AAPS PharmSciTech (2019) 20: 205
DOI: 10.1208/s12249-019-1417-9

Research Article

Theme: Paul Myrdal Memorial Issue - Pharmaceutical Formulation and Aerosol Sciences
Guest Editors: Philip J. Kuehl and Stephen W. Stein

Efficacy of Ciprofloxacin and Its Copper Complex against *Pseudomonas aeruginosa* Biofilms

Frederic Tewes,[1,2,3] Tania F. Bahamondez-Canas,[2] and Hugh D. C. Smyth[2]

Received 6 November 2018; accepted 14 May 2019; published online 29 May 2019

Abstract. A limitation of antibiotic treatments for *P. aeruginosa* (PA) chronic pulmonary infections is the reduced efficacy due to sub-therapeutic concentrations at the infection site and the development of biofilm. A novel approach to sustain ciprofloxacin (CIP) in the lungs after inhalation is to reduce its pulmonary absorption rate by its complexation with copper (CIP-Cu). This study aimed to evaluate the antimicrobial action of cationic CIP-Cu complex in PA biofilms in terms of drug concentration and time. Two *PA* strains, PA01 and PA14, were grown to form biofilm layers in equilibrium with planktonic cells. Static parameters such as pyoverdine production by planktonic cells, enzymatic activity within biofilms, and biofilm biomass 24 h after the addition of CIP or CIP-Cu were evaluated. Also, the kinetic effects of CIP and CIP-Cu on biofilms were evaluated by bioluminescence kinetics using transgenic strains. No differences were observed between CIP and CIP-Cu in terms of efficacy against biofilms, validating the potential of using this complex to treat PA biofilms. Interestingly, CIP concentrations slightly below the MIC value against planktonic bacteria stimulated both virulence and biofilm PA01 production. These results support the need to accurately achieve high CIP concentration in the lungs, which can be more easily achieved by pulmonary delivery of advanced CIP formulations (CIP-metal complexes or liposomal CIP) instead of the oral administration of free CIP.

KEY WORDS: ciprofloxacin; copper-based antimicrobials; pulmonary delivery; Pseudomonas aeruginosa; biofilm infections.

INTRODUCTION

In order to improve antibiotic efficacy and reduce antibiotic resistance development, copper-based antimicrobials have been developed as a novel class of therapeutics (1). We recently developed microparticles loaded with positively charged ciprofloxacin-copper complex (CIP-Cu) for the treatment of chronic lung infections by pulmonary inhalation (2,3). The antibacterial activities of CIP and CIP-Cu against planktonic *P. aeruginosa*, one of the main pathogen bacterium in chronic lung infections, were found to be equivalent (2).

However, the major challenge associated with the therapy against chronic pulmonary infections is the formation in the lung of *P. aeruginosa* biofilms that lead to treatment-resistant infections. Bacteria within biofilms can be over 1000-fold more resistant to antimicrobials compared to planktonic bacteria (4,5). These bacteria are embedded in an extracellular polymeric substance (EPS), composed of polysaccharides, proteins, and DNA (6–9). Most of these polymers are negatively charged at physiological conditions. One of the several proposed mechanisms that contributes to the increased resistance to antimicrobials is the binding and sequestration of positively charged antibiotics by these negatively charged polymers (5,10,11). This drug binding results in a slower antibiotic concentration increase rate within the biofilm than in the surrounding media, giving more time for the bacteria within biofilms to adapt to the presence of antibiotic (12–14).

We have previously demonstrated that a sustained CIP concentration in the lung epithelial lining fluid (ELF) of rats was best achieved by pulmonary delivery of CIP-Cu-loaded microparticles compared to the pulmonary delivery of a CIP solution or CIP-loaded microparticles (3). This sustained local concentration was due to the reduction of the CIP apparent permeability across the lung epithelium, reducing the CIP lung-blood absorption rate, when CIP was delivered as a positively charged CIP-Cu complex (2,3,15).

Guest Editors: Philip J. Kuehl and Stephen W. Stein

[1] INSERM, U1070, UFR de Médecine Pharmacie, Université de Poitiers, 1 rue Georges Bonnet, TSA 51106, 86073, Poitiers Cedex 9, France.
[2] Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712, USA.
[3] To whom correspondence should be addressed. (e–mail: ftewes@univ-poitiers.fr)

Based on these preliminary data showing promising lung delivery pharmacokinetics and planktonic bacteria killing efficacy, the aim of this study was to evaluate the effect of positively charged CIP-Cu complex on biofilms formed by two well-characterized *P. aeruginosa* strains, PA01 and PA14.

MATERIALS AND METHODS

Bacteria Strains and Materials

Two different *P. aeruginosa* strains were used. The first strain was PA01 (ATCC® 15692™), used as received from ATCC or tagged by chromosomal integration of p16Slux construction (16) to obtain a bioluminescent PA01 (PA01::p16Slux, gift from Pr. Shawn Lewenza from the University of Calgary, Canada). The second strain was a PA14 constitutively bioluminescent from intergration of the plasmid pQF50-lux (17) (UCBPP-PA14, gift from Marvin Whiteley from The University of Texas, USA). BHI medium, ciprofloxacin, copper sulfate, fluorescein diacetate (FDA), and crystal violet (CV) were purchased from Sigma-Aldrich (St Louis, USA).

Biofilm Susceptibility Testing

PA01 and PA14 strains were grown overnight in BHI in an orbital shaker at 37°C. Then, each bacterial suspension was adjusted to 3×10^7 CFU/mL. For biofilm formation, 100 μL of this suspension was added into a clear round-bottomed 96-well plate treated for optimal cell attachment (Corning®) and incubated at 37°C in aerobic water-saturated atmosphere. After 24 h, the supernatants were removed, and the wells were rinsed four times with 150 μL of PBS. Then, the plates were filled with 100 μL of two-fold dilutions of CIP, Cu^{2+}, or CIP/Cu^{2+} mix at (1/1) molar ratio in BHI. In a 1/1 (mol/mol) CIP/Cu^{2+} mix, close to 100% of the CIP molecules are complexed with copper (II) (2). CIP concentrations ranged from 32 to 0.031 μg/mL. These concentrations were equivalent to 128–0.128 times the CIP minimum inhibitory concentration (MIC) against these two strains when planktonically grown. The plates were again incubated for 24 h at 37°C and the BHI broth turbidity, reflecting the planktonic bacteria growth, was measured by recording the optical density at 600 nm (OD_{600}) in each well. Simultaneously, the pyoverdine relative concentration variation was assessed by measuring its fluorescence intensity in the wells. Then, the supernatants were removed, and the wells rinsed four times with 150 μL of PBS to eliminate the planktonic bacteria. Finally, the metabolic activity within the biofilms was assayed using a fluorescein diacetate (FDA)-based test and the total organic mass of the biofilms was evaluated by crystal violet (CV) staining.

Pyoverdine Fluorescence Intensity

Pyoverdine is a siderophore and virulence factor produced by planktonic *P. aeruginosa* in order to invade his host. Pyoverdine fluorescence intensity was measured at λ_{em} = 460 nm after excitation at λ_{ex} = 400 nm using a 96-well plate reader (Infinite M200, Tecan Systems Inc., CA, USA). The specificity of the fluorescence for pyoverdine in this condition was determined by Zaborin *et al.* (18) by verifying that a mutant deficient in pyoverdine was not fluorescent. In order to distinguish between an increase in pyoverdine fluorescence due to an increase in pyoverdine production per bacteria and an increase in pyoverdine fluorescence due to simple increase in the number of bacteria (information already obtain from the OD_{600} value), the pyoverdine fluorescence intensity was normalized by the OD_{600} value as done by Zaborin *et al.* (18) ($n = 3$).

Biofilm Enzymatic Activity Determination by FDA Assay

The enzymatic activity within bacteria-forming biofilms was measured by the hydrolysis of the fluorescein diacetate (FDA) substract into fluorescein. FDA is a cell-permeant substrate that is hydrolyzed by several enzyme classes including lipases, esterases, and proteases. A FDA stock solution in acetone (10 mg/mL) was stored at − 20°C for 2 months. For each assay, freshly prepared FDA working solutions were made by diluting to 1:400 the stock solution in PBS buffer (pH 7.4–0.05 M). Then, 200 μL of FDA working solutions was added to each rinsed well. The plates were incubated at 37°C in the plate reader (Infinite M200, Tecan Systems Inc., CA, USA) to measure fluorescence after 30 min (λ_{ex}: 494 nm and λ_{em}: 518 nm). Fluorescence intensities were normalized with respect to the intensity measured for biofilms in the absence of CIP ($n = 3$).

Biofilm Biomass Evaluation by CV Staining

Biofilms in round-bottomed plates were washed 3–4 times with PBS and fixed with 150 μL of absolute methanol for 20–30 min. Then, biofilms were stained with 150 μL of CV solution at 0.1% (*w*/*v*) in water for 30 min. After rinsing 4–5 times with deionized water, the wells were allowed to air-dry overnight. The CV content was solubilized in 200 μL of 30% (*v*/*v*) acetic acid in water and transferred after 30 min to flat-bottomed 96-well plates (Greiner®). After adequate dilution, the CV absorbance was measured at 550 nm using a plate reader (Infinite M200, Tecan Systems Inc., CA, USA). CV calibration curves were made using two-fold serially diluted CV standard solutions (65 to 0.5 μg/mL) made in 30% acetic acid in water. These calibration curves were used to calculate the amount (μg) of CV per well ($n = 3$).

Time-Luminescence Intensity Kinetics

To characterize the dynamics of the CIP and CIP-Cu effect over time, we measured the time-resolved bioluminescence obtained from luminescent PA01::p16Slux and UCBPP-PA14 biofilms growing in the presence of various concentrations of CIP or CIP-Cu complex. Biofilms were formed in TC-treated black 96-well plates with flat clear bottom (Corning®). For UCBPP-PA14, biofilms were grown in BHI supplemented with carbenicillin (100 μg/mL) to maintain the presence of the pQF50-lux plasmid, as previously described (17). Then, the plates were washed three times with PBS and filled with 100 μL of two-fold dilution of CIP or CIP/Cu^{2+} mix at (1/1) molar ratio in BHI broth containing no carbenicillin. After addition of the treatment solutions, peripheral wells of the plates were filled with sterile PBS. Then, the plates were

sealed using an air-permeable membrane to prevent evaporation during the assay and were incubated at 37°C in the plate reader (SpectraMax M3, molecular devices, USA). Luminescence intensity emitted from the bottom of the wells was measured during 1 s per well every 20 min. Plates were shaken before each measurement ($n = 3$). Curves were obtained from the set of data points with the statistical technique called LOESS (locally weighted scatterplot smoothing) using the R software.

RESULTS

Susceptibility of Planktonic Bacteria in Contact with the Biofilm

The susceptibility of PA01 and PA14 biofilms, two *P. aeruginosa* strains classically used to study biofilms (19), to CIP and CIP-Cu complex was evaluated in wells of microtiter plates. In this model, biofilms initially formed on the bottom of the wells are submerged with the growing medium that can be colonized by planktonic bacteria released from the biofilms through various dispersal processes (20). Depending on their susceptibility to the antibiotic tested, these planktonic bacteria can multiply in the growth medium, which increases turbidity. Also, by measuring the turbidity, *i.e.*, measuring the optical density at 600 nm (OD_{600}) of the medium in each microtiter well, it was possible to assess the susceptibility of the planktonic bacteria ejected from the biofilm to CIP and the CIP-Cu complex (Fig. 1a, b). For PA01 and PA14, a gradual decrease in CIP concentration induced a gradual increase of OD_{600}, with the higher values obtained for the control without CIP. Similar results were obtained with CIP alone or with the CIP-Cu complex, while Cu^{2+} alone, at the same range of concentrations, had no effect (data not shown). Based on these data, the minimum inhibitory concentrations (MIC) of CIP, free or complexed with Cu^{2+}, were 0.25 μg/mL for PA01 and PA14 (Fig. 1a, b–black arrows). These MIC values were close to those previously obtained for free CIP against PA01 (17,21).

Pyoverdine Production

The second parameter measured was the fluorescence of the pyoverdine produced by *P. aeruginosa*. This siderophore is essential for pathogenesis of *P. aeruginosa* in mammalian infections. Some studies have shown that the production of pyoverdine in *P. aeruginosa* biofilms is much lower than that in planktonic cells (20). Thus, the pyoverdine present in the microtiter plates' wells was mainly produced by planktonic cells ejected from the biofilms, and a normalization of the pyoverdine fluorescence intensity by the OD_{600} was performed (Fig. 1c, d). This ratio illustrates the proverdine production per bacteria and thus their virulence. For the two strains of *P. aeruginosa*, the pyoverdine fluorescence/OD_{600} ratios *versus* CIP or CIP-Cu complex concentrations profiles were similar. Ratios increased slightly with CIP concentrations, then increased sharply to reach a maximum value for CIP concentration just before the MIC, *i.e.*, at sub-MIC (0.125 μg/mL). Then, a further increase in the CIP concentration above the MIC greatly decreased the value of the ratios. The maximum value of the ratio was higher for PA14 (pyoverdine fluorescence/$OD_{600} \approx 20$) than for PA01 (pyoverdine fluorescence/$OD_{600} \approx 6$), suggesting a higher virulence of PA14 compared to PA01.

Enzymatic Activity in the Biofilm

The first two sets of data (Fig. 1) were mostly linked to the planktonic bacteria in contact with the biofilms. The next two sets (Fig. 2) were specific to biofilms since planktonic bacteria were carefully removed by several washing before doing the experiments. The enzymatic activity within the bacteria forming the biofilms was evaluated by measuring the fluorescence intensity of fluorescein produced by FDA hydrolysis (Fig. 2a, b). An increase in the value of the FDA test describes an increase in the metabolic activity of bacteria or an increase in the number of bacteria. No differences were observed between the experiments performed with CIP or CIP-Cu complex. For PA14 biofilms (Fig. 2b), the enzymatic activity decreased from the 100% obtained in the absence of CIP to around 55% for a CIP concentration of 0.125 μg/mL, then plateaued without further changes up to 32 μg/mL of CIP. For PA01 biofilms (Fig. 2a), the changes in metabolic activity with the CIP concentrations were more variable than for PA14. It decreased from the 100% obtained in the absence of CIP down to 71 ± 14% for a CIP concentration of 0.063 μg/mL. Then, it unexpectedly increased up to 138 ± 4% when the CIP concentration was increased to a sub-MIC value of 0.125 μg/mL. A further increase in CIP concentration to 0.5 μg/mL led to a new decrease in enzymatic activity to a minimum of around 20% that did not change with a further increase of CIP concentration up to 32 μg/mL.

Biofilm Mass Determination

The last test performed in the microtiter plate screening study was the staining of biofilms with crystal violet (CV) (Fig. 2c, d). This test is based on CV dye binding to all the organic content, *i.e.*, the polymeric matrix forming the biofilm and both viable and nonviable bacteria, allowing quantification of the total biofilm biomass (22). Once more, no differences were observed between the experiments performed with CIP or CIP-Cu complex for both strains. The profiles showing the variation of CV (μg) per well *versus* CIP concentrations (Fig. 2c, d) were comparable to trends observed for the enzymatic activity (FDA test) of the biofilms. The maximal amount of CV found within the PA01 biofilm was 4 μg, obtained for a CIP concentration of 0.125 μg/mL (Fig. 2c). This CIP sub-MIC value was also the concentration for which the highest enzymatic activity was measured, suggesting an increase in the number of bacteria more than an increase in the amount of biofilm matrix. This amount of CV was twice the amount found in the CIP-untreated PA01 biofilm. For PA14 (Fig. 2d), the quantity of CV assayed per well was much higher than those measured for the PA01 strain, *i.e.*, 40 μg of CV within the CIP-untreated PA14 biofilm *versus* 2 μg within the PA01-untreated biofilm. The minimum amount of CV assayed in the PA14 biofilm was around 15 μg and was obtained in the presence of CIP concentrations comprised between 0.125 and 32 μg/mL.

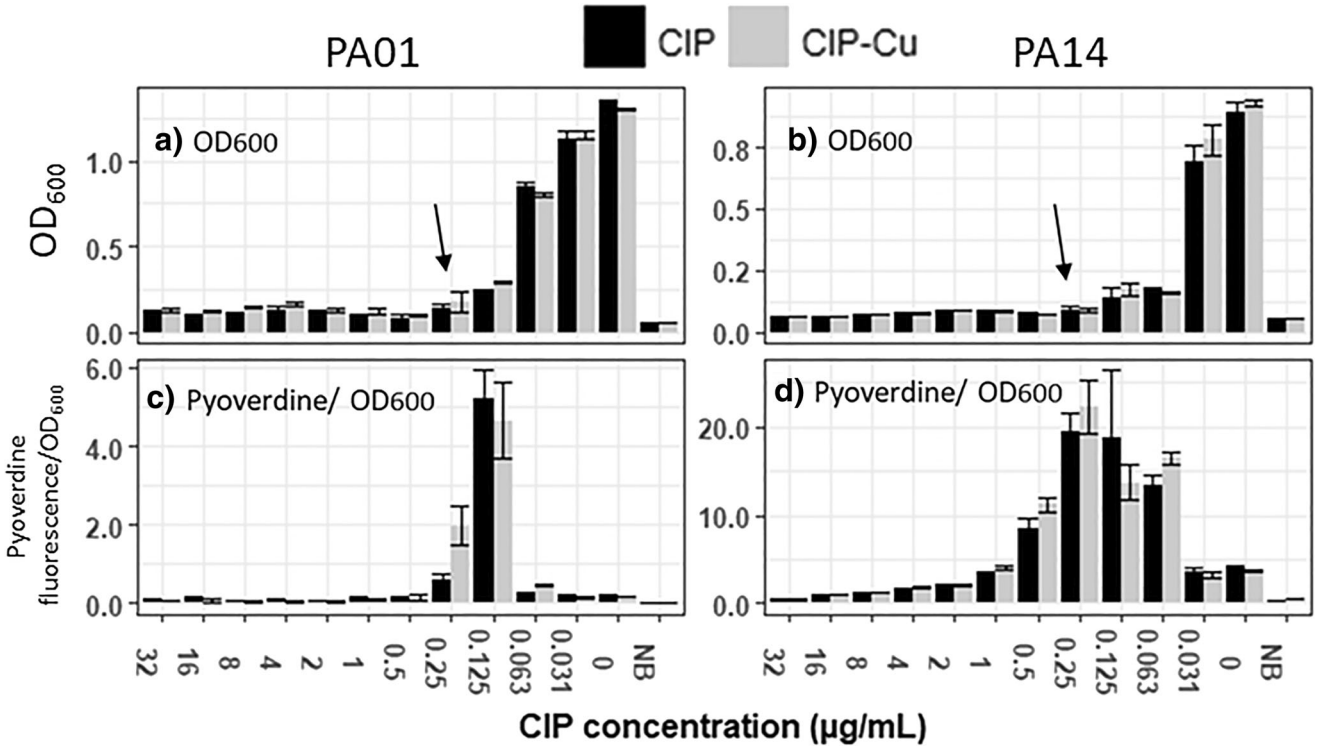

Fig. 1. *In vitro* evaluation of the response of planktonic *P. aeruginosa* PAO1 strain (left side) and PA14 strain (right side) to various concentrations of CIP and CIP-Cu complex after 20 to 24 h. **a**, **b** OD_{600} variation in the medium above the biofilms due to planktonic bacteria growth. The arrows indicate the minimum inhibitory concentration (MIC). **c**, **d** Pyoverdine fluorescence intensities normalized against the OD_{600} (Em 460/OD_{600}). Importantly, the same range of Cu^{2+} concentrations tested without CIP did not show any effect on the various tests performed. NB: no bacteria ($n = 3$; mean ± SD)

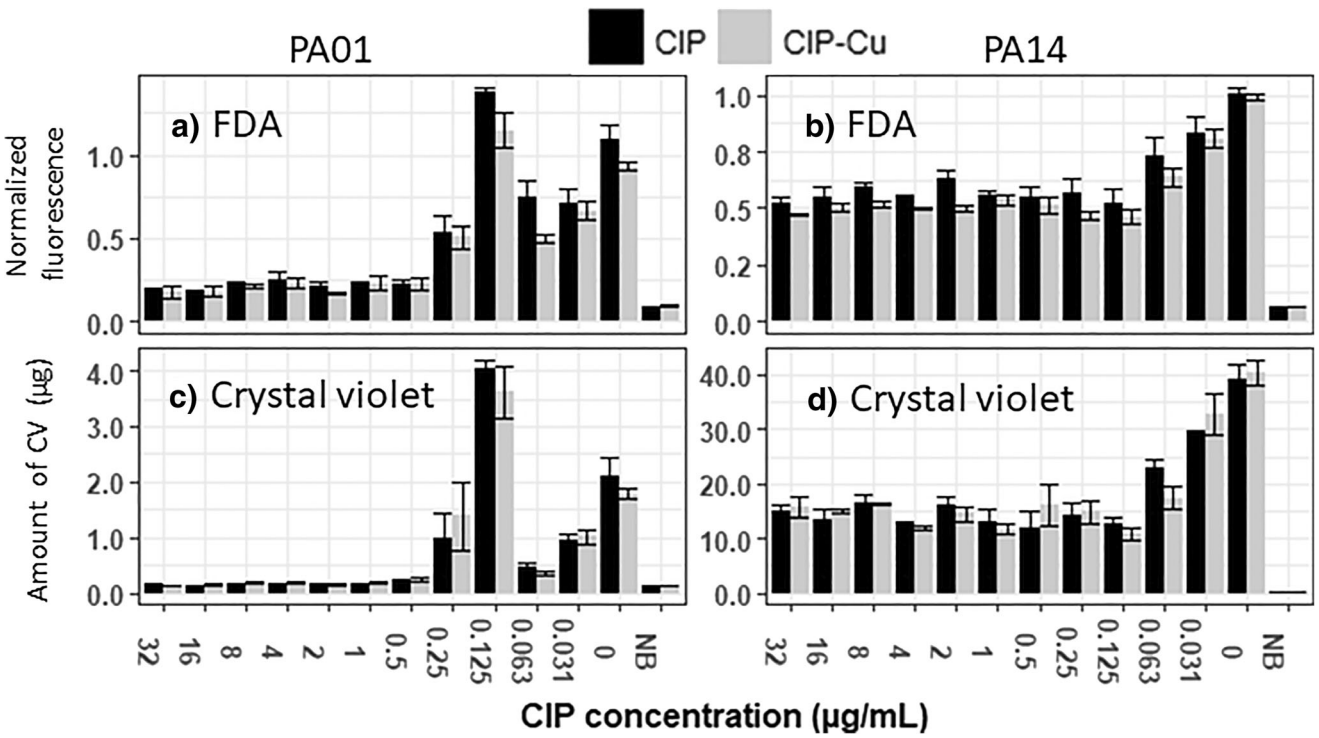

Fig. 2. *In vitro* evaluation of the response of biofilms of *P. aeruginosa* PAO1 strain (left side) and PA14 strain (right side) to various concentrations of CIP and CIP-Cu complex after 20 to 24 h. **a**, **b** Normalized enzymatic activity in the biofilms measured by the production of fluorescein from the fluorescein diacetate (FDA) hydrolysis. **c**, **d** Amount of crystal violet (CV) retained in the biofilm (µg). Importantly, the same range of Cu^{2+} concentrations tested without CIP did not show any effect on the various tests performed. NB: no bacteria ($n = 3$; mean ± SD)

Kinetic Assessment of Anti-biofilm Effects

To further explore the susceptibility of the two strains of *P. aeruginosa* to CIP and CIP-Cu, we compared the time-resolved bioluminescence curves obtained from PA01::p16Slux and UCBPP-PA14 biofilms growing in the presence of various concentrations of CIP or CIP-Cu complex (Fig. 3). No differences were observed between CIP and the CIP-Cu complex on the bioluminescence kinetic profiles of the two *P. aeruginosa* biofilms systems, suggesting that the diffusion rates of both species are similar or are not the limiting factor controlling the killing rate, *e.g.*, the electrostatic interactions between the CIP-Cu complex and the biofilm EPS matrix do not provide a strong reduction of the complex diffusion. The kinetic profiles obtained with PA01 showed a similar final effect on the whole range of CIP concentrations tested (128 to 1 times MIC). The highest CIP concentration (128 times MIC) caused a first-order kinetic decrease in luminescence. In this condition, a decrease of 95 to 99% of luminescence was achieved after 20 h (Fig. 3–blue lines). After 24 h of incubation with CIP, the same total luminescence decrease was achieved with the CIP concentration equal to the MIC. However, for CIP concentrations lower than 64 times MIC, a rebound of the luminescence signal was observed after 1–2 h. The magnitude of this rebound increased with the decrease in CIP concentration and led to a shift of 5 h in the kinetic profiles. It is interesting to highlight that for the CIP MIC, a second rebound was observed at time 18 h. This rebound could be due to a mechanism of adaptation such as the overexpression of Mex efflux pumps for example (23).

For PA14, all the bioluminescence *versus* time profiles started with an increase in luminescence until a peak was reached, followed by a slow signal decrease. In the presence of CIP, the peak relative intensity increased with a decrease in CIP concentration. For the highest CIP concentration of 128 times the MIC, a maximal decrease in luminescence of 70% was reached after 20 h.

DISCUSSION

There have been a few programs investigating the impact of inhaled antibiotics on patients with chronic infections with *P. aeruginosa*, some of which resulted in product approvals, notably solution and dry powder formulations of tobramycin (TOBI®, solution; TOBI-podhaler®, powder), colistin (Colimycine®, solution; Colobreathe®, powder), and a solution formulation of aztreonam. In 2015, a solution of levofloxacin (Quinsair®), a fluoroquinolone like CIP, was authorized for use by inhalation in Europe (24). Quinsair® has been investigated in two main clinical studies in patients who had cystic fibrosis (*CF*) with *P. aeruginosa* chronic lung infection. In the first study, Quinsair® was shown to be better than placebo at improving the patients' forced expiratory volume in 1 s (FEV1) after 28 days of treatment (1.73% *versus* 0.43% for placebo). The second study showed that Quinsair® was at least as good as tobramycin at improving FEV1 following one to three treatment cycles (25). Similarly to our approach, Quinsair® technology involved the use of a divalent cation (Mg^{2+}) to reduce the systemic absorption rate of levofloxacin after its nebulization (26,27). Mg^{2+} forms complexes with fluoroquinolones such as CIP and levofloxacin, which are less stable than the complexes formed with Cu^{2+}. Therefore, 138 times more Mg^{2+} than Cu^{2+} (in moles) is required to reduce the CIP apparent permeability by 50% across a pulmonary epithelium model (2). Also, by using the CIP-Cu^{2+} complex, one can expect higher CIP pulmonary concentration for a longer time than if using an Mg^{2+}-based complex. In fact, CIP-Ca^{2+}, which has a stability comparable to that of the CIP-Mg^{2+} complex, is five times less efficient at maintaining the CIP in rat lung after inhalation than CIP-Cu^{2+} (3).

Other CIP formulations for pulmonary inhalation are promising. Mainly, two liposomal formulations of CIP (Lipoquin® and Pulmaquin®) have been shown to be particularly efficient against intracellular bacteria that develop in lung macrophages, such as *F. tularensis* and *Y. pestis*, by concentrating in these cells (28). These inhaled CIP formulations were developed to provide a sustained CIP release profile in the lung with CIP concentrations above the MIC for a longer period of time compared to what is achieved by *i.v.* or pulmonary administration of free CIP. Also, they have been clinically evaluated for the management of *P. aeruginosa* infections in *CF* and non-cystic fibrosis bronchiectasis (BE) patients (28,29). Both formulations performed very well in phase 2b study in BE patients and Pulmaquin®, a mix of free and liposomal CIP, was taken into phase 3 clinical trials (28). In March 2018, a marketing authorization application was submitted to the European Medicines Agency for EU marketing approval of Linhaliq® (formerly called Pulmaquin®) for the treatment of BE patients with chronic lung infections with *P. aeruginosa.* One of the main reasons for the failure of the antibiotic treatments in eradicating *P. aeruginosa* from the lung of *CF* patients is the presence of biofilms associated to viscous mucus, forming a barrier to antibiotic diffusion (30). These liposomes that are able to penetrate sputum have comparable *in vitro* efficacy than free CIP against *P. aeruginosa* biofilms. The aim of this study was to evaluate the effect of positively charged CIP-Cu complex on biofilms formed by two well-characterized *P. aeruginosa* strains, PA01 and PA14. Both free CIP and its copper complex were tested *in vitro* using *P. aeruginosa* biofilms grown in microtiter plates. This method allows for a high-throughput screening of different treatments and assays on biofilms but also on planktonic cells associated to biofilms (22).

High CIP Concentration Was Required to Avoid an Increase in *P. aeruginosa* Virulence

In this study, we evaluated the production of pyoverdine as an indicator of bacterial virulence (Fig. 1c, d). In fact, for *P. aeruginosa*, pyoverdine is necessary for infection (31–33) and is mainly produced by planktonic bacteria (20), which are able to move and invade their host. Also, we normalized the pyoverdine fluorescence intensity by the OD_{600}, a parameter reflecting the planktonic population. We found that the maximal pyoverdine production was measured at sub-MIC CIP concentrations for both *P. aeruginosa* strains. In response to environmental changes, *P. aeruginosa* can activate the expression of a virulent pathogen phenotype responsible for acute infections. Shen *et al.* (34) showed that the activation of

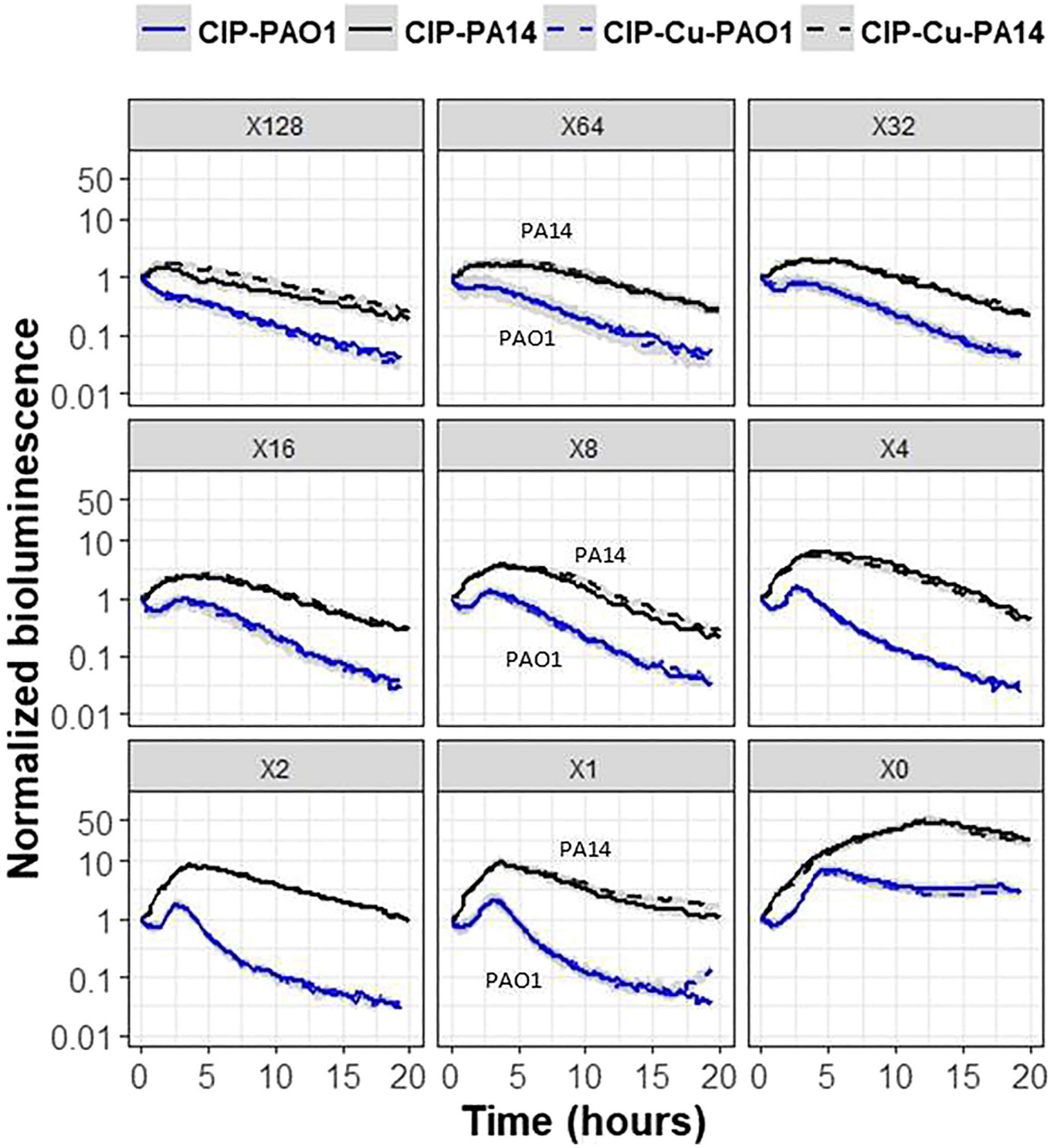

Fig. 3. PAO1::p16Slux (blue) and UCBPP-PA14 biofilm (black) time-bioluminescence kill curves obtained in the presence of various CIP (solid lines) or CIP-Cu (dashed lines) concentrations (expressed as X-times MIC). Luminescence intensities were normalized to the values measured at time zero. The gray band is the 95% confidence level interval for predictions from the LOESS smoothing model ($n = 3$)

the PA01 virulent phenotype can be induced by sub-optimal concentrations of antibiotics such as vancomycin. Similarly, our data suggest that sub-MIC CIP concentrations, which are not high enough to prevent planktonic *P. aeruginosa* growth, can stimulate the production of pyoverdine by PA14 and PA01, and therefore, possibly their virulence. Zaborin *et al.* (18) showed that such a PA01 phenotype was activated by a change in phosphate concentration, leading to an increase in pyoverdine production and in the formation of pyoverdine-Fe^{3+} complex that was lethal to mice. Additionally, they showed that the pyoverdine production was increased by a decrease in iron concentration. Also, it is interesting to highlight that CIP can form a very stable complex with iron (having a stability similar to the complex formed with copper) that can limit the free iron concentration (35). This could be a mechanism by which CIP or CIP-Cu complex stimulate the

pyoverdine production when the concentrations of the drug were not high enough to kill the bacteria. Accordingly, adequate CIP pulmonary concentrations (mainly the absence of low CIP concentrations) appear important in the treatment of *P. aeruginosa* infections; otherwise, a detriment in the patient condition could result as consequence of virulence induction.

When Treating CIP Susceptible *P. aeruginosa* Biofilms, High CIP Concentration Should Be Achieved in the Vicinity of the Biofilm as Low CIP Concentration Stimulates Biofilm Production

PA14 biofilms had a low susceptibility to CIP or CIP-Cu complex compared to PA01 biofilms. In fact, both the enzymatic activity and the total biomass were reduced, due to the presence of CIP, only up to a factor of 2 within PA14 biofilms. In comparison, the enzymatic activity within PA01 biofilms was reduced by 5-fold and the biomass by 10-fold. Also, this study suggests that for *P. aeruginosa* biofilms sensitive to CIP, such as PA01 biofilms, CIP concentrations around MIC stimulate their enzymatic activity and biomass production (Fig. 2a, c). This result is in agreement with the study of Linares *et al*. (36), which showed that CIP at sub-MIC concentrations induced the formation of PA01 biofilm. Interestingly, other antibiotics can also stimulate PA01 biofilm matrix production. For instance, alginate expression was enhanced by treatment of PA01 biofilms with sub-MIC concentrations of imipenem (37). Several studies have found that sub-MIC aminoglycoside concentrations enhance PA01 biofilm formation (36,38). Thus, several studies showed that antibiotic concentrations usually used to treat planktonic bacteria can stimulate their activity when they are assembly as a biofilm.

In our study, the sub-MIC CIP concentration that stimulated the formation of PA01 biofilms and its enzymatic activity also enhanced the pyoverdine production from the planktonic cells above the biofilms. It has been reported that pyoverdine is involved in the formation of the specific structure of PA01 biofilms (39). Moreover, it was shown that PA01 can create normal biofilms only if there is a functional pyoverdine system and only if there is a sufficient level of iron for the pyoverdine system to function (40). Also, CIP concentrations slightly below the MIC value against planktonic bacteria seem stimulate both the virulence and biofilm production of PA01.

The Formation of a CIP Cationic Complex with Copper Does Not Inhibit CIP Activity Against *P. aeruginosa* Biofilms

No differences were observed between the parameters measured after the treatment of the two *P. aeruginosa* strains with CIP or the CIP-Cu complex. Both forms of the CIP produced the same kinetic profiles with the two *P. aeruginosa* strains (Fig. 3), suggesting that both biofilm matrices had no significant impact on their diffusion within the biofilms despite the differences of electric charge between the zwitterionic $CIP^{\pm}$ (neutral) and the bicationic $^{+}CIP\text{-}Cu^{+}$ complex that was responsible for the reduction by 80% of the CIP apparent permeability across a pulmonary epithelium model made of an air/liquid cultured Calu-3 monolayer (2).

Currently, CIP is used by the oral route to treat *P. aeruginosa* pulmonary infections in *CF* patients. We have shown that sub-MIC concentrations of CIP may enhance *P. aeruginosa* biofilm formation and virulence. These results support the need to develop advanced inhalable forms of CIP that allow sustained CIP concentrations in the lungs at values higher than the MIC. This could be achieved by the new CIP inhalation systems currently in late stage development (28,41,42). For example, Lipoquin®, Pulmaquin®, and free CIP have comparable efficacy against *P. aeruginosa* biofilms *in vitro* at concentrations of 1.0–2.0 μg/mL. In this *in vitro* study, in contrast to the *in vivo* rapid systemic absorption of free CIP, CIP has no clearance pathway and its concentration in contact with the biofilm remains stable, maximizing its effect (28). Also, both liposome formulations, which sustain CIP in the lung after pulmonary administrations, are expected to produce better *in vivo* results than free CIP (28). Similarly, we have previously shown that CIP can be sustained in the lung epithelial lining fluid by forming a cationic complex with copper that reduces CIP apparent epithelium permeability and lung absorption rate (3). Our results show that the complexation with copper does not reduce its efficacy against biofilms of two different strains of *P. aeruginosa* (PA01 and PA14) producing different kind of biofilm matrices. Inhaled tobramycin is currently used to treat chronic lung infections. This antibiotic stays a much longer time in the lungs than free CIP after its pulmonary administration due to its intrinsic low systemic absorption rate compared to free CIP. For example, tobramycin concentration, measured at distribution equilibrium (around 4 h after administration), in rats pulmonary epithelial lining fluid (ELF) is 250 times higher after nebulization than after *i.v.* administration of the same dose (43). In comparison, CIP concentrations measured in pulmonary ELF 0.5 h after nebulization or *i.v.* administration of free CIP are the same, showing no benefit of nebulization over *i.v.* administration for the free CIP (3). However, due to the presence of positive charges at physiological pH, tobramycin diffusion within EPS matrix of biofilms is reduced, which limits its efficacy against pulmonary biofilms (30,44,45). Therefore, CIP-Cu complex could be a promising alternative to current inhaled antibiotics to treat pulmonary *P. aeruginosa* biofilm infections.

CONCLUSIONS

The positively charged complex made between CIP and copper that previously showed a reduced epithelial apparent permeability and reduced systemic absorption rate after pulmonary delivery has now demonstrated the same activity as free CIP against two different strains of *Pseudomonas aeruginosa*, grown planktonically or as biofilms adhered to an abiotic surface. However, the two different strains had different concentration-dependence responses to these two formulations. Mainly, PA14 biofilm was less sensitive than PA01 biofilm to both formulations.

CIP is currently used by the oral route to treat *P. aeruginosa* lung infections in *CF* patients. We observed that this antibiotic, at sub-MIC concentrations, could stimulate the production of pyoverdine, a virulence factor, in both strains and also enhance biofilm formation in PA01. These results support the need to accurately achieve high CIP

concentration in the lungs, which can be more easily realized by pulmonary delivery instead of oral administration.

FUNDING INFORMATION

This work was supported by a financial support from the Region Poitou-Charentes (grant to F. Tewes), the University of Poitiers, and an EU FP7 grant (Cofund PRESTIGE-2015-3-0022 under grant agreement no PCOFUND-GA-2013-609102).

REFERENCES

1. Wehbe M, Leung AW, Abrams MJ, Orvig C, Bally MB. A perspective–can copper complexes be developed as a novel class of therapeutics? Dalton Trans. 2017;46(33):10758–73.
2. Brillault J, Tewes F, Couet W, Olivier J. In vitro biopharmaceutical evaluation of ciprofloxacin/metal cation complexes for pulmonary administration. Eur J Pharm Sci. 2017;97:92–8.
3. Lamy B, Tewes F, Serrano DR, Lamarche I, Gobin P, Couet W, *et al*. New aerosol formulation to control ciprofloxacin pulmonary concentration. J Control Release. 2018;271:118–26.
4. Wagner VE, Iglewski BH. P. aeruginosa biofilms in CF infection. Clin Rev Allergy Immunol. 2008;35(3):124–34.
5. Bahamondez-Canas TF, Zhang H, Tewes F, Leal J, Smyth HD. PEGylation of tobramycin improves mucus penetration and antimicrobial activity against Pseudomonas aeruginosa biofilms in vitro. Mol Pharm. 2018;15(4):1643–52.
6. Geller DE. Aerosol antibiotics in cystic fibrosis. Respir Care. 2009;54(5):658–70.
7. van Tilburg Bernardes E, Charron-Mazenod L, Reading D, Reckseidler-Zenteno SL, Lewenza S. Small molecules with antibiofilm, antivirulence and antibiotic synergy activities against Pseudomonas aeruginosa. bioRxiv. 2016:067074.
8. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms. PLoS Pathog. 2008;4(11):e1000213.
9. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, *et al*. The in vivo biofilm. Trends Microbiol. 2013;21(9):466–74. https://doi.org/10.1016/j.tim.2013.06.002.
10. Wilton M, Charron-Mazenod L, Moore R, Lewenza S. Extracellular DNA acidifies biofilms and induces aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2016;60(1):544–53.
11. Du J, Bandara H, Du P, Huang H, Hoang K, Nguyen D, *et al*. Improved biofilm antimicrobial activity of polyethylene glycol conjugated tobramycin compared to tobramycin in pseudomonas aeruginosa biofilms. Mol Pharm. 2015;12(5):1544–53.
12. Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9(1):34–9. https://doi.org/10.1016/S0966-842X(00)01913-2.
13. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents. 2010;35(4):322–32.
14. Jolivet-Gougeon A, Bonnaure-Mallet M. Biofilms as a mechanism of bacterial resistance. Drug Discov Today Technol. 2014;11:49–56. https://doi.org/10.1016/j.ddtec.2014.02.003.
15. Tewes F, Brillault J, Smyth H. Inhalable microparticles loaded with a fluoroquinolone/metal cation complex for the treatment of respiratory diseases. International Application No.: PCT/IB2016/001866. Pub. No.: WO/2018/104759. 2018.
16. Riedel CU, Casey PG, Mulcahy H, O'Gara F, Gahan CG, Hill C. Construction of p16Slux, a novel vector for improved bioluminescent labeling of gram-negative bacteria. Appl Environ Microbiol. 2007;73(21):7092–5.
17. Murray JL, Kwon T, Marcotte EM, Whiteley M. Intrinsic antimicrobial resistance determinants in the superbug Pseudomonas aeruginosa. mBio. 2015;6(6). https://doi.org/10.1128/mBio.01603-15.
18. Zaborin A, Romanowski K, Gerdes S, Holbrook C, Lepine F, Long J, *et al*. Red death in Caenorhabditis elegans caused by Pseudomonas aeruginosa PAO1. Proc Natl Acad Sci. 2009;106(15):6327–32.
19. Wozniak DJ, Wyckoff TJ, Starkey M, Keyser R, Azadi P, O'Toole GA, *et al*. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms. Proc Natl Acad Sci. 2003;100(13):7907–12.
20. Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC, *et al*. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. Nat Commun. 2014;5.
21. Tewes F, Brillault J, Lamy B, O'Connell P, Olivier J-C, Couet W, *et al*. Ciprofloxacin-loaded inorganic–organic composite microparticles to treat bacterial lung infection. Mol Pharm. 2015;13(1):100–12.
22. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods. 2008;72(2):157–65. https://doi.org/10.1016/j.mimet.2007.11.010.
23. Grégoire N, Raherison S, Grignon C, Comets E, Marliat M, Ploy M-C, *et al*. Semimechanistic pharmacokinetic-pharmacodynamic model with adaptation development for time-kill experiments of ciprofloxacin against Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2010;54(6):2379–84.
24. Elborn JS, Vataire A-L, Fukushima A, Aballea S, Khemiri A, Moore C, *et al*. Comparison of inhaled antibiotics for the treatment of chronic Pseudomonas aeruginosa lung infection in patients with cystic fibrosis: systematic literature review and network meta-analysis. Clin Ther. 2016;38(10):2204–26.
25. Stuart Elborn J, Geller DE, Conrad D, Aaron SD, Smyth AR, Fischer R, *et al*. A phase 3, open-label, randomized trial to evaluate the safety and efficacy of levofloxacin inhalation solution (APT-1026) versus tobramycin inhalation solution in stable cystic fibrosis patients. J Cyst Fibros. 2015;14(4):507–14. https://doi.org/10.1016/j.jcf.2014.12.013.
26. Loutit JS, Morgan EE, Dudley MN, Griffith DC, Lomovskaya O. Use of aerosolized levofloxacin for treating cystic fibrosis. U.S. Patent Application No. 15/623,168. 2018.
27. Griffith DC, Dudley MN, Surber MW, Bostian KA, Rodny O. Aerosol fluoroquinolone formulations for improved pharmacokinetics. U.S. Patent 8,815,838. 2014.
28. Cipolla D, Blanchard J, Gonda I. Development of liposomal ciprofloxacin to treat lung infections. Pharmaceutics. 2016;8(1):6.
29. Cipolla D, Shekunov B, Blanchard J, Hickey A. Lipid-based carriers for pulmonary products: preclinical development and case studies in humans. Adv Drug Deliv Rev. 2014;75:53–80. https://doi.org/10.1016/j.addr.2014.05.001.
30. Braun A, Sewald K, Müller L, Wronski S, Murgia X, Lehr C-M, *et al*. Human airway mucus alters susceptibility of Pseudomonas aeruginosa biofilms to tobramycin, but not colistin. J Antimicrob Chemother. 2018;73(10):2762–9. https://doi.org/10.1093/jac/dky241.
31. Visca P, Imperi F, Lamont IL. Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. 2007;15(1):22–30. https://doi.org/10.1016/j.tim.2006.11.004.
32. Meyer J-M, Neely A, Stintzi A, Georges C, Holder IA. Pyoverdin is essential for virulence of Pseudomonas aeruginosa. Infect Immun. 1996;64(2):518–23.
33. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D. Pseudomonas aeruginosa pyocyanin is critical for lung infection in mice. Infect Immun. 2004;72(7):4275–8.
34. Shen L, Shi Y, Zhang D, Wei J, Surette MG, Duan K. Modulation of secreted virulence factor genes by subinhibitory concentrations of antibiotics in Pseudomonas aeruginosa. J Microbiol. 2008;46(4):441–7.
35. Uivarosi V. Metal complexes of quinolone antibiotics and their applications: an update. Molecules. 2013;18(9):11153–97.
36. Linares JF, Gustafsson I, Baquero F, Martinez J. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci. 2006;103(51):19484–9.
37. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, *et al*. Pseudomonas aeruginosa biofilms exposed

to imipenem exhibit changes in global gene expression and β-lactamase and alginate production. Antimicrob Agents Chemother. 2004;48(4):1175–87.
38. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005;436(7054):1171–5.
39. Yang L, Nilsson M, Gjermansen M, Givskov M, Tolker-Nielsen T. Pyoverdine and PQS mediated subpopulation interactions involved in Pseudomonas aeruginosa biofilm formation. Mol Microbiol. 2009;74(6):1380–92.
40. Banin E, Vasil ML, Greenberg EP. Iron and Pseudomonas aeruginosa biofilm formation. Proc Natl Acad Sci U S A. 2005;102(31):11076–81.
41. Aksamit T, Bandel T-J, Criollo M, De Soyza A, Elborn JS, Operschall E, *et al*. The RESPIRE trials: two phase III, randomized, multicentre, placebo-controlled trials of ciprofloxacin dry powder for inhalation (ciprofloxacin DPI) in non-cystic fibrosis bronchiectasis. Contemp Clin Trials. 2017;58:78–85. https://doi.org/10.1016/j.cct.2017.05.007.
42. Sugianto TD, Chan H-K. Inhaled antibiotics in the treatment of non-cystic fibrosis bronchiectasis: clinical and drug delivery perspectives. Expert Opin Drug Deliv. 2016;13(1):7–22.
43. Marchand S, Grégoire N, Brillault J, Lamarche I, Gobin P, Couet W. Biopharmaceutical characterization of nebulized antimicrobial agents in rats: 3. Tobramycin. Antimicrob Agents Chemother. 2015;59(10):6646–7. https://doi.org/10.1128/aac.01647-15.
44. Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, *et al*. Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function. J Bacteriol. 2001;183(18):5395–401.
45. Khan W, Bernier SP, Kuchma SL, Hammond JH, Hasan F, O'Toole GA. Aminoglycoside resistance of Pseudomonas aeruginosa biofilms modulated by extracellular polysaccharide. Int Microbiol. 2010;13(4):207–12.