MAJOR ARTICLE

Inhibition of Daptomycin by Pulmonary Surfactant: In Vitro Modeling and Clinical Impact

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The lipopeptide daptomycin has been approved for use in skin and skin-structure infections but has failed to meet statistical noninferiority criteria in a clinical trial for severe community-acquired pneumonia. Daptomycin exhibited an unusual pattern of activity in pulmonary animal models: efficacy in *Staphylococcus aureus* hematogenous pneumonia and inhalation anthrax but no activity against *Streptococcus pneumoniae* in simple bronchial-alveolar pneumonia. Daptomycin was shown to interact in vitro with pulmonary surfactant, resulting in inhibition of antibacterial activity. This effect was specific to daptomycin and consistent with its known mechanism of action. This represents the first example of organ-specific inhibition of an antibiotic.

It is recognized that the pharmacology of the lung differs from that of the rest of the body and that this difference can affect antibiotic efficacy in the treatment of pneumonia [1]. Research has focused on antibiotic penetration into the lungs and, specifically, epithelial lining fluid (ELF), as well as on accumulation within alveolar macrophages [2]. Less attention has been paid to unique aspects of lung physiology, including pulmonary surfactant, a primary component of ELF. Surfactant is a complex lipid-and-protein mixture that coats the interior surface of the airway, reducing surface tension within the alveoli [3]. Antibiotic concentrations within ELF have been routinely measured, but the effect of surfactant on antibiotic function has rarely been investigated [4, 5]. We report on the selective inhibition of an antibiotic by surfactant, resulting in lack of efficacy in a specific clinical indication, bronchial-alveolar pneumonia (BAP).

Daptomycin is the first approved member of a new class of antibiotics, the lipopeptides [6]. It is rapidly bactericidal against gram-positive bacteria, including antibiotic-resistant isolates. The activity of daptomycin is strictly dependent on the presence of physiological levels of free calcium. Daptomycin has a novel mechanism of action—insertion into and disruption of the functional integrity of the G^+ plasma membrane—which results in rapid loss of membrane potential, cessation of macromolecular synthesis, and cell death [7].

Daptomycin was approved in September 2003 for treatment of complicated skin and skin-structure infections. Phase 3 clinical trials were also conducted for the treatment of hospitalized patients with community-acquired pneumonia (CAP). Despite potent bactericidal activity (MIC_{90}, 0.06 μg/mL) against *Streptococcus pneumoniae,* the most common gram-positive pathogen in this indication, daptomycin (4 mg/kg every 24 h) failed to achieve statistical noninferiority against the comparator, ceftriaxone (2 g every 24 h). Clinical efficacy in the pneumonia trial was 79% for daptomycin and 87% for ceftriaxone. The potential reasons for this outcome were investigated in a series of rodent-infection models, leading to the development of a novel in vitro susceptibility assay designed to better mimic the physiology of the lung.

METHODS

Animal experiments were performed in the Cubist Pharmaceuticals vivarium in accordance with guidelines cre-

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Table 1. Daptomycin efficacy in pulmonary infection models.

Model	Organism	Daptomycin		Comparator	
		Dose, mg/kg	Log reduction	Drug (dose, mg/kg)	Log reduction
Mouse BAP	*S. pneumoniae*	100	0.1 ± 0.13	Ceftriaxone (50)	4.5 ± 0.28
Mouse BAP	MRSA	100	0 ± 0.4	…	…
Rat HP	MRSA	50	2.1 ± 0.56	Vancomycin (100)	1.3 ± 1.19
Rat HP	*S. aureus*	75	2.2 ± 1.0	Nafcillin (150)	1.5 ± 0.62

NOTE. Data are expressed as reduction in bacterial burden in the infected organ, compared with that in untreated, infected controls. For bronchial-alveolar pneumonia (BAP), mice were treated at 1 and 4 h after infection, and lungs were harvested 24 h after infection. For hematogenous pneumonia (HP), rats were treated once daily (daptomycin and vancomycin) or twice daily (nafcillin) for 6 days after infection, and lungs were harvested on day 7. MRSA, methicillin-resistant *Staphylococcus aureus*; *S. pneumoniae, Staphylococcus pneumoniae*.

ated by the Cubist Pharmaceuticals Animal Care and Use Committee. For BAP, CD-1 mice (Charles River Laboratories) were infected intranasally with 1×10^8 cfu of *S. pneumoniae* (ATCC 6303) or *Staphylococcus aureus* (ATCC 43300). Antibiotic treatment was provided at 1 and 4 h after infection, by subcutaneous injection. At 24 h after inoculation, mice were killed by CO_2 asphyxiation. Lungs were removed aseptically and homogenized in sterile, distilled water. Homogenates were serially diluted and plated on tryptic soy agar supplemented with 5% lysed sheep blood. The bacterial burden was measured as colony-forming units per gram of lung tissue. Hematogeneous pneumonia infections were produced, as described by Sawai et al. [8]. Rats were treated on days 1–6 after infection; on day 7, rats were killed, and the bacterial burden was determined as above.

S. aureus ATCC 29213 was used for all susceptibility testing. MICs were determined by use of Mueller-Hinton broth supplemented with 50 mg/L Ca^{2+} (MHBc). Assays were performed in accordance with NCCLS methodologies [9], except that cultures were incubated at 37°C with agitation. For surfactant MIC testing, MHBc was supplemented with Survanta (Beractant; Ross Laboratories) to the indicated concentration; all other conditions were as above. Survanta contains 25 mg/mL phospholipids, including 11.0–15.5 mg/mL disaturated phosphatidylcholine (PC), 0.5–1.75 mg/mL triglycerides, 1.4–3.5 mg/mL free fatty acids, and <1.0 mg/mL protein in 0.9% NaCl.

For insertion studies, surfactant was added, to a final concentration of 1%, to 5 mmol/L HEPES–0.2% glucose (pH 7.4), in a polystyrene fluorimeter cuvette (VWR International) containing a stir bar, and was placed in the heated (37°C) sample chamber of an AMINCO-Bowman Series 2 luminescence fluorimeter (Thermo Spectronic). Samples were excited at 385 nm, and emission was measured at 400–540 nm (scan rate, 3 nm/s). Daptomycin was added, to a final concentration of 1 μg/mL; $CaCl_2$ and $MgCl_2$ were added, to a final concentration of 1 mmol/L, from 1 mol/L stocks.

RESULTS

In multiple animal models, daptomycin has been demonstrated to have efficacy against both *S. aureus* and *S. pneumoniae*, including skin and soft tissue (thigh) infections [10], meningitis [11], and endocarditis [12], but has specifically failed in a simple model of BAP (table 1). No detectable reduction in the bacterial burden was observed at 24 h after infection, even at 100 mg/kg, a dose that produces drug exposure significantly greater than that produced in clinical settings, whereas ceftriaxone reduced the bacterial burden by >5 orders of magnitude during the same period. In contrast, daptomycin was effective in a model of hematogenous pneumonia (HP) meant to mimic infections that frequently develop as a consequence of bacteremia. Daptomycin was more effective than was either nafcillin or vancomycin in this system; it reduced methicillin-susceptible *S. aureus* and methicillin-resistant *S. aureus* bacterial loads by >100-fold. Additionally, daptomycin was active in a mouse model of inhalation anthrax (IA) [13]. In this model, untreated controls died within 1–4 days of infection. Treatment with daptomycin or ciprofloxacin for 21 days protected 90% of the mice tested; results of pathological examination were normal for surviving mice.

The pathophysiology of BAP differed significantly from that of hematogenous and anthrax infections. The infection produced in the 24-h BAP model was confined to the interior of the airway, with no notable tissue disruption and minimal inflammatory response [14]. Importantly, the epithelial lining of the airspace remained intact and largely unperturbed. In contrast, the infections produced by HP and IA resulted in significant tissue involvement and disseminated bacteremia. Daptomycin-treatment failure appeared to be specific to infections limited to the interior airway with minimal histopathological disruption.

Because of the presence of pulmonary surfactant, the interior of the airway represents a unique environment within the body. Surfactant is composed primarily of dipalmitoylphosphatidylcholine (~80% in all mammalian species), along with significant amounts of phosphatidylglycerol (PG) and smaller amounts of minor phospholipids, neutral lipids, and cholesterol [3]. There are 4 protein components: hydrophilic proteins SP-A and SP-D and hydrophobic proteins SP-B and SP-C. Daptomycin has previously been shown to be inserted into artificial membrane vesicles composed of PC and PC/PG [15, 16]. We reasoned that daptomycin could interact with pulmonary surfactant, in-

cluding insertion into lipid aggregates, and that this would be expected to sequester and inhibit the antibiotic.

The antistaphylococcal activity of daptomycin was tested in MHBc supplemented with increasing amounts of bovine-derived surfactant (Survanta). This preparation is used clinically to treat respiratory distress syndrome in premature infants; its composition differs slightly from that of human surfactant [3], but it is functionally equivalent. As shown in figure 1*A*, daptomycin was significantly inhibited by the inclusion of even small amounts of surfactant in the test media, with a 16–32-fold loss of potency in 1% surfactant and a >100-fold loss in 10% surfactant. By contrast, ceftriaxone was unaffected by surfactant. Activity against *S. pneumoniae* was similarly inhibited (data not shown). Physiological concentrations of phospholipids in alveolar surfactant are significantly higher than the 2.5 mg/mL in our 10%-surfactant sample [17]. Daptomycin concentrations in mouse lungs are 1–5 μg/g of tissue (data not shown), which is well below the MIC for *S. pneumoniae* or *S. aureus* in the presence of surfactant.

The inhibition of daptomycin by pulmonary surfactant is directly related to its novel mechanism of action: calcium-dependent insertion into the bacterial membrane. Insertion of daptomycin into surfactant aggregates in vitro could be demonstrated by means of a fluorescence assay (figure 1*B*). The fluorescence emission of daptomycin is highly dependent on its environment. Transition from an aqueous to a hydrophobic environment is accompanied by a significant increase in fluorescence intensity and a shift in emission maximum (E_{max}) from 460 to 440 nm [15]. We monitored daptomycin fluorescence in the presence of surfactant and demonstrated a calcium-dependent increase in fluorescence intensity (with an E_{max} at 445 nm), which is consistent with calcium-induced insertion into lipid aggregates. Addition of magnesium ions produced no increase in fluorescence signal, indicating that this process is calcium specific. Daptomycin incubated in the presence of surfactant and calcium was removed from solution by a simple centrifugation step, which also removed surfactant aggregates; this finding is consistent with sequestration of the drug (data not shown).

Direct insertion into the bacterial membrane is a unique feature of the daptomycin mechanism. Consistent with this, no other marketed class of antibiotics was affected by the inclusion of 1% surfactant in the microdilution assay (data not shown). Inclusion of a low level of detergent (0.1% Triton X-100) in the assay restored daptomycin activity, presumably by disrupting surfactant aggregates and preventing antibiotic sequestration.

DISCUSSION

The development of antibacterial agents for the treatment of respiratory infections has traditionally focused on antibacterial potency against the target pathogens. More recently, pharmacokinetic considerations—specifically, penetration of antibiotics into lung tissue, ELF, and alveolar macrophages—have played a major role. Relatively little attention has been paid to the influence of lung-specific components on the activity of antibacterial agents. The failure of daptomycin in clinical trials for CAP highlights the importance of pulmonary physiology in antibiotic function.

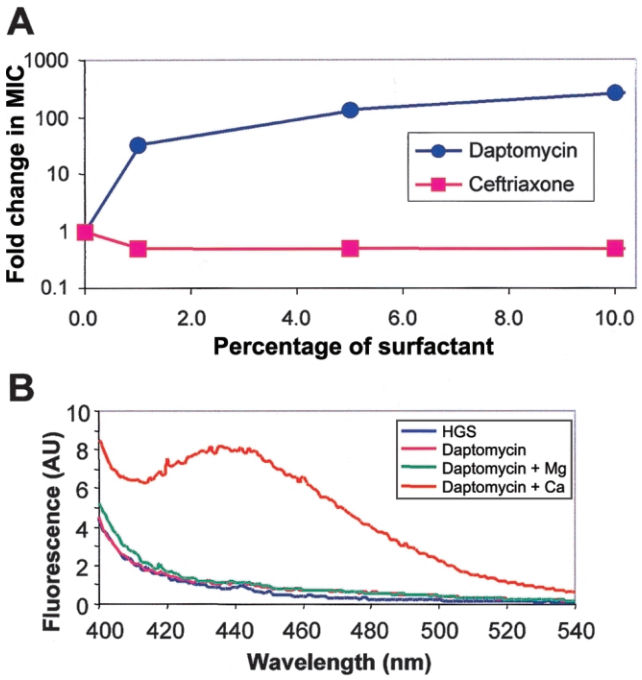

Figure 1. *A,* Inhibition of daptomycin by pulmonary surfactant. Antistaphyloccal activity of daptomycin and ceftriaxone was determined by broth microdilution in Mueller-Hinton broth supplemented with Ca^{2+} (MHBc) in the presence of increasing concentrations of pulmonary surfactant. Data are expressed as ratios of activity in the presence or absence of surfactant. *B,* Insertion of daptomycin into surfactant aggregates in vitro. The fluorescence emission of daptomycin (1 μg/mL) was determined in 5 mmol/L HEPES–0.2% glucose supplemented with 1% surfactant (HGS) after sequential addition of 1 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$. Excitation was at 385 nm. AU, arbitrary units.

Animal models of pulmonary infection were studied to provide a suitable explanation for the clinical failure. The potency of daptomycin in models producing significant tissue damage (HP and IA) contrasted sharply with the lack of efficacy in the acute pneumonia model. In this model, there is little time for an inflammatory response to develop, for the infection to spread from the airway space, or for the infection to greatly alter airway structure and function. The model may not precisely represent the clinical situation for patients with severe CAP, and it remains possible that the results of our animal studies do not yield an accurate picture of the clinical results. However, the data strongly indicate that the interior of the airway is different vis-à-vis the activity of daptomycin, and we believe that the results are highly predictive of behavior in humans.

The interaction between daptomycin and pulmonary surfactant is a direct consequence of its mechanism of action. Daptomycin is inserted into the membrane of gram-positive bacteria and artificial lipid vesicles [7, 15, 16]. Data to date are consistent with direct membrane interaction, with no requirement for a protein or other macromolecular receptor. The composition of pulmonary surfactant is different from that of most eukaryotic membranes; it primarily contains PC and only low levels of cholesterol and sphingolipids [3]. Surfactant is also ~10% PG, a prominent component of the gram-positive plasma membrane [18]. Importantly, although daptomycin will interact with lipid vesicles composed of pure PC, the presence of negatively charged PG significantly enhances insertion into the membrane [16]. The calcium dependence of daptomycin-surfactant interactions, the absence of surfactant inhibition of other classes of antibiotic, and the demonstration of direct insertion of daptomycin into surfactant aggregates in vitro all support a model whereby daptomycin in the lungs is unable to efficiently distinguish between the vast surfactant layer and the relatively small surface area of target pathogens. Insertion into the surfactant layer is predicted to be an essentially irreversible process resulting in sequestration of the antibiotic, rendering it inactive.

The in vitro model described here is not a perfect mimic of the in vivo situation. The surfactant used differs from natural surfactant, largely because of the absence of proteins critical to surfactant function. Additionally, in the context of pulmonary infection, there can be a localized breakdown in surfactant function, which is not accounted for in our model. Finally, surfactant is typically present in the lungs as a monolayer or film on the surface of an aqueous layer, with its upper surface exposed to the air. In our model, surfactant, which is immiscible in standard antibiotic testing media, existed as large aggregates. However, daptomycin has been shown to be inserted into lipid monolayers at air-water interfaces [19]. By extension, we believe that the interaction between daptomycin and surfactant aggregates should accurately predict interactions with natural surfactant layers.

The failure of daptomycin in clinical trials for CAP and murine BAP was striking in light of its efficacy in other clinical settings and multiple animal models. We have provided here a plausible mechanistic basis for the lack of efficacy in the airway that is directly linked to the mechanism of action of the antibiotic. To our knowledge, this is the first example of a pulmonary-specific mechanism resulting in the failure of an antibiotic in a clinical trial, and, this article may be the first report of organ-specific inactivation of an antibacterial agent.

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