

Effect of Pulmonary Surfactant on Antimicrobial Activity *In Vitro*

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Time-kill curve experiments were performed with linezolid, doripenem, tigecycline, moxifloxacin, and daptomycin against *Staphylococcus aureus* and with colistin, moxifloxacin, and doripenem against *Pseudomonas aeruginosa* to evaluate the effect of porcine pulmonary surfactant on antimicrobial activity. Pulmonary surfactant significantly impaired the activities of moxifloxacin and colistin. When antibiotics are being developed for respiratory tract infections, the method described here might be used to preliminarily quantify the effect of pulmonary surfactant on antimicrobial activity.

Generally, the susceptibilities of bacterial strains to antimicrobial agents are determined by comparing the MICs in standard nutrient solutions, i.e., Mueller-Hinton broth (MHB), with established breakpoints. This information is used to predict the clinical efficacy of an antimicrobial agent against a bacterial strain. However, one must consider that, apart from antimicrobial susceptibility, the characteristics of different infection sites may also affect the pharmacodynamic activity of antibiotics. This, for example, has already been described for the activity of fosfomycin in cerebrospinal fluid (1).

Until today, only a few studies investigated the effect of pulmonary surfactant (PS) on the activity of antibiotics (2, 3). In these studies, the efficacy of some antibiotics was negatively affected by PS. Indeed, it was observed that daptomycin and tobramycin, both highly effective *in vitro* against pathogens responsible for respiratory tract infections (RTI), preclinically or clinically failed in the presence of PS (2, 3). Drug binding either to surfactant-associated proteins or to phospholipids was blamed for the reduced antimicrobial activity. In contrast, other antibiotics, such as telavancin, vancomycin, ceftazidime, and amoxicillin, remain unaffected by surfactant *in vitro* (3–5). This fact bears importance for the choice of antibiotics to successfully treat lower RTI.

Although there may be appropriate methods to obtain human alveolar surfactant, it is difficult to obtain and probably impossible to obtain in large amounts for *in vitro* experiments due to ethical concerns. Therefore, we had to use a surrogate for our experiments. While recent *in vitro* studies used bovine surfactant to evaluate its effect on antimicrobial activity (2, 3), this is the first study using porcine surfactant. Porcine surfactant was used because clinical studies showed superiority of porcine surfactant over other types of mammalian surfactant for treatment of respiratory distress syndrome (6, 7).

In general, MHB (Merck, Darmstadt, Germany) was cation adjusted to a level of 25 mg/liter Ca²⁺ and 12.5 mg/liter Mg²⁺ and served as the reference medium. For experiments with daptomycin, MHB was supplemented with Ca²⁺ to a final level of 50 mg/liter as recommended by the CLSI (8).

For settings testing the influence of PS, cation-adjusted MHB was enriched with porcine surfactant (Curosurf; Chiesi Pharmaceuticals GmbH, Vienna) to a final concentration of 1 mg/ml (MHB_{surf}). This concentration was used in accordance with other *in vitro* studies, even though the concentration of PS in epithelial lining fluid has been described to be lower (0.01 mg/ml), in order

TABLE 1 MICs of tested antibiotics against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 in MHB

Strain	Antibiotic	MIC (μg/ml)
<i>S. aureus</i>	Daptomycin	0.125
	Doripenem	0.03
	Moxifloxacin	0.06
	Tigecycline	0.5
	Linezolid	2
<i>P. aeruginosa</i>	Colistin	0.25
	Doripenem	0.5
	Moxifloxacin	2

to ensure that even slight effects of surfactant on antibiotic activity would be revealed (4).

The MICs of daptomycin (Novartis Pharma GmbH, Vienna, Austria), doripenem (Janssen Pharmazeutika, Belgium), linezolid (Pfizer Corporation Austria, Vienna, Austria), moxifloxacin (Bayer Austria, Vienna, Austria), and tigecycline (Pfizer Corporation Austria, Vienna, Austria) against *Staphylococcus aureus* ATCC 29213 and the MICs of colistin (Forest Laboratories Oesterreich, Vienna, Austria), doripenem, and moxifloxacin against *Pseudomonas aeruginosa* ATCC 27853 were determined. *S. aureus* (Gram positive) and *P. aeruginosa* (Gram negative) were chosen because of their high frequency in complicated cases of pneumonia (9). MICs were determined in MHB according to CLSI guidelines (Table 1). Then time-kill curve experiments were performed in MHB and MHB_{surf} over 24 h with the combinations of antibiotics and bacterial strains given above. Time-kill curve experiments were performed in triplicate at static drug concentrations at and above the respective MICs. Antibiotic concentrations were increased in different time-kill curve experiments until a concentration was reached at which bacterial killing was observed in both media.

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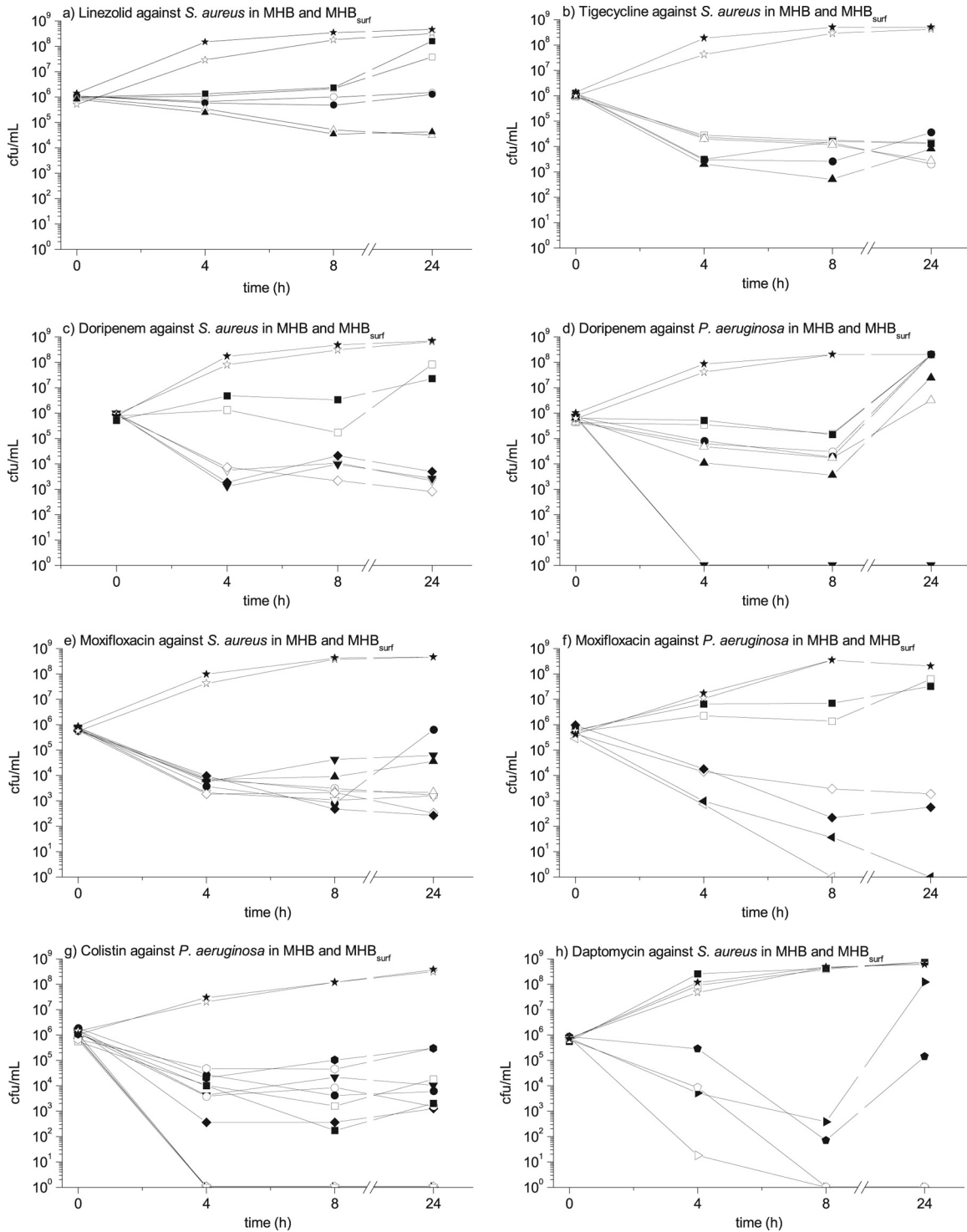


FIG 1 Time-kill curves for linezolid (a), tigecycline (b), doripenem (c and d), moxifloxacin (e and f), colistin (g), and daptomycin (h) against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 in MHB (empty symbols) and in MHB with surfactant (MHB_{surf}) (filled symbols) at different antibiotic concentrations represented by the following symbols: empty hexagon, 0.5× MIC; filled hexagon, 0.5× MIC_{surf}; empty square, 1× MIC; filled square, 1× MIC_{surf}; empty circle, 2× MIC; filled circle, 2× MIC_{surf}; empty triangle, 4× MIC; filled triangle, 4× MIC_{surf}; empty inverted triangle, 8× MIC; filled inverted triangle, 8× MIC_{surf}; empty rhombus, 16× MIC; filled rhombus, 16× MIC_{surf}; empty left-pointing triangle, 32× MIC; filled left-pointing triangle, 32× MIC_{surf}; empty right-pointing triangle, 64× MIC; filled right-pointing triangle, 64× MIC_{surf}; empty pentagon, 256× MIC; filled pentagon, 256× MIC_{surf}; empty star, GC; filled star, GC_{surf}. Mean bacterial counts at baseline and after 4, 8, and 24 h are shown as CFU/ml.

In detail, 2 ml medium, i.e., MHB or MHB_{surf} was inoculated with one of the bacterial strains. The final inoculum was 1×10^5 to 5×10^5 CFU per ml. After 30 min of incubation on a stirrer in a water bath at 37°C, one of the six antibiotics was added in a concentration at or above the MIC previously determined in MHB at standardized conditions (CLSI). Before the addition of the antibiotic and after 4, 8, and 24 h of incubation, each tube was vortex mixed for 15 s and samples of 100 μ l were drawn to determine bacterial counts. The samples were diluted with 0.9% sodium chloride solution in five log₁₀ steps, and 20 μ l of each dilution was poured on sheep blood-supplemented Mueller-Hinton agar plates (bioMérieux, Marcy-l'Étoile, France). Consecutively, the plates were incubated at 37°C for approximately 24 h. After overnight incubation, colonies were counted and the number was back extrapolated to the original volume. Growth controls (GC) were included with every experiment; daptomycin served as a positive control.

This study revealed effects of PS on antimicrobial agents that have not been described before. PS virtually had no effect on linezolid or tigecycline in the time-kill curve experiments against *S. aureus* (Fig. 1a and b). Similarly, the killing kinetics of doripenem were slightly altered, but no relevant effect on bacterial counts of either *S. aureus* or *P. aeruginosa* was observed after 24 h by the addition of PS (Fig. 1c and d). This is in line with a previous study that showed no effect of PS on amoxicillin or ceftazidime, two other β -lactam antibiotics (3).

In contrast, PS had distinct effects on the activity of moxifloxacin against the two tested strains (Fig. 1e and f). While the activity of moxifloxacin against *P. aeruginosa* was not affected by the addition of PS, the activity of moxifloxacin against *S. aureus* was reduced. In experiments using concentrations between 2 \times and 8 \times the MIC of moxifloxacin for *S. aureus*, bacterial killing was profoundly diminished by the addition of PS. This difference might be attributed to the divergent concentrations of moxifloxacin required to sustainably reduce the bacterial counts of *S. aureus* (MIC, 0.06 μ g/ml) and *P. aeruginosa* (MIC, 2 μ g/ml). It might be speculated that the distinct effect of PS on the activity of moxifloxacin against *S. aureus* and *P. aeruginosa* occurred because the binding capacity of PS for moxifloxacin was saturated at "high" but not at "low" moxifloxacin concentrations (i.e., at 2 μ g/ml but not at 0.06 μ g/ml). This assumption is supported by a similar effect observed with daptomycin in which increasing PS levels resulted in decreasing *in vitro* activity of daptomycin (4). A systematic study of the relationship between different concentrations of PS and the activity of antimicrobial agents was beyond the scope of this project, but this topic deserves further investigation.

Similarly, the activity of colistin against *P. aeruginosa* was notably reduced by the addition of PS at colistin concentrations of 2 \times to 32 \times the MIC (Fig. 1g). Since colistin's mechanism of action involves binding to and forming complexes with phospholipids, among other bacterial cell membrane particles (10), it seems plausible that colistin may also be bound by PS. At higher colistin concentrations ($\geq 64\times$ the MIC), the inhibitory effects of PS were overcome, leading to equal levels of bacterial killing in MHB and MHB_{surf}. This result is in line with the clinical efficacy of high concentrations of colistin achieved when inhaled in the treatment of complicated pneumonia (11).

As previously described, daptomycin (positive control) was not bactericidal in the presence of PS at concentrations of $\leq 256\times$ the MIC (Fig. 1h). The mechanism of inhibition, the insertion of

daptomycin into lipid aggregates, has been described by Silverman et al. (2).

To our knowledge, this is the first study that used MHB enriched with natural porcine PS to evaluate the effect of pulmonary surfactant on antimicrobial activity by means of time-kill curve experiments. Porcine surfactant was used because recent studies showed its clinical advantages over other types of surfactant supplements in the treatment of respiratory distress (6, 7). While the clinical relevance of these *in vitro* findings should be evaluated cautiously, the method used in this study may be of profound interest. During development of a drug for treatment of pneumonia, this method could be used to test whether the activity of the substance is affected in the presence of PS due to binding of antimicrobial molecules to phospholipids or other components of PS. Thus, binding of antimicrobials to PS is dependent on physicochemical properties and might receive more attention in the future in analogy to plasma protein binding.

The inhibitory effects of PS on the activity of moxifloxacin and colistin observed at concentrations above the MIC in this study are similar to the effects described for tobramycin in another study (3). The influence of PS on antimicrobial activity appears to be dependent on the antibiotic concentration, and antimicrobial activity may also differ at other concentrations of PS, which should be explored in future studies in more detail.

The intrinsic limitations of this study have to be considered. On one hand, porcine surfactant was used instead of human surfactant, and on the other hand, the concentration of PS used in this study was approximately 100 times higher than the concentration of PS at the infection site, i.e., epithelial lining fluid. The possibility cannot be excluded that the inhibition of antibiotic activity by PS might be dependent on the concentration of PS *in vivo*. Nevertheless, this study is the first that systematically evaluates whether there is any impact of PS on the activity of antibiotics of different classes by using PS concentrations corresponding to those previously used in experiments with bovine lung extract-based surfactant (5). Beyond that, regardless of what concentrations of PS are used *in vitro* for revealing its principal impact on the activity of select antibiotics, the clinical relevance of such findings for *in vivo* conditions must be examined thoroughly in separate studies.

In conclusion, in the presence of PS, the antimicrobial efficacy of moxifloxacin and colistin is affected at concentrations above the MIC. Therefore, when antibiotics are being developed for treatment of RTI, it might be reasonable to evaluate drug binding to PS and possible effects on antimicrobial efficacy early during *in vitro* investigations.

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