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Anti-biofilm activity of murepavadin against cystic fibrosis *Pseudomonas aeruginosa* isolates

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Objectives: To determine the activity of murepavadin in comparison with tobramycin, colistin and aztreonam, against cystic fibrosis (CF) *Pseudomonas aeruginosa* isolates growing in biofilms. The biofilm-epidemiological cut-off (ECOFF) values that include intrinsic resistance mechanisms present in biofilms were estimated.

Methods: Fifty-three CF *P. aeruginosa* isolates from respiratory samples were tested using the Calgary (closed system) device, while 4 [2 clinical (one smooth, one mucoid) and 2 reference strains] were tested using the BioFlux, a microfluidic open model of biofilm testing. Biofilm was stained with SYTO9® and propidium iodide. The minimal biofilm inhibitory concentration (MBIC) and the minimal biofilm eradication concentration (MBEC) were determined. The MBIC-ECOFF and the MBEC-ECOFF were calculated.

Results: Colistin, tobramycin and murepavadin presented similar $MBIC_{50}$/$MBIC_{90}$ values (4/32, 8/64 and 2/32, respectively). Murepavadin exhibited the lowest $MBEC_{90}$ (64 mg/L). Aztreonam MBIC and MBEC values were higher than those of the other antibiotics tested. Tobramycin and murepavadin had the lowest MBEC-ECOFF (64 and 128 mg/L, respectively), while those of aztreonam and colistin exceeded 512 mg/L. Using the BioFlux, for the PAO1, PAO *mutS* and the smooth clinical strain, a significant difference ($P < 0.0125$) was observed when comparing the fluorescence of treated and untreated biofilms. For the mucoid strain, only the biofilm treated with aztreonam (MBIC and MBEC) and tobramycin (MBEC) showed differences with respect to the untreated biofilm.

Conclusions: Murepavadin demonstrated good activity against *P. aeruginosa* biofilms both in open and closed systems. The MBIC-ECOFF and the MBEC-ECOFF are proposed as new parameters to estimate the activity of antibiotics on biofilms.

Introduction

Pseudomonas aeruginosa is a major cause of morbidity and mortality in people with cystic fibrosis (CF). The biofilm mode of growth is directly involved in the pathogenesis of this microorganism, which makes its eradication extremely difficult.[1]

Currently, three antibiotics are registered for inhaled administration: tobramycin, colistin, and aztreonam-lysine. Murepavadin (formerly POL7080, Polyphor Ltd.) is a novel antibiotic targeting an outer-membrane protein (LptD) that exhibits selective anti-*P. aeruginosa* activity; it could therefore be a potential alternative for CF treatment without perturbing the respiratory microbiota.[2]

Two types of assays are available to evaluate the *in vitro* antibiotic activity of compounds against biofilms: open and closed systems. The closed or static systems analyse biofilm formation in the wells of microtitre plates and are suitable for high-throughput analysis, obtaining pharmacodynamics (PD) parameters. In contrast, the open or dynamic systems resemble *in vivo* conditions more closely. The BioFlux system (Fluxion, San Francisco, CA) is an open high-throughput model based on microfluidics to create biofilms grown in continuous flow.[3,4]

The biofilm mode of growth confers increased tolerance towards antibiotics. Higher concentrations and longer exposures are required to kill bacteria growing in biofilm than those growing planktonically. Various factors contribute to this tolerance, in particular the binding of antibiotics to matrix constituents, the anaerobic environment and the presence of persister cells.[5,6]

No breakpoints for biofilm-growing bacteria and for inhalation therapy have yet been established. However, PD parameters such as the minimal biofilm inhibitory concentration (MBIC) or the minimal biofilm eradication concentration (MBEC) provide an indication of the antibiofilm activity of an antibiotic. The latest EUCAST recommendation for the topical use of antimicrobials is to apply the epidemiological cut-off (ECOFF) values to indicate susceptibility to these types of agents (EUCAST Guidance Document—Breakpoints for topical agents, 29 March 2014). The ECOFF separates the wild-type bacterial population from that which has acquired mechanisms of resistance, but this breakpoint is based on planktonically growing bacteria and does not consider biofilm-associated antimicrobial activity.

In the present study, the effect of the new antimicrobial murepavadin, and the comparators tobramycin, aztreonam and colistin, was tested against CF *P. aeruginosa* isolates growing in biofilms in the Calgary and BioFlux devices, a closed and an open model, respectively. The MBIC-ECOFF and the MBEC-ECOFF were determined, as indicators that could potentially discriminate strains possessing acquired mechanisms of resistance and tolerance to antibiotics from the wild-type population when growing in biofilm.

Methods

Strains

To perform biofilm tests with the Calgary device, 53 *P. aeruginosa* isolates from respiratory samples of people with CF were selected from the 414 *P. aeruginosa* isolates of the iABC-collection.[7] A representative number of isolates from Northern Ireland, Spain, the Netherlands and Australia were chosen, including different morphotypes: 18 smooth, 11 mucoid, 11 rough, 10 metallic, and 3 small colony variants. PAO1 and its hypermutator derivative, PAO *mutS*, were used as control strains.[8]

Two CF isolates, *PA*34 (smooth phenotype) and *PA*40 (mucoid phenotype), and the two reference strains PAO1 and PAO *mut*S were tested with the BioFlux device. None of these two clinical strains were hypermutators.

MIC values were determined by standard broth microdilution. For the susceptibility categorization of the strains, EUCAST breakpoints were used.[9]

Biofilm assays performed with the Calgary static device

This assay was performed as previously described, but with minor variations[3] (Figure S1, available as Supplementary data at *JAC* Online). Briefly, a 0.5 McFarland culture was transferred to a flat-bottom 96-well microtitre plate (Nunc International, Rochester, NY). Bacterial biofilm was formed around the pegs of a modified polystyrene microtitre lid. This lid with pegs was immersed into a growth plate and incubated for 20 h at 37°C. After rinsing the pegs three times in saline solution, they were placed onto the antimicrobial in BBL Mueller-Hinton II cation-adjusted broth (MHB)[Becton, Dickinson (BD), Sparks, MD] and incubated for 20 h at 37°C. Two-fold increasing concentrations (0.5–512 mg/L) of colistin sulfate (Sigma–Aldrich Chemical Co., St Louis, MO), tobramycin (Sigma–Aldrich Chemical Co.), aztreonam (Azactam®, Bristol-Myers Squibb), and murepavadin (Polyphor Ltd) were used. After this incubation, the biofilm was recovered by centrifuging (800 RPM/110 RCF for 10 min) the peg lid in an antibiotic-free MHB filled-microtitre plate. Experiments were done in triplicate. The MBIC was calculated after measuring the OD (450 nm) before and after a 6 h incubation. Biofilm growth was defined as a mean OD difference of $\geq$0.05. MBIC was defined as the lowest antibiotic concentration that resulted in an OD difference at or below 10% of the OD positive control. The MBEC was defined as the lowest concentration that prevents visible growth in the biofilm recovery medium after 18 h incubation of the microtitre plate.[10,11] Results were graphed as the cumulative percentage of inhibited strains against the antibiotic concentrations.

Biofilm assays performed by the BioFlux microfluidic open system

Several biofilms were simultaneously grown in a 48-well plate in the BioFlux 200 system, following an adapted protocol from Benoit *et al.*[4] (Figure S2). Microchannels were filled with 100 μL of prewarmed diluted (0.1×) LB media (Oxoid, LTD., Basingstoke, Hampshire, UK) through the input wells (5 min, 1 dyne/cm^2). For the cell attachment, 85 μL of a 10^8–10^9 cfu/mL bacterial suspension was inoculated into the output wells for 5 s at 2 dyne/cm^2 and incubated without flow for 2 h at 30°C. After these 2 h, biofilm was formed during 18 h of continuous flow at 30°C (0.15 dyne/cm^2). Biofilm formation was checked after the incubation and antibiotic was added to the inlet wells, (except for the inoculum control), incubating the biofilm under continuous flow for 18 h (30°C, 0.15 dyne/cm^2). The tested antibiotic concentrations corresponded to those of antibiotic MBIC and MBEC values obtained with the Calgary device. Tests were performed in triplicate. After incubation, biofilms were washed by injecting saline solution from the input reservoir for 10 min at 0.5 dyne. The biofilm was stained with the *Bac*Light LIVE/DEAD stain (Invitrogen, ThermoFisher Scientific, Paisley, UK), which consists of SYTO9® and propidium iodide (PI) prepared according to the manufacturer's instructions. These stains were pumped through the channels (0.7 dyne, 15 min), and afterwards the channels were washed for 20 min with saline solution to remove excess stain. Biofilm images were taken using a Nikon Eclipse Ti microscope and analysed with the Image J program. The mean percentage of live (green) and dead (red) fluorescence signal from biofilms were represented in a bar chart. Between groups, comparisons of untreated and treated biofilms were analysed using one-way ANOVA tests with post-hoc tests using Scheffe multiple comparison procedure. We used a Bonferroni correction and we lowered our significance level to 0.0125 to correct the type I error rate adjusting for multiple comparison (i.e. PAO1, PAO *mutS*, PA34 and PA40). Stata statistical software was used (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

Biofilm-ECOFF estimation

The ECOFF values were calculated with the ECOFFinder program. It is implemented in a Microsoft Excel® workbook (ECOFFinder) that can be downloaded from the CLSI website (http://clsi.org/standards/micro/ecoffinder/). The 97.5% subset ECOFF was chosen as it is the one that comes closest to the conventional visual ('eyeball') method.[12] The MIC, MBIC and the MBEC distributions were compared (with the MIC, MBIC and MBEC based-ECOFF represented).

Results

Calgary results

Thirty-two (60.4%) isolates were biofilm producers; 8 were classified as weak producers (<25th percentile), 16 as moderate producers (25th–75th percentile) and 8 as strong producers (>75th percentile). Between these biofilm producers, all the morphotypes commonly observed in CF isolates were represented: 6 metallic, 6 mucoid, 7 rough, 12 smooth and 1 small colony variant. MBIC and MBEC values were determined in these 32 biofilm-producing isolates.

$MIC_{50/90}$, $MBIC_{50/90}$, and $MBEC_{50/90}$ and MIC, MBIC and MBEC range results for each antibiotic are described in Table 1. These results are also graphically represented in Figure 1 and Figure 2.

Table 1. Planktonic (MIC_{50}/MIC_{90}) and biofilm ($MBIC_{50}$/$MBIC_{90}$, $MBEC_{50}$/$MBEC_{90}$) activities (in mg/L) for each tested antibiotic

Substance	MIC_{50}/MIC_{90}	MIC range	$MBIC_{50}$/$MBIC_{90}$	MBIC range	$MBEC_{50}$/$MBEC_{90}$	MBEC range
Murepavadin	≤0.5/1	≤0.5–32	4/32	≤0.5–64	16/64	1–>512
Colistin	1/4	≤0.5–16	8/64	≤0.5–256	64/128	≤0.5–256
Tobramycin	2/16	≤0.5–256	2/32	≤0.5–512	8/256	≤0.5–512
Azithromycin	4/256	0.5–512	32/>512	1–>512	512/>512	1–>512

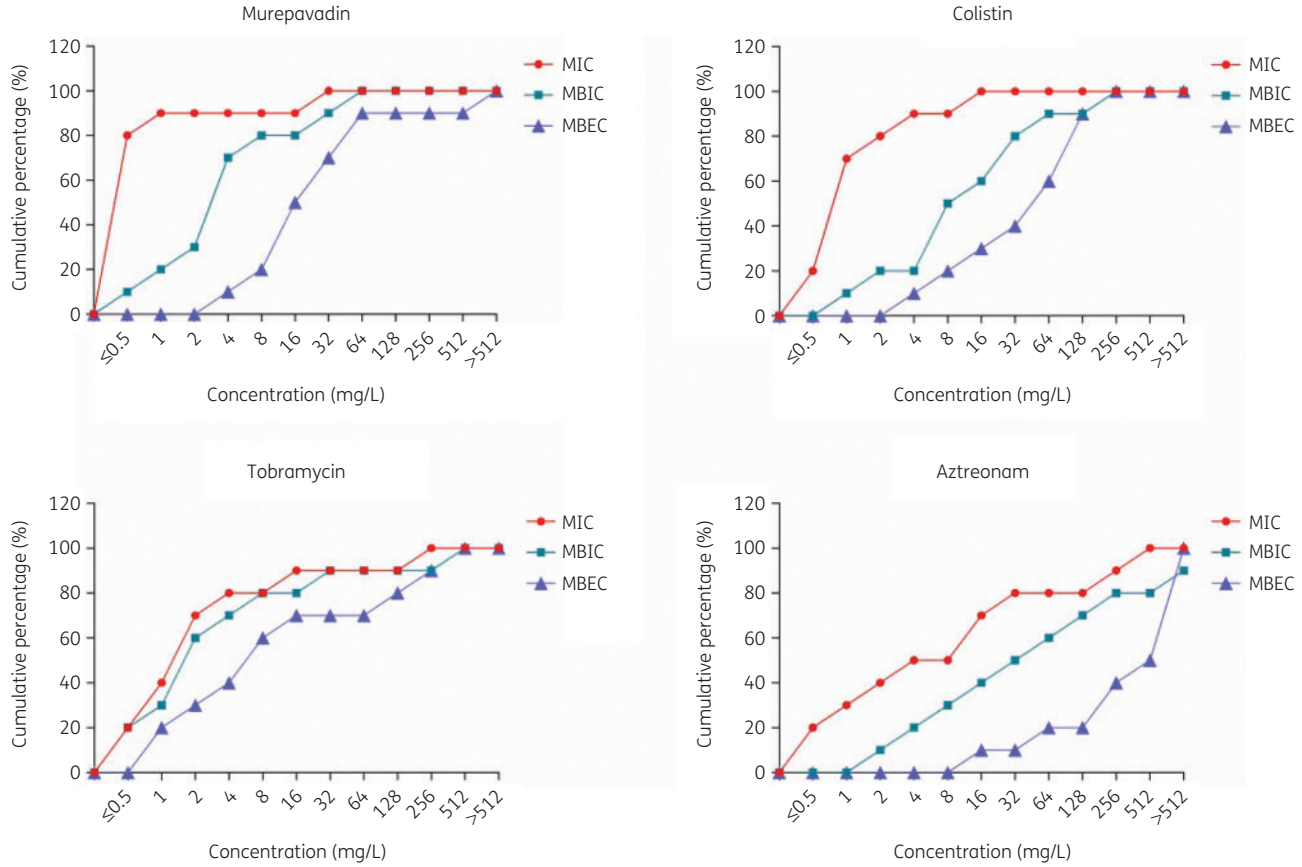

Figure 1. Antimicrobial activity of murepavadin, colistin, tobramycin and aztreonam according to their respective MIC, MBIC and MBEC values. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

As seen in Figure 1, the higher the line is located on the left side, the more active the antibiotic. While tobramycin and murepavadin showed the best efficacy against biofilm growth, colistin and murepavadin presented the best activity against planktonic cells. Tobramycin presented the lowest variations between planktonic and biofilm activity, as the cumulative percentage of inhibited strains with the MIC and the MBIC and MBEC was close all over the concentrations range. For this antibiotic, the $MBIC_{50}$ and $MBEC_{50}$ were the same and two 2-fold dilutions higher than the MIC_{50}, respectively. A larger difference between planktonic and biofilm activity was observed for colistin and murepavadin. For colistin $MBIC_{50}$ and $MBEC_{50}$ were three and six 2-fold dilutions higher than the MIC_{50}, respectively. For murepavadin $MBIC_{50}$ and $MBEC_{50}$ were three and five 2-fold dilutions higher than the MIC_{50}, respectively. Aztreonam showed a large difference between the MIC and the biofilm PD parameters (MIC_{50}=4 mg/L versus $MBEC_{50}$=512 mg/L, seven 2-fold dilutions difference).

Differences were observed between the PAO1 and PAO *mutS* murepavadin biofilm susceptibility, with an increase of three and five 2-fold dilutions in the MBIC and the MBEC, respectively, for the PAO *mutS* compared with the PAO1 values (Table 2).

The biofilm antimicrobial activity was also analysed according to biofilm production (weak, moderate and strong biofilm producers), but no clear differences in the antibiotic activity linked to the biofilm production were observed (Table S1).

BioFlux results

The planktonic and biofilm susceptibility of the strains used in the BioFlux device are described in Table 2. All the tested strains were

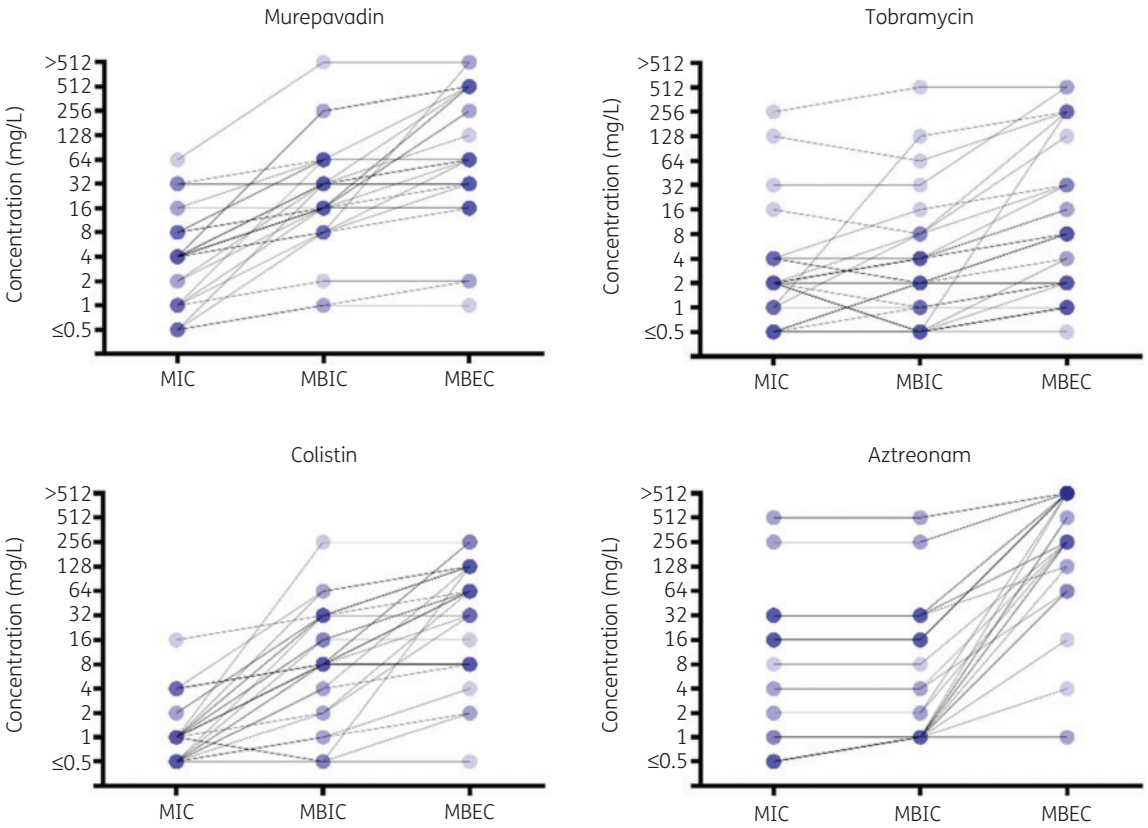

Figure 2. Variations in MIC, MBIC and MBEC values for murepavadin, tobramycin, colistin and aztreonam for the individual *P. aeruginosa strains*. Superimposed points and lines are represented by a darker shade. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 2. Susceptibility (mg/L) of the strains used in the BioFlux device

Substance	PAO1	PAO *mutS*	*PA40*	*PA34*
Murepavadin				
MIC	0.06	0.12	0.06	0.06
MBIC	4	32	2	2
MBEC	8	256	8	4
Aztreonam				
MIC	4	64	16	0.5
MBIC	>256	>256	32	8
MBEC	>256	>256	>256	256
Tobramycin				
MIC	0.5	2	0.5	0.25
MBIC	2	16	2	2
MBEC	16	32	4	2
Colistin				
MIC	1	1	0.5	2
MBIC	64	256	32	32
MBEC	128	256	32	128

susceptible to colistin (MIC $\leq$2 mg/L) and tobramycin (MIC $\leq$2 mg/L). While the PAO1 and the two CF isolates were categorized as 'susceptible increased exposure' (I) to aztreonam, PAO *mutS* was resistant to this antibiotic (MIC >16 mg/L) (https://www.eucast.org/clinical_breakpoints/). The strains showed a murepavadin MIC range from 0.06 to 0.12 mg/L.

BioFlux results are shown in Figure 3. For the control (PAO1 and PAO *mutS*) and *PA34* clinical strains, a statistically significant difference was observed ($P < 0.0125$) when comparing the fluorescence registered in the control channel (without antibiotic) with those treated with the antibiotics at the MBIC and MBEC concentrations. However, for the mucoid clinical *PA40* strain, only the MBIC and the MBEC of aztreonam and the MBEC of tobramycin showed differences when compared with the inoculum.

Bacterial cells within the biofilm that were treated with aztreonam displayed filamentous structures (Figure S3).

Experiments were also conducted at the MIC values of the different antibiotics, but, due to the inefficacy of these concentrations against the biofilm, the overgrowth collapsed the microchannel, precluding the staining and, consequently, the collection of numerical results.

Biofilm-ECOFF results

The ECOFF results can be observed in Figure 4. Murepavadin displayed the lowest MIC-ECOFF value. Tobramycin and colistin showed similar MIC-ECOFF values to those published by EUCAST. However, the aztreonam MIC-ECOFF was higher (256 mg/L) than

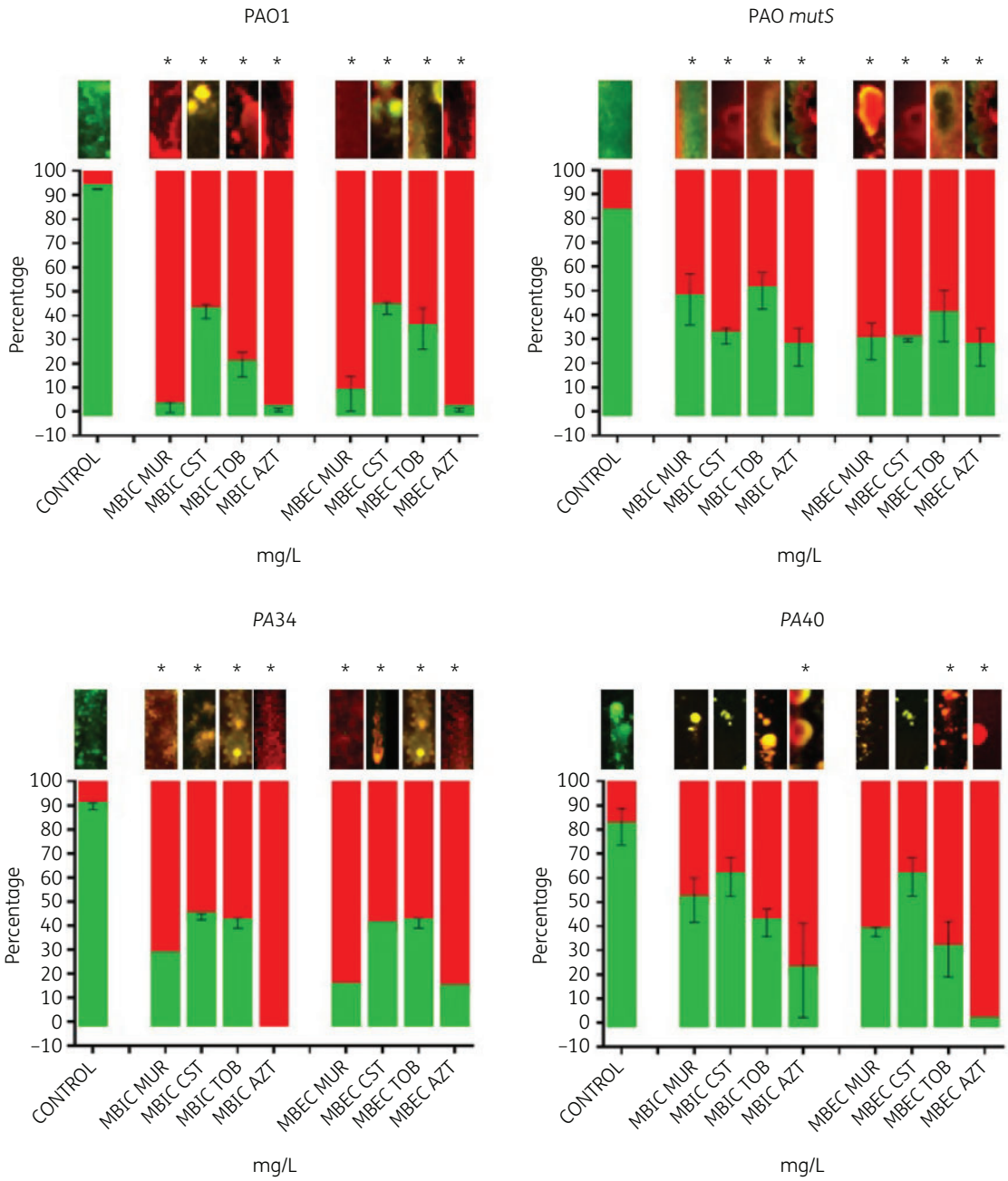

Figure 3. Bar chart showing BioFlux results. The percentage of intensity of red (dead cells) and green (live cells) fluorescence within the biofilm are expressed for each antibiotic and tested strain. In the upper part of the bars the microscope images are displayed. *=$P < 0.0125$ versus no antibiotic, ANOVA test with Scheffe correction. MUR, murepavadin; CST, colistin; TOB, tobramycin; AZT, azithromycin.

the one established by EUCAST (16 mg/L). Also, an aztreonam MIC-ECOFF of 256 mg/L was obtained for the iABC-complete collection.[7] This result indicates differences in aztreonam activity between CF and non-CF *P. aeruginosa* isolates.

Considering all the tested antibiotics, the ECOFF-MBIC values were between one and nine 2-fold dilutions higher than the ECOFF-MICs, and the ECOFF-MBECs were from three to ten 2-fold dilutions higher than the ECOFF-MICs. Murepavadin and colistin presented the highest differences between planktonic and biofilm ECOFF values. Tobramycin and murepavadin presented the lowest MBEC-ECOFF values (64 and 128 mg/L, respectively), while those of aztreonam and colistin exceeded 512 mg/L.

Discussion

Although neither the EUCAST nor the CLSI has standardized end-point parameters in biofilm antibiotic susceptibility, the MBIC and the MBEC can be used to determine the anti-biofilm antibiotic *in vitro* efficacy. MBIC is the lowest concentration at which there is no time-dependent increase in the mean number of biofilm viable

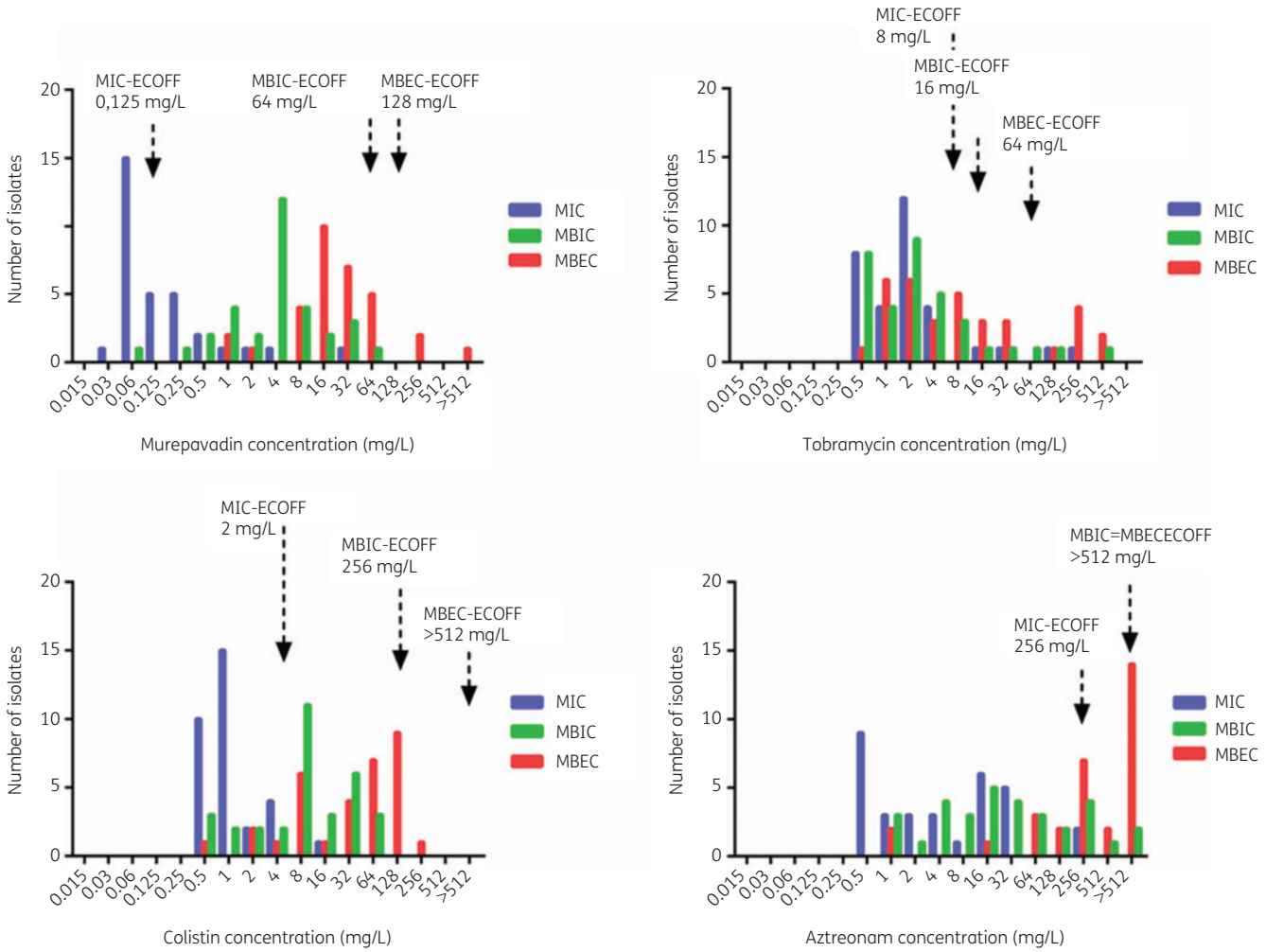

Figure 4. MIC, MBIC and MBEC distributions (with the MIC, MBIC and MBEC based-ECOFF) for the tested antibiotics.

cells comparing an early with a later exposure time, while the MBEC indicates the eradication (partly or complete) of biofilm viable cells.[13]

There is controversy as to whether treatment decisions should be based on MBIC or MBEC values because studies supporting the clinical validity of the *in vitro* models are lacking. However, biofilm testing increases the knowledge of how an antibiotic acts on bacteria in chronic infections and future standardization of methodologies may allow the screening of large libraries for potential compounds with anti-biofilm activity.[14,15]

In this study, two ECOFFs based on the MBIC and MBEC values are proposed to establish the activity of antimicrobials within the biofilm. These parameters would differentiate the wild-type biofilm bacterial population and the population with acquired resistance mechanisms, including those concerning biofilm mode of growth, as antimicrobial tolerance. With the combination of MBIC, MBEC, ECOFFs, pharmacokinetics data on antibiotic lung exposure with inhaled therapy and clinical efficacy data, it may ultimately be possible to establish pharmacokinetics and pharmacodynamics targets for inhaled therapy, to determine whether an inhaled antibiotic will be active, and to predict therapeutic success in individual patients. These parameters are a novel approach that would require further studies with a large collection of strains to investigate their usefulness for clinical prediction. Also, future studies will need to establish which of the two (MBIC or MBEC) if either, is the better predictor.

The novel antibiotic murepavadin has demonstrated a high *in vitro* activity against CF *P. aeruginosa* isolates, with a MIC_{50} and MIC_{90} of 0.12 and 2 mg/L, respectively.[7] Also, its activity is affected neither by artificial sputum nor by lung surfactant, which supports its development for CF inhaled therapy.[16]

Murepavadin was also highly effective against *P. aeruginosa* growing in biofilms, both in open and in closed biofilm-testing systems, although less activity against the hypermutator derivative PAO *mutS* than against the one produced by the PAO1 control strain was observed. This decrease in the activity of murepavadin against hypermutator strains was previously demonstrated in time–kill and mutant prevention concentration assays, in which PAO *mutS* second step mutants were obtained.[16] The high antibiotic resistance rate and the resistance development during antibiotic exposure are common features described for hypermutable strains, which are frequently encountered strains in people with CF, and more generally in chronic respiratory infections.[8]

The MBECs and MBICs of the tested antibiotics confirmed their effectiveness to reduce the biofilm developed in the continuous fluid system for the control and the smooth phenotype (*PA*34) strains. It should be noted that murepavadin concentrations used for the PAO *mutS* were up to five 2-fold dilutions higher than those used for the PAO1 control strain.

However, for the mucoid phenotype strain (*PA*40) only aztreonam (MBIC and MBEC) and tobramycin (MBEC) were effective. Also, this strain presented different biofilm architecture, developing a microcolony-type biofilm with less attachment surface. An increase in antibiotic resistance for mucoid phenotypes of CF *P. aeruginosa* has been previously described. The mucoid phenotype favours persistence and its appearance is generally associated with deterioration of patient's prognosis.[17]

The discrepancy observed in aztreonam activity when using the Calgary or the BioFlux device was an unexpected result. This antibiotic presented the lowest activity according to its MBIC and MBEC values. However, it was highly effective in the dynamic model, even against the mucoid *PA*40 strain. The filamentous structures observed in aztreonam-treated biofilms indicate the high affinity of this antibiotic for penicillin-binding protein 3 (PBP-3), which is involved in bacterial septation.[18]

Discrepancies in aztreonam *in vitro* susceptibility according to the tested methodology have been previously described.[19] MIC results differed from aztreonam biofilm activity on a biotic surface, as aztreonam-resistant strains (defined by MIC > 256 mg/L), were susceptible to this antibiotic when grown as a pre-formed biofilm on airway cells.

Conclusions

Murepavadin has demonstrated high activity against biofilms of CF *P. aeruginosa*. This characteristic together with its preserved activity in lung surfactant and artificial sputum[16] make this antibiotic a candidate for use in inhalation therapy. The MBIC-ECOFF and the MBEC-ECOFF are proposed as new parameters to estimate antibiotic activity on biofilms.

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Transparency declarations

F.B. and D.O. are employed by Polyphor AG, the patent holder of murepavadin. All other authors have none to declare.

Supplementary data

Table S1 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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