Effect of pulmonary surfactant on antimicrobial activity in-vitro

Schwameis R.1, Erdogan-Yildirim Z.1, Manafi M.2, Zeitlinger M.A.1, Strommer S.1, Sauermann R.1,3 #

1 Department of Clinical Pharmacology, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria;

2 Institute of Hygiene and Applied Immunology, Medical University of Vienna, Kinderspitalgasse 15, 1090 Vienna, Austria;

3 Main Association of Austrian Social Security Institutions, Kundmannngasse 21, 1031 Vienna, Austria;

E-mail: richard.schwameis@meduniwien.ac.at, zeynep.e.yildirim@gmail.com, mohammad.manafi@meduniwien.ac.at, markus.zeitlinger@meduniwien.ac.at, robert.sauermann@meduniwien.ac.at # correspondence and reprints
1. Abstract

Time-kill curves 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 developing antibiotics for respiratory tract infections (RTI) the presented method might be used to preliminary quantify the effect of pulmonary surfactant on antimicrobial activity.
2. Introduction

Generally, the susceptibility of bacterial strains to antimicrobial agents is determined by comparing the minimal inhibitory concentration (MIC) in standard nutrient solutions, i.e. in 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 regard that, apart from antimicrobial susceptibility, also the characteristics of different infection sites may affect the pharmacodynamic activity of antibiotics. This, for example was already described for the activity of fosfomycin in cerebrospinal fluid [1].

Until today only 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 RTI, preclinically or clinically failed in this indication [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 respiratory tract infections (RTI).

Although there would be appropriate methods, human alveolar surfactant is difficult to obtain and probably impossible to obtain in high amounts for in-vitro experiments due to ethical concerns. Hence 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.\cite{6, 7}
3. Methods

In general, MHB (Merck, Darmstadt, Germany) was cation-adjusted to a level of 25 mg/L Ca++ and 12.5 mg/L Mg++ and served as reference medium. For experiments with daptomycin MHB was supplemented with Ca++ to a final level of 50 mg/L as recommended by 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 (MHBsurf). This concentration was used in accordance with other in-vitro studies, even though the concentration of PS in epithelial lining fluid was described to be lower (0.01 mg/mL), in order to ensure that even slight effects of surfactant on antibiotic activity would be revealed [4].

The MICs of daptomycin (DAP; Novartis Pharma GmbH, Vienna, Austria), doripenem (DOR, Janssen Pharmaceutica, Belgium), linezolid (LZD, Pfizer Corporation Austria, Vienna, Austria), moxifloxacin (MOX, Bayer Austria, Vienna, Austria) and tigecycline (TGC, Pfizer Corporation Austria, Vienna, Austria) against *Staphylococcus aureus* ATCC 29213 and the MICs of colistin (CST, 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 pneumonia [9]. MICs were determined in MHB according to CLSI guidelines. Then time-kill curves were performed in MHB and MHBsurf over 24 hours with combinations of antibiotics and bacterial strains given above. Time-kill curves 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 reaching a concentration at which bacterial killing was observed in both media.
In detail, 2mL medium, i.e. MHB or MHBsurf were inoculated with one of the bacterial strains. The final inoculum was 1-5x10^5 colony-forming units (cfu) per mL. After 30 minutes 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 addition of the antibiotic and after 4, 8, and 24 hours of incubation, each tube was vortex-mixed for 15 seconds and samples of 100 µL were drawn to determine bacterial counts. The samples were diluted with 0.9% sodium chloride solution in five log10 steps and 20 µL of each dilution were 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 hours. After overnight incubation, colonies were counted and back extrapolated to the original volume. Growth controls (GC) were included into every experiment, daptomycin served as positive control.
4. Results and Conclusion:

This study revealed effects of PS on antimicrobial agents that have not been described before. PS did virtually not affect linezolid and tigecycline in the time kill curve experiments against \textit{S. aureus} (Fig. 1a+b). Similarly, killing kinetics of doripenem was slightly altered, but no relevant effect on bacterial counts of both, \textit{S. aureus} and \textit{P. aeruginosa} was observed after 24 h by addition of PS (Fig. 1c+d). This is in line with a previous study that showed no effect of PS on amoxicillin and ceftazidime, two other \textit{β}-lactam antibiotics [3]. In contrast, PS had distinct effects on the activity of moxifloxacin against the two tested strains (Fig. 1e+f). While the activity of moxifloxacin against \textit{P. aeruginosa} was not affected by addition of PS, the activity of moxifloxacin against \textit{S. aureus} was thoroughly reduced. In experiments using concentrations between 2 and 8x MIC of moxifloxacin for \textit{S. aureus}, bacterial killing was profoundly diminished by addition of PS. This difference might be contributed to the divergent concentrations of moxifloxacin required to sustainably reduce the bacterial counts of \textit{S. aureus} (MIC 0.06 µg/mL) and \textit{P. aeruginosa} (MIC 2 µg/mL). It might be speculated that the distinct effect of PS on the activity of moxifloxacin against \textit{S. aureus} and \textit{P. aeruginosa} occurred because the binding capacity of PS for moxifloxacin was saturated only at ‘high’ but not at ‘low’ moxifloxacin concentrations (i.e. at 2 µg/mL, but not at 0.06 µg/mL). This assumption is supported by a similar effect observed with daptomycin where increasing PS levels resulted in decreasing in-vitro activity of daptomycin[4]. Studying systematically the relationship between different
concentrations of PS and the activity of antimicrobial agents was beyond the scope of this project, but deserves further investigations.

Similarly, the activity of colistin against *P. aeruginosa* was notably reduced by addition of PS at colistin concentrations of 2-32 fold MIC. 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 (≥64 fold MIC) inhibiting effects of PS were overcome leading to equal bacterial killing in MHB and MHBsurf. This is in line with 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 presence of PS at concentrations below and equal to 256x the MIC (Fig. 1g). The mechanism of inhibition, the insertion of daptomycin into lipid aggregates was described by Silverman et al [2].

To our knowledge this is the first study that used Mueller-Hinton broth enriched with natural porcine PS to evaluate the effect of pulmonary surfactant on antimicrobial activity by means of time kill curves. Porcine surfactant was used because recent studies showed clinical advantages compared to 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 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 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 on the infection site, i.e. epithelial lining fluid. It cannot be excluded that inhibition of antibiotic activity by PS might be dependent on the concentration of PS in vivo. Nevertheless, this study is the first that systematically evaluated if there is any impact of porcine surfactant on the activity of antibiotics of different classes 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 has to be examined thoroughly in separate studies.

In conclusion, in presence of PS antimicrobial efficacy of moxifloxacin and colistin is affected at concentrations above the MIC. Therefore, it might be reasonable for
antibiotics developed for treatment of RTIs, to evaluate drug binding to PS and possible effects on antimicrobial efficacy early during in-vitro investigations. (Parts of these results were presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy, 2012).
5. Funding

The Department of Clinical Pharmacology of Medical University Vienna sponsored this study. Supplies were in part received from the Institute of Hygiene and Applied Immunology, Medical University of Vienna.

6. Transparency declaration

RS: The contents presented here reflect personal opinion. They are not necessarily identical with those of the Main Association of Austrian Social Security Institutions, its Advisory Committees or its management.

Rest of the authors: nothing to declare
7. References


Fig. 1) Time kill curves of a) linezolid, b) tigecycline, c+d) doripenem, e+f) moxifloxacin g) colistin and h) daptomycin against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 in MHB (empty symbols) and in MHB with surfactant (MHBsurf, full symbols) at different antibiotic concentrations; (empty hexagon) 0.5x MIC, (full hexagon) 0.5x MICsurf, (empty square) 1x MIC, (full square) 1x MICsurf, (empty circle) 2x MIC, (full circle) 2x MICsurf, (empty triangle pointing upwards) 4x MIC, (full triangle pointing downwards) 4x MICsurf, (empty triangle pointing downwards) 8x MICsurf, (empty rhombus) 16x MIC, (full rhombus) 16x MICsurf, (empty triangle to the left) 32x MIC, (full triangle to the left) 32x MICsurf, (empty triangle to the right) 64x MIC, (full triangle to the right) 64x MICsurf, (empty pentagon) 256x MIC, (full pentagon) 256x MICsurf, (empty star) GC, (full star) GCsurf.

Mean bacterial counts at baseline and after 4, 8 and 24 h are shown as colony forming unites per ml (cfu/mL).
Table 1. MICs of tested antibiotics against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 determined in MHB (µg/mL); Daptomycin (DAP), Doripenem (DOR), Moxifloxacin (MOX), Tigecycline (TGC), Linezolid (LZD), Colistin (CST)

<table>
<thead>
<tr>
<th>Strain</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td>DAP</td>
<td>DOR</td>
</tr>
<tr>
<td>MHB</td>
<td>0.125</td>
<td>0.03</td>
</tr>
</tbody>
</table>