Subinhibitory concentrations of moxifloxacin decrease adhesion and biofilm formation of *Stenotrophomonas maltophilia* from cystic fibrosis

A. Pompilio,^{1,2} C. Catavittello,³ C. Picciani,^{1,2} P. Confalone,^{1,2}
R. Piccolomini,^{1,2} V. Savini,³ E. Fiscarelli,⁴ D. D'Antonio³
and G. Di Bonaventura^{1,2}

Correspondence

G. Di Bonaventura
gdibonaventura@unich.it

¹Clinical Microbiology Unit, Department of Biomedical Sciences, 'G. D'Annunzio' University of Chieti-Pescara, Chieti, Italy

²Center for Excellence on Aging, 'G. D'Annunzio' University Foundation, Chieti, Italy

³Clinical Microbiology and Virology Unit, Department of Transfusion Medicine, 'Spirito Santo' Hospital, Pescara, Italy

⁴Laboratory of Clinical Microbiology, 'Bambino Gesù' Pediatric Hospital, Rome, Italy

Stenotrophomonas maltophilia is an emerging nosocomial bacterial pathogen that is currently isolated with increasing frequency from the airways of cystic fibrosis (CF) patients. In this study the effect of subinhibitory concentrations (subMICs) of moxifloxacin on adhesion, biofilm formation and cell-surface hydrophobicity of two strains of *S. maltophilia* isolated from CF patients were evaluated. Adhesion and biofilm formation assays were carried out on polystyrene and quantified by colony counts. Cell-surface hydrophobicity was determined by a test for adhesion to n-hexadecane. Moxifloxacin at 0.03× and 0.06× MIC caused a significant decrease in adhesion and biofilm formation by both strains tested. A significant reduction in cell-surface hydrophobicity following exposure to subMICs of moxifloxacin was observed for one strain only. The results of the present study provide an additional rationale for the use of moxifloxacin in CF patients and more generally in biofilm-related infections involving *S. maltophilia*.

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INTRODUCTION

Stenotrophomonas maltophilia is an emerging nosocomial pathogen, especially in the immunocompromised host. Although recent clinical evidence suggests an increase in the frequency of isolation of the micro-organism from the respiratory tract of patients with cystic fibrosis (CF) (Conway *et al.*, 2003), the role of *S. maltophilia* in the pathophysiology of CF lung disease has not yet been clearly elucidated (O'Sullivan & Freedman, 2009; Ryan *et al.*, 2009). However, the reported isolation of *S. maltophilia* from the sputa of CF patients has become a cause for concern in the CF community, as the organism is resistant to many of the antibiotics prescribed in the management of CF (Nicodemo & Paez, 2007).

The effective therapeutic result of antibiotics is best when the concentration is above the MIC. However, after a certain period of time following a dose, antibiotic concentrations within many tissues become lower than

the MIC and are called subinhibitory concentrations (subMICs). Micro-organisms often grow in the presence of subMICs, which, although not able to inactivate micro-organisms, are potentially capable of altering the chemical and physical cell-surface characteristics and consequently the functionality and expression of some virulence properties such as adhesion, biofilm formation, hydrophobicity and motility (Fonseca *et al.*, 2004; Wojnicz & Jankowski, 2007).

Most studies on the effects of subMICs of antibiotics have focused largely on *Escherichia coli* (Wojnicz & Jankowski, 2007), *Staphylococcus* spp. (Chisari *et al.*, 2002; Cerca *et al.*, 2005), and *Pseudomonas aeruginosa* (Fonseca *et al.*, 2004), and, to the best of our knowledge, there is no such information in the literature concerning *S. maltophilia*.

Infections caused by *S. maltophilia* are particularly difficult to eradicate because it is usually highly resistant to many of the currently available broad-spectrum antibiotics (Avison *et al.*, 2001; Gould & Avison, 2006). Moxifloxacin, a recent fluoroquinolone used mainly in the treatment of respiratory infections, exhibits significant *in vitro* activity against *S. maltophilia* (Galles *et al.*, 2008), including strains

Abbreviations: ANOVA, analysis of variance; CF, cystic fibrosis; MATH, microbial adherence to n-hexadecane; subMIC, subinhibitory concentration.

resistant to trimethoprim/sulfamethoxazole (Giamarellos-Bourboulis *et al.*, 2002), the drug of choice for the treatment of infections, thus indicating that this quinolone should be evaluated further as a therapeutic option for *S. maltophilia* infections. Considering the potential clinical implications, we performed the present study in an attempt to evaluate the effect of moxifloxacin subMICs against a number of virulence properties (adhesion, biofilm formation and cell-surface hydrophobicity) expressed by *S. maltophilia* isolated from CF patients.

METHODS

Bacterial strains and culture conditions. The *S. maltophilia* Sm132 and Sm144 strains used in this study were collected from the respiratory tract of two different patients with CF admitted to the Bambino Gesù Pediatric Hospital, and who had not been treated previously with moxifloxacin. Identification of *S. maltophilia* was performed by biochemical tests (BBL Crystal E/NF; Becton Dickinson) and confirmed by rRNA-directed PCR (Whitby *et al.*, 2000). Both strains were selected for their ability to form significant amounts of biofilm on polystyrene. Stock cultures were maintained at -80°C in a Microbank preservation system (Pro-Lab Diagnostics) until use. Stocks were thawed as needed and subcultured twice on Mueller–Hinton agar (MHA; Oxoid) for 24 h at 37°C .

Antimicrobial susceptibility. Reagent-grade moxifloxacin powder of known potency was kindly supplied by Bayer (Milan, Italy). Moxifloxacin MICs for the *S. maltophilia* Sm132 and Sm144 strains were determined using a broth microdilution technique according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). Results were observed after overnight incubation at 37°C , with the MIC defined as the lowest concentration that inhibited visible growth. *P. aeruginosa* ATCC 27853 was included in each run of experiments as a quality control.

Bacterial exposure to moxifloxacin at subMICs. Briefly, colonies from an overnight culture grown on MHA were suspended in trypticase soy broth (TSB; Oxoid) at a concentration of approximately 1×10^8 c.f.u. ml^{-1} , diluted 1:10 in cation-adjusted Mueller–Hinton broth (Becton Dickinson) and then exposed (37°C , 18 h), under static conditions, to moxifloxacin at concentrations of $0.06 \times$ and $0.03 \times$ MIC. Control samples consisted of bacteria that were not exposed to moxifloxacin but were otherwise treated identically. Bacteria were then washed three times in PBS (10 500 g, 5 min, 4°C) to remove the antibiotic and resuspended in PBS (for the hydrophobicity assay) or TSB (for adhesion and biofilm formation assays) to an OD_{550} of 0.80 or 0.13, respectively. The inoculum concentration was determined by making serial dilutions in PBS and performing colony counts in duplicate on MHA after overnight incubation at 37°C .

Adhesion to and biofilm formation on polystyrene. One millilitre of the standardized inoculum was dispensed into each well of a sterile, flat-bottomed, polystyrene, 24-well, tissue culture-treated culture plate (Iwaki Glass). After 3 h (adhesion) or 24 h (biofilm formation) of static incubation at 37°C , the medium containing non-adherent cells was removed and each well was washed carefully three times with 1 ml sterile PBS (pH 7.3; Sigma-Aldrich), previously warmed at room temperature, using a micropipette. To avoid inter-operator variability, sample washings were carried out by the same operator. Adherent and biofilm cells were then harvested by scraping and quantified by colony counts.

Cell-surface hydrophobicity. Evaluation of hydrophobicity was carried out by using a microbial adherence to n-hexadecane (MATH) test (Mattos-Guaraldi *et al.*, 1999). Briefly, 4 ml moxifloxacin-exposed inoculum prepared in PBS at an OD_{550} of 0.8 was overlaid with 400 μl n-hexadecane (Sigma-Aldrich). After 1 min agitation by vortexing, the phases were allowed to separate for 15 min at room temperature. The OD_{500} value of the aqueous phase was then measured. The results were expressed as the proportion of cells that were excluded from the aqueous phase, determined by the equation: $[(A_0 - A)/A_0] \times 100$, where A_0 and A are, respectively, the initial and final OD_{500} of the aqueous phase. Strains were considered as strongly hydrophobic when the values obtained were $>50\%$, moderately hydrophobic for values ranging between 20 and 50%, and hydrophilic when values were $<20\%$ (Mattos-Guaraldi *et al.*, 1999).

Bacterial morphology. The effects of moxifloxacin subMICs on *S. maltophilia* cell morphology were assessed by scanning electron microscopy. Briefly, samples were fixed for 3 h at 4°C with 2.5% (v/v) glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4), with the cationic dye ruthenium red (0.15%, w/v; Polysciences Europe). Samples were then post-fixed in 1.5% (v/v) OsO_4 , rinsed in 0.15 M cacodylate buffer and dehydrated in an ascending ethanol series (30, 50, 70, 80, 95 and 100%, v/v) before critical-point drying. Examination of samples was performed at 15 kV in a Hitachi S-500 scanning electron microscope.

Data analysis. Each experiment was carried out in triplicate and repeated on at least two different occasions; the results are presented as means $\pm$ SD. Data from the adhesion and biofilm assays were normalized to the control, which was taken as 100%. All data were statistically analysed by one-way analysis of variance (ANOVA), followed by pairwise comparisons using the Bonferroni test. The difference was considered statistically significant for $P < 0.05$. Analyses were performed using the Prism 5.0 software package (GraphPad Software).

RESULTS AND DISCUSSION

Susceptibility to moxifloxacin and determination of subMICs

Both strains of *S. maltophilia* tested were susceptible to moxifloxacin (MIC $0.5 \mu\text{g ml}^{-1}$ for both strains). Preliminary studies were conducted in order to assess the killing of *S. maltophilia* following exposure to moxifloxacin at different subMICs ($0.5 \times$, $0.25 \times$, $0.12 \times$, $0.06 \times$ and $0.03 \times$ MIC). Exposure to moxifloxacin at $0.5 \times$, $0.25 \times$ and $0.12 \times$ MIC caused significant killing (ranging from 43.0 to 94.0% for the Sm132 strain and from 45.8 to 81.7% for the Sm144 strain), compared with the control (without moxifloxacin). No killing was observed after exposure to $0.06 \times$ and $0.03 \times$ MIC. Thus, all experiments carried out in this study were performed using a standardized *S. maltophilia* inoculum exposed to moxifloxacin at $0.06 \times$ and $0.03 \times$ MIC, corresponding to 0.03 and $0.015 \mu\text{g ml}^{-1}$, respectively.

Effect of moxifloxacin on bacterial adherence

The mean adherence of *S. maltophilia* to polystyrene after exposure to subMICs of moxifloxacin is shown in Fig. 1. Adhesion by Sm132 strain was, in comparison with the

Fig. 1. Effect of moxifloxacin subMICs on adherence of *S. maltophilia* strains Sm132 and Sm144 to polystyrene. The control contained bacteria that were not exposed to moxifloxacin. Results are expressed as means $\pm$ SD. *, $P < 0.05$; **, $P < 0.001$ (versus control) (ANOVA + Bonferroni's post-test). White bars, control; hatched bars, $0.03 \times$ MIC; grey bars, $0.06 \times$ MIC.

control, significantly lower ($P < 0.001$) in the presence of $0.03 \times$ and $0.06 \times$ MIC, with inhibition levels of 97.0 ± 2.9 and 98.7 ± 0.2 %, respectively. Adhesion by Sm144 strain was, in comparison with the control, also significantly lower ($P < 0.05$) in the presence of $0.03 \times$ and $0.06 \times$ MIC, with inhibition levels of 72.9 ± 11.8 and 80.7 ± 16.6 %, respectively. No statistically significant differences were observed between inhibition levels caused by $0.06 \times$ and $0.03 \times$ MIC exposure for either strain.

The ability to adhere to abiotic and biotic substrates is a prerequisite for infection so that the micro-organism can colonize and thus cause infection in the host. One of the major risk factors associated with *S. maltophilia* is the presence of prosthetic devices (Muder *et al.*, 1996). We recently demonstrated that the ability to adhere to polystyrene is highly conserved in *S. maltophilia* isolates from CF patients (Di Bonaventura *et al.*, 2007).

SubMICs of antibiotics could inhibit bacterial adhesion through different mechanisms. They may inhibit the synthesis or expression of adhesins on the bacterial cell surface, lead to the formation of functionally aberrant adhesins, cause the release of adhesins from the surface of bacterial cells or modify the bacterial shape in a such way as to interfere with the ability of the micro-organisms to approach host cell-surface receptors (Lorian & Ernst, 1987; Lorian *et al.*, 1989).

These data confirm the anti-adhesive properties of fluoroquinolones in general (Baskin *et al.*, 2002; Wojnicz & Jankowski, 2007). Indeed, moxifloxacin caused a significant reduction in *S. maltophilia* adherence up to concentrations equal to $0.03 \times$ MIC, unlike previous studies with *E. coli* in which the effect of fluoroquinolones was limited at concentrations equal to or not less than $0.125 \times$ MIC (Baskin *et al.*, 2002; Wojnicz & Jankowski, 2007).

Effect of moxifloxacin on biofilm formation

The effects of subMICs of moxifloxacin on *S. maltophilia* biofilm formation on polystyrene are shown in Fig. 2. Biofilm production by Sm132 strain was, in comparison with the control, significantly lower ($P < 0.05$) in the presence of $0.03 \times$ and $0.06 \times$ MIC, with inhibition levels of 65.4 ± 11.2 , and 65.3 ± 37.1 %, respectively. Biofilm production by Sm144 strain was, in comparison with the control, also significantly lower ($P < 0.001$) in the presence of $0.03 \times$ and $0.06 \times$ MIC, with inhibition levels of 74.8 ± 14.2 , and 72.3 ± 7.3 %, respectively. No statistically significant differences were observed between inhibition levels caused by $0.06 \times$ and $0.03 \times$ MIC exposure for either strain.

A number of studies have demonstrated that, in CF patients, the inefficiency of antibiotics used to treat infections is related to the ability of the micro-organisms to form biofilms (Costerton, 2001; Ebert & Olivier, 2002). The finding that adhesion and biofilm formation are highly conserved in *S. maltophilia* isolates from CF patients (Di Bonaventura *et al.*, 2007, 2008b) has led to the suggestion that *in vivo* this micro-organism may also develop biofilms, which may protect it from natural or acquired immune defences and from the action of antibiotics. Recently, we observed that *S. maltophilia* is able to form biofilms on CF bronchial epithelial cells (Di Bonaventura *et al.*, 2008a), thus providing a rationale for the persistence of the bacterium in CF patients. The results in Fig. 2 demonstrate that exposure to moxifloxacin subMICs is associated with a significant decrease in the ability of *S. maltophilia* to form biofilms on polystyrene, which may leave cells more susceptible to immune clearance or antimicrobial drug therapy.

Although having the same mechanism of action as that of moxifloxacin, other quinolones show anti-biofilm prop-

Fig. 2. Effect of moxifloxacin subMICs on biofilm formation of *S. maltophilia* strains Sm132 and Sm144 on polystyrene. The control contained bacteria that were not exposed to moxifloxacin. Results are expressed as means $\pm$ SD. *, $P < 0.05$; **, $P < 0.001$ (versus control) (ANOVA + Bonferroni's post-test). White bars, control; hatched bars, $0.03 \times$ MIC; grey bars, $0.06 \times$ MIC.

erties to a lesser extent at subMICs. In fact, we found previously that moxifloxacin was the most active fluoroquinolone in affecting *S. maltophilia* biofilm formation at $0.5 \times$ and $0.25 \times$ MIC, being about twice as active as levofloxacin and grepafloxacin, whilst norfloxacin was less active among the fluoroquinolones tested (Di Bonaventura *et al.*, 2004). Thus, we hypothesize that the significant anti-biofilm activity of moxifloxacin subMICs is a specific property not shared by other antibiotics with similar structures and mechanisms of action.

Effect of moxifloxacin on cell-surface hydrophobicity

The effect of moxifloxacin subMICs on cell-surface hydrophobicity is summarized in Fig. 3. In particular, the presence of moxifloxacin did not significantly affect the MATH of strain Sm132, which preserved its original hydrophilicity (MATH 6.6 ± 5.0 , 2.9 ± 2.9 and 4.8 ± 3.4 % for the control, $0.03 \times$ and $0.06 \times$ MIC, respectively; $P > 0.05$). Strain Sm144, which started off with a hydrophobic MATH in the absence of moxifloxacin (25.9 ± 3.2 %), preserved its hydrophobicity in the presence of moxifloxacin at $0.03 \times$ MIC (21.1 ± 4.8 %; $P > 0.05$ vs control), but became hydrophilic in the presence of moxifloxacin at $0.06 \times$ MIC, as indicated by the statistically significant decrease in MATH with respect to the control (16.9 ± 3.7 %; $P < 0.001$ vs control).

As soon as micro-organisms reach a living (epithelial mucosa) or non-living (prostheses) surface, they will be attracted or repelled by it, depending on the sum of the different non-specific interactions, especially by hydrophobic interactions (Doyle, 2000; Costa *et al.*, 2006). Recently, we observed a positive correlation between hydrophobicity and levels of both adhesion and biofilm

formation in *S. maltophilia* isolates from neutropenic patients (Pompilio *et al.*, 2008), thus suggesting that hydrophobicity is a significant determinant of adhesion and biofilm formation by *S. maltophilia*.

The results of the present study suggest that moxifloxacin can modulate the level of hydrophobicity in *S. maltophilia*, although this effect appears to be strain-dependent.

Moxifloxacin could decrease cell-surface hydrophobicity by interfering with the synthesis and expression of outer-membrane proteins, LPS or fimbriae, structures known to affect bacterial hydrophobicity (Doyle, 2000; Costa *et al.*, 2006; Pompilio *et al.*, 2008). However, further studies are needed to clarify whether the reduction in hydrophobicity levels induced by moxifloxacin can be extended to a larger number of strains.

Effect of moxifloxacin on bacterial morphology

SubMICs of antibiotics, particularly fluoroquinolones, can reduce the virulence of some bacteria (Gram-negative bacilli, in particular) by inducing an elongation of the cell soma along its longitudinal axis, a phenomenon also known as filamentation. These morphologically altered cells generally show reduced pathogenicity in terms of lower levels of adhesion, altered susceptibility to phagocytosis and decreased release of bacterial enzymes (Labro *et al.*, 1987; Chen *et al.*, 2005). Recently, Drago *et al.* (2005) observed that moxifloxacin subMICs ($0.125 \times$ and $0.06 \times$ MIC) induced filamentation in a remarkable portion of *Klebsiella pneumoniae* in an animal experimental model of pulmonary infection.

On this basis, we examined the effect of exposure to moxifloxacin at $0.06 \times$ and $0.03 \times$ MIC on *S. maltophilia* morphology. Electron microscopic analysis revealed that moxifloxacin subMICs did not induce morphological abnormalities, as showed by the absence of such extrusions in the cell envelope in the form of blebs or bacterial filamentation (Fig. 4).

In conclusion, our results showed that moxifloxacin subMICs were effective in preventing initial adherence and subsequent biofilm formation by *S. maltophilia*, possibly by at least two mechanisms: a cell-surface hydrophobicity-dependent and a cell-surface hydrophobicity-independent mechanism.

After a daily oral dose of 400 mg, moxifloxacin reaches a peak serum level of $3.3 \mu\text{g ml}^{-1}$, attaining $5.5 \mu\text{g ml}^{-1}$ in bronchial mucosa and $24.4 \mu\text{g ml}^{-1}$ in the epithelial lining fluid (Schubert *et al.*, 2005). As we found that the anti-adhesive effect produced by moxifloxacin persists up to $0.015 \mu\text{g ml}^{-1}$ (corresponding to $0.03 \times$ MIC for both strains tested), it is plausible that clinically attainable moxifloxacin concentrations would inhibit *S. maltophilia* adherence and biofilm formation, even if the cells are resistant to killing at these concentrations.

Thus, results of the present study could have important clinical implications, providing an additional rationale for

Fig. 3. Effects of moxifloxacin subMICs on hydrophobicity of *S. maltophilia* strains Sm132 and Sm144. The dotted line indicates the cut-off point for hydrophobicity (MATH > 20 %). The control contained bacteria that were not exposed to moxifloxacin. Results are expressed as means $\pm$ SD. **, $P < 0.001$ (versus control) (ANOVA $\pm$ Bonferroni's post-test). White bars, control; hatched bars, $0.03 \times$ MIC; grey bars, $0.06 \times$ MIC.

Fig. 4. Scanning electron microscopy of *S. maltophilia* Sm132 strain in the absence (a) and presence of 0.03× MIC (b) or 0.06× MIC (c) moxifloxacin for 18 h. Bars, 5 µm. Micrographs are also representative of the results obtained for *S. maltophilia* Sm144.

the use of moxifloxacin in CF patients and more generally in biofilm-related infections involving *S. maltophilia*. Future work is needed to confirm our findings in an animal model.

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