the values of $OD_{570\ nm}$ with and without drug, adopting the following formula: [($OD_{570\ nm}$ with drug/$OD_{570\ nm}$ without drug) × 100], as previously described [16]. The results reported were derived from three separate experiments and $OD_{570\ nm}$ values were expressed as mean ± standard deviation.

2.5. Statistical analysis

Student's *t*-test was employed to evaluate any significant differences between the $OD_{570\ nm}$ values obtained without drug (controls) and those observed in the presence of different drug concentrations. A paired *t*-test was used to compare the mean between each control/drug-treated group. Differences were considered statistically significant at $P < 0.05$. Analysis was performed using SPSS statistical software.

2.6. Counting of viable cells

After 24 h exposure to daptomycin, bacteria were harvested from the surface of each well. Daptomycin-containing growth medium was discarded and the wells were washed three times and filled with PBS. The surfaces of the wells were vigorously scraped with a sterile bacteriological loop, as previously described [16,23]. Plates were placed in a sonicator bath for 15–30 min to aid dissolution of bacterial clumps. The number of viable cells was estimated by plate count after transferring 100 μL of the mixture and further dilutions to