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Evaluation of antibiofilm activity of cefiderocol alone and in combination with imipenem against *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: The main aim of this study was to evaluate the antibiofilm activity of cefiderocol alone and in combination with imipenem vs. sessile cells of *Pseudomonas aeruginosa*, assessing a potential synergistic bactericidal effect.

Methods: Ten *P. aeruginosa* clinical isolates from infected implants and bloodstream were included in the study. Cefiderocol was tested alone and in combination with imipenem on 24-h-old *P. aeruginosa* biofilm formed on porous glass beads. For each antibiotic formulation, minimum bactericidal biofilm concentration (MBBC), defined as the lowest concentration that determined a reduction of at least 3 log10 CFU/mL compared with the untreated control, was evaluated. Scanning electron microscopy (SEM) was used to investigate the biofilm of *P. aeruginosa* treated with cefiderocol, imipenem, or their combination.

Results: Cefiderocol and imipenem were tested alone on *P. aeruginosa* biofilm and a reasonable reduction in the number of viable cells was observed, especially at high drug concentrations tested. The synergistic effect of cefiderocol in combination with imipenem was evaluated for five selected isolates. Cotreatment with the two drugs led to a remarkable reduction of cell viability by resulting in synergistic bactericidal activity in all tested strains and in synergistic eradicating activity in only one isolate. SEM analysis revealed that, in cefiderocol-treated biofilm, bacterial cells became more elongated than in the untreated control, forming filaments in which bacterial division seems to be inhibited.

Conclusions: Cefiderocol exhibited an encouraging antibiofilm activity against tested strains, representing a valid option for the treatment of *P. aeruginosa* biofilm-associated infections, especially when administered in combination with imipenem.

1. Introduction

The excessive and often inappropriate use of antibiotics has spurred antimicrobial resistance and multidrug-resistant bacteria, leading to a high risk of treatment failure and relapse of infections [1,2]. In this scenario, nosocomial infections caused by Gram-negative bacteria are increasingly problematic to treat [3]. In addition, the increased use of indwelling medical devices has led to a higher incidence of biofilm-associated infections, in which bacteria such as *Pseudomonas aeruginosa* can grow in an aggregate form embedded in a self-produced extracellular matrix, forming a sessile community [4]. This structure makes bacteria resistant not only to the action of the host immune system but also to antibiotics, rendering the treatment of biofilm-related infections highly complicated [5]. Given the emergence of bacterial resistance and biofilm-associated infections, much effort is required to develop novel and effective antibacterial and antibiofilm agents [1]. Part of the research and development of new antibiotics is focused on modifying active antibiotic structures (e.g. beta-lactams) by adding siderophores, iron-chelating molecules produced by bac-

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Table 1
List of *P. aeruginosa* strains and their antibiotic resistance profile.

ID	Infection site	Geographical location	Antimicrobial resistance
PAO1	/	/	FEP; CAZ; CZA; C/T; MEM; TZP
PA1	Joint prosthesis	Naples	No resistance
PA2	Joint prosthesis	Berlin	FEP; CAZ; CZA; C/T; CIP; MEM; TZP
PA3	Joint prosthesis	Berlin	FEP; IPM; CAZ; C/T; CIP; MEM; TZP; TOB
PA4	Blood	Naples	No resistance
PA5	Blood	Naples	No resistance
PA6	Joint prosthesis	Verona	FEP; CAZ; C/T; TZP
PA7	Joint prosthesis	Rome	FEP; CAZ; CZA; C/T; CIP; TZP
PA8	Blood	Naples	FEP; CAZ; CZA; C/T; TZP
PA9	Blood	Naples	FEP; CAZ; TZP
PA10	Cardiothoracic prosthesis	Naples	No resistance

CAZ, ceftazidime; CIP, ciprofloxacin; CZA, ceftazidime-avibactam; C/T, ceftolozane-tazobactam; FEP, cefepime; IPM, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; TOB, tobramycin.

teria to capture iron, which is useful for the survival of their own cells. None of the siderophore antibiotics developed before 2019 progressed to clinical development, due to poor correlation between in vitro activity and in vivo efficacy or because of cardiovascular toxicity. On 14 November 2019, the Food and Drug Administration approved cefiderocol, a novel parenteral catechol-substituted siderophore cephalosporin indicated for the treatment of multidrug-resistant Gram-negative infections in adult patients with limited therapeutic options [6]. Cefiderocol is a synthetic conjugate composed of a cephalosporin moiety and a catechol-type siderophore [7]. The catechol moiety allows the molecule to work as a siderophore by forming a chelating complex with ferric iron and by using the bacterial ferric iron transport system to cross the outer membrane of Gram-negative organisms and get into the periplasmatic space. Once cefiderocol successfully enters the periplasmic space, it dissociates from iron and, as with other beta-lactams, the cephalosporin moiety binds to penicillin-binding proteins (PBPs) and disrupts the synthesis of the peptidoglycan layer of bacterial cell walls, inducing cell death [6]. This mechanism is called a 'Trojan horse' strategy, allowing successful penetration by evading some intrinsic or acquired antibiotic resistance mechanisms. The active transport mechanism through iron transport channels contributes not only to the delivery of cefiderocol efficiently into the periplasmic space, where the target PBPs are located, but it also overcomes permeability-related drug resistance due to porin channel loss and overexpression of efflux pumps [8,9].

The main aim of this work was to evaluate the ability of cefiderocol to kill biofilm-embedded *P. aeruginosa* clinical strains. The synergistic bactericidal and/or eradicating effect of cefiderocol in combination with imipenem was also evaluated for five selected isolates in sessile form.

2. Materials and methods

2.1. Pseudomonas aeruginosa strains

The 10 *P. aeruginosa* clinical strains included in the study were isolated from infected implants and bloodstream with different susceptibility patterns. A reference strain, PAO1, was included in the study. Table 1 shows the bacterial strains' ID, their geographical origin, the site of infection, and antimicrobial resistance profile. Each strain was aliquoted and stored at –80°C until used. According to the European Committee on Antimicrobial Susceptibility Test (EUCAST) guidelines [10], all strains were cultured in iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB). Each strain was inoculated from the stock into 5 mL of ID-CAMHB to grow overnight at 37°C shaking. Bacteria were plated onto LB agar. Phosphate-buffered saline (PBS) was used for dilutions.

2.2. Antibacterial molecules

Cefiderocol powder was provided by Shionogi and Co., Ltd. (Osaka, Japan), and it was maintained and preserved according to the company's guidelines. The master stock solution was reconstituted in a vial by solubilizing the whole amount of drug product (1 g/vial) with 10 mL of sterile H_2O, aliquoted and stored at –20°C. Imipenem (Sigma-Aldrich, St. Louis, Missouri, USA) was weighed prior to each use and diluted in sterile H_2O because it could not be frozen, according to the guidelines.

2.3. In vitro P. aeruginosa biofilm formation

The ability of *P. aeruginosa* strains to form biofilm in vitro was evaluated on porous glass beads following a previously established protocol [11]. Bacteria from overnight culture were diluted up to 10^7 CFU/mL and incubated with porous glass beads in ID-CAMHB for 24 h at 37°C to allow the bacteria to adhere to the beads and form biofilm. The ratio between beads and the diluted overnight bacterial culture (mL) was 1:1. After 24 h, the beads were washed three times with PBS, and each of them was transferred into a 2 mL Eppendorf tube containing 1 mL of PBS using a sterile inoculation loop. Next, beads were vortexed for 30 s, sonicated for 1 min in a sonication bath, and vortexed for a further 30 s to detach the biofilm and disaggregate the bacterial cells. After this, the sonication fluid was 10-fold serially diluted in PBS, and the different dilutions were plated onto LB agar. Plates were incubated for 24 h at 37°C for colony counting. Quantification of biomass formed by *P. aeruginosa* strains was performed by crystal violet (CV) assay [12]. Overnight, growing bacteria (100 µL) diluted 1:100 in fresh LB broth were incubated at 37°C for 24 h in a flat-bottom microtiter plate to allow cells to attach to the polystyrene surface. The same volume of LB broth was added as a blank. After incubation, the content of the wells was removed by turning the plate upside down on absorbent paper and tapping it vigorously to encourage as much liquid as possible to flow out. After, each well was washed with 125 µL of PBS. The biofilms were stained for 15 min at room temperature by adding 125 µL of 0.1% CV solution. Then, each well was washed again with PBS three times, and the plate was left to dry for 15 min. Finally, 200 µL of 96% ethanol was added to each well for 15 min, allowing the stain to solubilize. Optical density (OD) values of the CV/ethanol solutions were measured at a wavelength of 570 nm in a microplate reader (ThermoScientific Multiskan FC Microplate Photometer, Waltham, Massachusetts, U.S). The final OD value of each tested strain was indicated as the average OD value of the strain [13]. The interpretation of obtained results was performed according to Stepanović and colleagues [13] as follows: $OD \leq ODc$ = no biofilm producer; $ODc < OD \leq 2 \times ODc$ = weak biofilm producer; $2 \times ODc < OD \leq$

4 × ODc = moderate biofilm producer; 4 × ODc < OD = strong biofilm producer. The cut-off value (ODc) was defined as three standard deviations (SD) above the mean OD of the negative control (ODc = average OD of negative control + 3 × SD of negative control) [13].

2.4. In vitro activity of cefiderocol and imipenem against planktonic and biofilm-embedded P. aeruginosa

The antimicrobial susceptibility testing of cefiderocol and imipenem was performed by broth microdilution assay, according to EUCAST guidelines [10]. The experiments for determining the minimum inhibitory concentration (MIC) were performed in a 96-round-bottom-well plate containing 100 µL suspension of bacteria at the final load of 10^6 CFU/mL and twofold serial dilutions of the drugs. The cefiderocol and imipenem were tested, and ranged from 0.06 mg/L to 4 mg/L and from 0.5 mg/L to 8 mg/L, respectively. The well containing bacterial suspension (10^6 CFU/mL) without antibiotics was used as growth control (GC). Samples were incubated at 37°C. After 24 h, each bacterial suspension was 10-fold serially diluted and plated onto LB agar for colony counting. The minimum bactericidal concentration (MBC) was defined as the lowest antibiotic concentration able to reduce by at least 3 log10 CFU/mL in comparison with the CFU/mL value of the initial inoculum.

In order to perform the antibiofilm assay with antibiotics, biofilm of *P. aeruginosa* strains was formed on porous glass beads, as described in paragraph 2.3. After biofilm formation, beads were washed with PBS three times and each of them was transferred, using a sterile inoculation loop, into a 48-well plate, in which antibiotics were serially diluted in ID-CAMHB. Cefiderocol was tested from 512 mg/L to 0.06 mg/L, while imipenem was tested from 512 mg/L to 1 mg/L. Cefiderocol was also tested in the presence of imipenem to evaluate the potential synergic bactericidal and/or eradicating effect. The drugs in combination were tested at concentrations lower than 2 mg/L and below 4 mg/L for cefiderocol and imipenem, respectively, and 24-h-old biofilm beads were incubated with 1 mL of fresh ID-CAMHB without drugs as the GC. Bead samples were incubated for 24 h at 37°C and treated as described in paragraph 2.3 for colony counting. For each tested strain, the minimum biofilm bactericidal concentration (MBBC), defined as the lowest antimicrobial concentration that killed 99.9% (i.e. 3 log10 CFU/mL) of the cells recovered from a biofilm culture compared with the GC, and the minimum biofilm eradication concentration (MBEC), defined as the lowest concentration of antimicrobial agent required to eradicate the biofilm (0 CFU/mL on plate) [14], were evaluated. In antibiotic combination tests, the fractional biofilm bactericidal concentration (FBBC) index and/or fractional biofilm eradication concentration (FBEC) index were used to investigate the synergistic bactericidal and/or eradicating activity of antibiotics tested in combination. The FBBC index was determined as follows: FBBC index = FBBC(C) + FBBC(I) = MBBC(C) in combination/MBBC(C) alone + MBBC(I) in combination/MBBC(I) alone; where (C) is cefiderocol and (I) is imipenem. The FBBC index was interpreted as follows: ≤0.5 indicates a synergistic effect (S); >0.5 to ≤4.0, no interaction (N); and >4.0, an antagonistic effect (A) [15]. The FBEC index was calculated using the same method, replacing MBBC with MBEC. The interpretation was the same as that for the FBBC index [15].

2.5. Evaluation of the antibiofilm effect by scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the PA10 colonization of porous glass beads following a previously established protocol [16]. Biofilm was formed on porous glass beads, as described in paragraph 2.3, and each sample was incubated for 24 h with different concentrations of antibiotics. Cefiderocol and imipenem were tested alone and in combination at 0.5 and 1 mg/L, respectively, and 24-h-old biofilm beads were incubated with ID-CAMHB as the untreated control. After incubation, each bead was washed in PBS three times and fixed with glutaraldehyde [16]. Bead samples were incubated with 2.5% glutaraldehyde solution (glutaraldehyde solution 25% was diluted 1:10 in sodium cacodylate 0.1 M buffer) for 1 h at room temperature. After incubation, samples were washed three times for 2–3 min with sodium cacodylate 0.1 M buffer. Then, samples were dehydrated with ethanol gradients, since the water content of the sample was not compatible with the vacuum needed for the electron beam functioning [16]. Beads were washed for 10 min with 30%, 50%, 70%, 85%, and 95% ethanol, and two times for 10 min with 100% ethanol. Then, dehydrated biofilm samples were treated with hexamethyldisilazane (HMDS) solvent to obtain an optimal resolution [16]. Samples were incubated with 100% ethanol and pure HMDS (1:1 v/v) for 4 min, then incubated with HMDS for a further 4 min at RT. Finally, samples were left to dry overnight on filter paper under a chemical hood. For sample investigation, beads were coated with a layer of platinum to increase their electrical conductivity. Samples were observed by a field emission scanning electron microscope (FEI Company Quanta 450 FEG, Hillsboro, Oregon, U.S) with 5.00 kV voltage.

3. Results

3.1. Evaluation of P. aeruginosa's ability to form biofilm

The 10 *P. aeruginosa* clinical strains included in the study were selected from patients with severe bloodstream or chronic prosthetic infections. In addition, the strains were chosen based on two patterns of resistance or susceptibility. All of the strains were selected for their ability to form biofilm in vitro, which was initially tested on porous glass beads by counting the CFU/mL of viable attached cells. A reference strain, PAO1, considered a strong biofilm producer [17], was also included. The CFU/mL numbers obtained by *P. aeruginosa* strains are reported in Fig. 1.

After 24 h, the PAO1 reference strain reached 4.5 × 10^7 CFU/mL, while 1.1 × 10^8 CFU/mL and 9.9 × 10^7 CFU/mL were the values observed for PA4 and PA5, respectively, indicating an increased capacity to form biofilm in vitro on porous glass beads. All other strains reached a lower number of CFU/mL compared with the reference strain, but still in a range of 5.5 × 10^6 to 4.3 × 10^7 CFU/mL. Next, the quantification of the whole biofilm biomass (including both cell and matrix components) of *P. aeruginosa* strains was performed by measuring the absorbance of CV extracted by stained adherent biomass. According to the OD value and classification described by Stepanović et al., strains were divided into four categories of biofilm producers (Fig. 1). PA3 and PA6 were weak biofilm producers, PA1 and PA7 were moderate biofilm producers, and all other isolates (including four strains isolated from blood) were strong biofilm producers.

3.2. Antimicrobial activity of cefiderocol and imipenem against planktonic P. aeruginosa

Tests of the susceptibility to antimicrobial activity of cefiderocol and imipenem were performed according to EUCAST guidelines [10]. Tables 2 and 3 showed the MIC values of cefiderocol and imipenem, respectively. For cefiderocol, MICs ranged from ≤0.06 to 0.25 mg/L, except for PA3 (whose MIC was 4 mg/L). According to EUCAST Breakpoint [18], cefiderocol was active against all the strains tested, with the exception of PA3, which was resistant to the antibiotic. The MBC values of cefiderocol required for reducing the original inoculum by >99.9% (i.e. 3 log10 CFU/ml) of tested

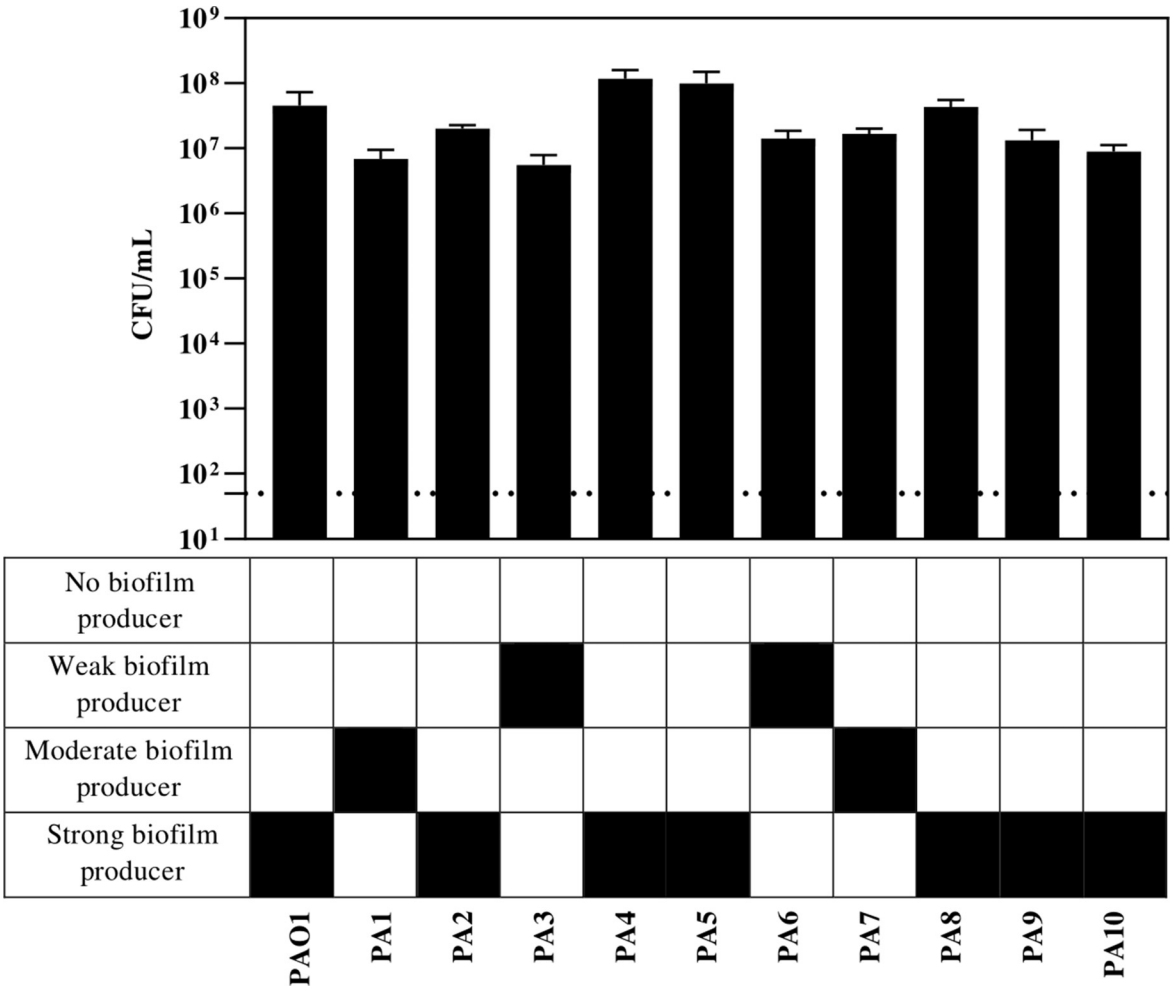

Fig. 1. *P. aeruginosa* strains biofilm formation on porous glass beads after 24 h incubation at 37°C. Biofilm growth was evaluated by CFU/mL counting of biofilm-forming viable cells. The detection limit (Y = 50 CFU/mL) is represented by the horizontal black dotted line. For each strain, media and SEM values are reported. The able indicates the quantification of bacterial biofilms formed by *P. aeruginosa* strains, measured according to Stepanović and colleagues [13].

Table 2
MIC and MBC values of cefiderocol (FDC) vs. *P. aeruginosa* isolates.

		PA01	PA1	PA2	PA3	PA4	PA5	PA6	PA7	PA8	PA9	PA10
FDC (mg/L)	MIC	≤ 0.06	≤ 0.06	0.25	4	≤ 0.06	0.125	≤ 0.06	0.125	≤ 0.06	≤ 0.06	≤ 0.06
	MBC	0.125	0.25	1	>8	0.125	0.25	0.25	1	0.25	0.25	0.06

isolates ranged from 0.06 to 1 mg/L, except for PA3 (>8 mg/L). Imipenem ranging from 0.5 to 8 mg/L was tested against PA6, PA7, PA8, PA9, and PA10. As shown in Table 3, imipenem's MIC values ranged from 1 to 4 mg/L and, according to EUCAST breakpoints [18], all tested strains were categorized as susceptible to increased exposure. Imipenem's MBCs were 4 mg/L for all isolates, except for PA8 (8 mg/L) and PA10 (2 mg/L).

3.3. The individually tested antibiofilm activity of cefiderocol and imipenem

According to their susceptibility profile to antibiotics and their ability to form biofilm in vitro, the PAO1 reference strain and seven clinical strains were selected from the panel of *P. aeruginosa* strains to evaluate the antibiofilm activity of cefiderocol. The siderophore cephalosporin was tested on 24-h-old biofilm formed on porous glass beads at different concentrations. The final numbers of CFU/mL, after 24 h of antibiotic treatment at 37°C, were determined. As shown in Fig. 2, in PAO1 and PA1, the obtained numbers of CFU/mL of the biofilm tested with different concentrations of cefiderocol were comparable to those of the untreated GC. In PA2 and PA7, a reduction of almost 2 log10 in the CFU/mL for tested concentrations compared with the GC was observed, maintaining all values higher than the MBBC. For half of the tested strains, the MBBC was observed. The MBBC values were 16 mg/L for PA6, 32 mg/L for PA8, 256 mg/L for PA9, and 2 mg/L for PA10. Eradication was only achieved in PA10, which was treated with 512 mg/L cefiderocol, the highest tested concentration.

Five clinical isolates (PA6, PA7, PA8, PA9, and PA10) were selected arbitrarily to evaluate the antibiofilm activity of imipenem alone and in combination with cefiderocol. The treatments with

Table 3
MIC and MBC values of imipenem (IPM) vs. *P. aeruginosa* isolates.

		PA6	PA7	PA8	PA9	PA10
IPM (mg/L)	MIC	2	1–2	4	2	2
	MBC	4	4	8	4	2

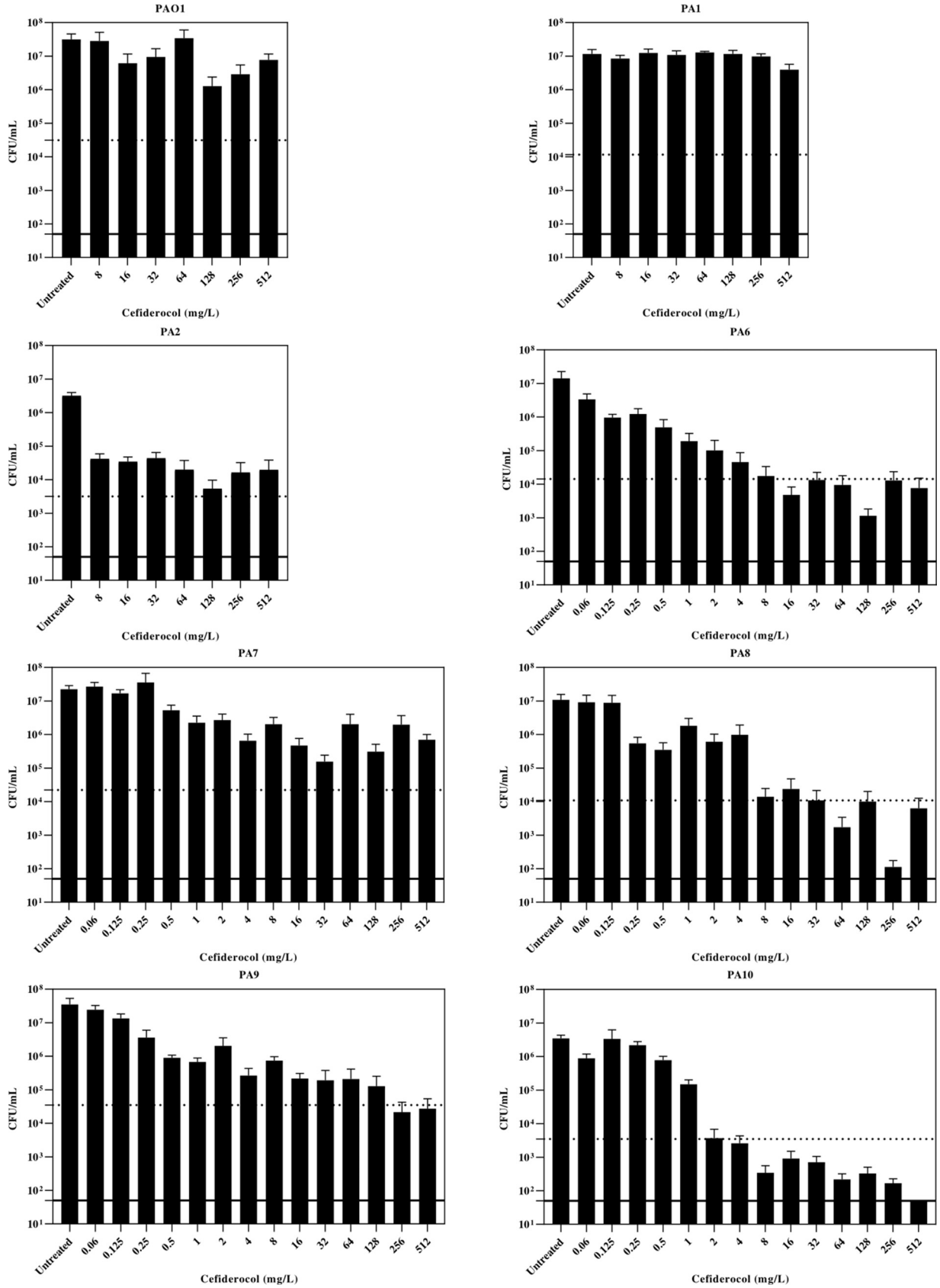

Fig. 2. Activity of cefiderocol on preformed 24-h-old *P. aeruginosa* biofilm. CFU/mL measurements are represented on the Y axis and concentrations of the drug are represented on the X axis. The bars represent the mean value of at least three independent biological replicates ($N \geq 3$) with the relative standard error of the mean (SEM). The detection limit ($Y = 50$) for CFU/mL count is represented by the horizontal continuous line, while MBBC (3 log10 under GC) is represented by the horizontal dotted line.

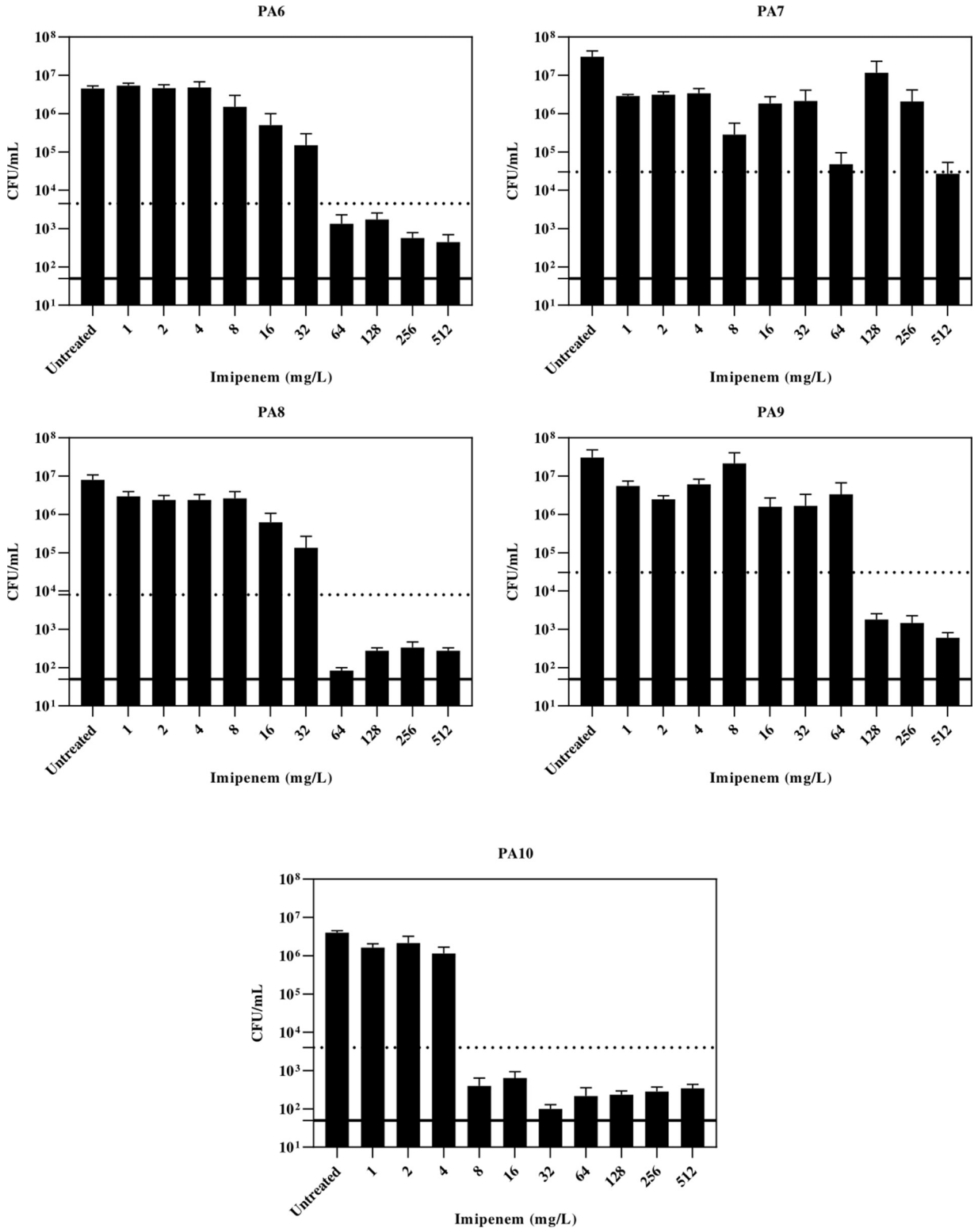

Fig. 3. Activity of imipenem on preformed 24-h-old *P. aeruginosa* biofilm. CFU/mL measurements are represented on the Y axis and concentrations of the drug are represented on the X axis. The bars represent the mean value of at least three independent biological replicates (N ≥ 3) with the relative standard error of the mean (SEM). The detection limit (Y = 50) for CFU/mL count is represented by the horizontal continuous line, while MBBC (3 log10 under GC) is represented by the horizontal dotted line.

imipenem were performed on 24-h-old biofilm formed on porous glass beads at concentrations ranging from 512 to 1 mg/L. As reported in Fig. 3, all tested clinical strains showed bactericidal activity at different drug concentrations, ranging from 64 to 512 mg/L. Only PA10 showed a very low MBBC (8 mg/L). No eradicating activity was observed in any tested strain.

3.4. In vitro antibiofilm assay of cefiderocol and imipenem tested in combination

The synergistic bactericidal and/or eradicating activity of cefiderocol in combination with imipenem was evaluated against 24-h-old *P. aeruginosa* biofilm formed on porous glass beads. Three sub-

Table 4
MBBC and FBBC index values for cefiderocol (FDC) and imipenem (IPM) alone and in combination against *P. aeruginosa* clinical strains.

Bacterial strain	Antibiotic	MBBC in isolation (mg/L)	MBBC in combination (mg/L)	FBBC index	FBBC index (interpretation)
PA6	FDC	16	1	0.0625	0.0937 (S)
	IPM	64	2	0.0312	
PA7	FDC	>512	0.5	<0.0009	<0.0028 (S)
	IPM	512	1	0.0019	
PA8	FDC	32	0.25	0.0078	0.0234 (S)
	IPM	64	1	0.0156	
PA9	FDC	256	2	0.0078	0.0156 (S)
	IPM	128	1	0.0078	
PA10	FDC	2	0.5	0.25	0.375 (S)
	IPM	8	1	0.125	

Interpretation of FBEC index: A = Antagonism; S = Synergism; NI = No interaction.

Table 5
MBEC and FBEC index values for cefiderocol (FDC) and imipenem (IPM) alone and in combination against PA7 strain.

Bacterial strain	Antibiotic	MBEC in isolation (mg/L)	MBEC in combination (mg/L)	FBEC index	FBEC index (interpretation)
PA7	FDC	>512	2	<0.0039	<0.0058 (S)
	IPM	>512	1	<0.0019	

Interpretation of FBEC index: A = Antagonism; S = Synergism; NI = No interaction.

bactericidal concentrations of each antibiotic were tested alone and in combination vs. PA6, PA7, PA8, PA9, and PA10. The results are summarized in Table 4. The combination of the two antibiotics resulted in synergistic bactericidal activity in all five clinical isolates tested. The highest FBBC index and, consequently, the lowest synergistic bactericidal effect, were obtained for PA10. The MBBCs of antibiotics tested alone were lower than those of the other strains.

The combination of the two antibiotics did not produce a synergistic eradicating effect vs. any isolates tested, except vs. PA7; the combination of cefiderocol (1 mg/L) and imipenem (2 mg/L) resulted in the eradication of the biofilm, despite the MBEC of both drugs alone being ≥512 mg/L (Table 5).

3.5. SEM imaging of biofilm-embedded P. aeruginosa treated with antibiotics

SEM can reveal the high-resolution 3D structure of biofilm-embedded bacteria attached to a surface at very high magnification [16]. PA10 was a *P. aeruginosa* clinical strain isolated from a thoracic prosthesis-associated infection in a patient with a critical medical history. To investigate the colonization of the porous glass beads by the clinical strain (and thus possible therapeutic strategies for the treatment of infections due to the formation of biofilms on prosthetic devices), SEM was employed. After the formation of a 24-h-old biofilm, samples were incubated with different concentrations of antibiotics. Cefiderocol and imipenem were tested alone and in combination at 0.5 and 1 mg/L (the MBBC values of both drugs when tested in combination), respectively. The PA10 cells attached to the beads, before being exposed to antibiotics, are shown in Fig. 4A. Aggregates of rod-shaped cells were observed, and they showed a length of ca. 1–2 µm and a diameter of ca. 0.5 µm, typical of *P. aeruginosa* strains. Differences in the size of the bacterial cells and the amount of the biofilm matrix were shown in drug-treated bead samples, as can be observed in Fig. 4B-C. In biofilm samples treated with cefiderocol alone, the bacterial cell took on a more elongated form than in the GC, while the amount of matrix remained unchanged. For some bacterial cells, complete lysis or severe damage (i.e. wrinkled surface) was observed. In addition, the bacteria formed elongated filaments, and bacterial division appeared to be inhibited. Treatment with imipenem alone induced an increase in the quantity of matrix and caused a change in the shape of bacterial cells, which became shorter and rounder than in the GC. Finally, with the combination of the two antibiotics, no bacterial growth was observed (Fig. 4D), confirming the synergistic activity of cefiderocol with imipenem against the PA10 strain.

4. Discussion

Biofilm-associated infections caused by *P. aeruginosa* pose a difficult challenge due to their inherent resistance to conventional antibiotics [19]. In particular, biofilm-embedded cells can be extremely tolerant to high concentrations of antibiotics, even though the planktonic form is sensitive to most antimicrobial molecules [20].

Here, 10 clinical isolates of *P. aeruginosa* with different antibiotic susceptibility profiles were collected from either prosthetic or bloodstream infections, and their ability to form biofilm was evaluated. All strains isolated from blood (4/10) were stronger biofilm producers in comparison with those isolated from indwelling medical devices (6/10), where more heterogeneous behaviour was noted.

Interestingly, the ability to form biofilm did not correlate with the resistance profile of the tested bacterial strains, in agreement with similar findings from other studies on various bacterial species [21–24].

All the tested strains exhibited a susceptibility profile to cefiderocol at the tested concentration. The obtained MIC values of cefiderocol were rather consistent with the data reported for *P. aeruginosa* by other authors in their in vitro studies, where they found MIC values ≤2 mg/L [25–27]. Only PA3 showed a cefiderocol MIC of 4 mg/L, demonstrating resistance to the drug. This strain was also resistant to all beta-lactam molecules, excluding CZA. In an experimental resistance frequency investigation, Simner and colleagues found higher MIC values of cefiderocol, and attributed the development of drug resistance to mutations in genes related to iron transport [28]. By contrast, none of the strains were resistant to imipenem. The MBC values observed for all the susceptible strains (except PA3) were rather low (ranging from 0.06 to 1 mg/L), suggesting that cefiderocol has potent bactericidal activity against all planktonic isolates.

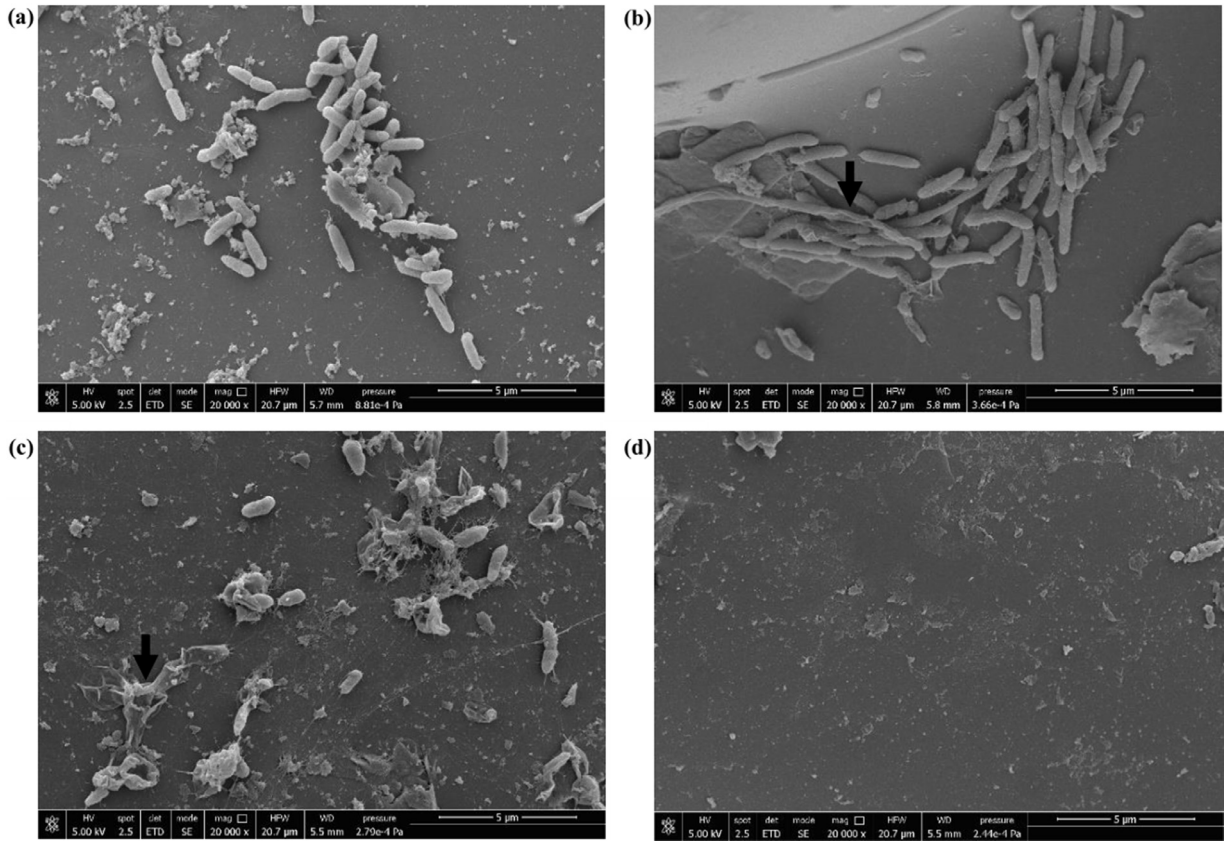

Fig. 4. SEM micrographs (20.000 ×) of PA10 biofilms formed in vitro on porous glass beads. Observation of untreated bead used as a growth control (**a**) and beads treated with cefiderocol (**b**), imipenem (**c**), and with their combination (**d**). The arrows indicate damaged bacterial cells.

As expected, both cefiderocol and imipenem showed higher bactericidal concentration when tested singularly against *P. aeruginosa* biofilms, more than 10–100 times higher than their planktonic counterparts. Although the two antibiotics alone appeared to have ineffective antibiofilm activity at the low concentrations tested for the majority of isolates, here, the in vitro data obtained suggested that the use of cefiderocol in combination with imipenem is a promising therapeutic strategy against *P. aeruginosa* biofilms, confirming the synergistic activity of the two molecules observed by the authors in a previously published case report [29]. In fact, the antibiotic combination can achieve therapeutic efficacy with a higher probability and selectivity than the drugs being administered singularly [30].

Interestingly, the stretched cell shape following treatment with cefiderocol was also observed by Bao and colleagues [31], through observation by a transmission electron microscope of cefiderocol-resistant *Klebsiella pneumoniae*. In this study, the cell shape-determining proteins *MreC* and *MreB* were upregulated when bacteria were incubated with cefiderocol, making the drug-resistant form of *K. pneumoniae* longer and thinner. The proteins are elongasomes belonging to the Rod system, which is associated with rod-shape determination in bacteria [31]. This effect is similar to that observed in *E. coli* cells following exposure to aztreonam [32]. Elongated and filamentous *P. aeruginosa* cells obtained after treatment with cefiderocol are likely the result of the inhibition of PBP3, a cefiderocol higher-affinity target. PBP3 is required for cell division, and its inhibition has been demonstrated to suppress cell division by preventing cell septation, thus leading to filamentation [32,33]. Ovoid cell formation occurs when bacteria, including *P. aeruginosa*, are exposed to antibiotics (including imipenem) that inhibit PBP2 [34]. Inhibition of PBP2 leads to cessation of lateral-wall peptidoglycan synthesis and permanent activation of septal wall peptidoglycan synthesis, resulting in daughter cells that comprise two poles with no cylindrical peptidoglycan to separate them [34]. The increase in the amount of PA10 biofilm matrix could be related to the fact that imipenem induces alginate production, one of the main exopolysaccharides forming the matrix of *P. aeruginosa* biofilm [35]. This effect has been reported by Bagge and colleagues [36], who observed a 20-fold increase in alginate in the biofilm matrix of imipenem-exposed PAO1 biofilms compared with an untreated biofilm control.

The target of PBPs 2 and 3 by imipenem and cefiderocol, respectively, may underlie the synergistic mechanism of action of the two drugs on *P. aeruginosa* biofilms. The effectiveness of beta-lactam combinations, based on a similar mechanism, has previously been reported for other difficult-to-treat pathogens [37,38]. The preliminary experimental evidence supporting the potential inhibition of dual beta-lactam combination in the synergistic effect of cefiderocol and imipenemrequires further investigation. However, another possible mechanism could be related to cefiderocol's iron-scavenging properties, which might disrupt the EPS structure, potentially weakening the biofilm barrier and enhancing the penetration of imipenem. This synergistic action could promote the eradication of both biofilm-residing bacteria and those in the planktonic state.

5. Conclusion

We confirm that the new siderophore cephalosporin cefiderocol exhibits encouraging antimicrobial activity against *P. aeruginosa*, and we demonstrate its antibiofilm activity when used in combination with imipenem. Although the present study was limited by

the small number of tested isolates with a narrow genetic diversity and geographical origin, the promising results suggest that cefiderocol in combination with imipenem might be a valid option for the treatment of biofilm-associated infections to be further investigated in clinical settings.

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References

[1] Annunziato G. Strategies to overcome antimicrobial resistance (AMR) making use of non-essential target inhibitors: a review. Int J Mol Sci 2019;20:5844. doi:10.3390/ijms20235844.
[2] Ventola CL. The antibiotic resistance crisis. P T 2015;40:277–83.
[3] Ito A, Sato T, Ota M, Takemura M, Nishikawa T, Toba S, et al. In vitro antibacterial properties of cefiderocol, a novel siderophore cephalosporin, against Gram-negative bacteria. Antimicrob Agents Chemother 2018;62:e0145417. doi:10.1128/AAC.01454-17.
[4] Abdelhamid AG, Yousef AE. Combating bacterial biofilms: current and emerging antibiofilm strategies for treating persistent infections. Antibiotics 2023;12:1005. doi:10.3390/antibiotics12061005.
[5] Yin R, Cheng J, Wang J, Li P, Lin J. Treatment of Pseudomonas aeruginosa infectious biofilms: challenges and strategies. Front Microbiol 2022;13:955286. doi:10.3389/fmicb.2022.955286.
[6] El-Lababidi RM, Rizk JG. Cefiderocol: a siderophore cephalosporin. Ann Pharmacother 2020;54:1215–31. doi:10.1177/1060028020929988.
[7] McCreary EK, Heil EL, Tamma PD. New perspectives on antimicrobial agents: cefiderocol. Antimicrob Agents Chemother 2021;65:e0217120. doi:10.1128/AAC.02171-20.
[8] Stracquadanio S, Bonomo C, Marino A, Bongiorno D, Privitera GF, Bivona DA, et al. Acinetobacter baumannii and cefiderocol, between cidality and adaptability. Microbiol Spectr 2022;10:e0234722. doi:10.1128/spectrum.02347-22.
[9] Sato T, Yamawaki K. Cefiderocol: discovery, chemistry, and in vivo profiles of a novel siderophore cephalosporin. Clin Infect Dis 2019;69:S538–43. doi:10.1093/cid/ciz826.
[10] The European Committee on Antimicrobial Susceptibility Testing – EUCAST. MIC determination of non-fastidious and fastidious organisms, https://www.eucast.org/ast_of_bacteria/mic_determination; 2024 [accessed].
[11] Butini ME, Abbandonato G, Di Rienzo C, Trampuz A, Di Luca M. Isothermal microcalorimetry detects the presence of persister cells in a Staphylococcus aureus biofilm after vancomycin treatment. Front Microbiol 2019;10:332. doi:10.3389/fmicb.2019.00332.
[12] Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol 2005 Chapter 1:Unit 1B.1. doi:10.1002/9780471729259.mc01b01s00.
[13] Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 2007;115:891–9. doi:10.1111/j.1600-0463.2007.apm_630.x.
[14] Macià MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clin Microbiol Infect 2014;20(10):981–90. doi:10.1111/1469-0691.12651.
[15] Dall GF, Tsang S-TJ, Gwynne PJ, MacKenzie SP, Simpson AHRW, Breusch SJ, et al. Unexpected synergistic and antagonistic antibiotic activity against Staphylococcus biofilms. J Antimicrob Chemother 2018;73:1830–40. doi:10.1093/jac/dky087.
[16] Vuotto C, Donelli G. Field emission scanning electron microscopy of biofilm-growing bacteria involved in nosocomial infections. Methods Mol Biol 2014;1147:73–84. doi:10.1007/978-1-4939-0467-9_6.
[17] da Costa Lima JL, Rodrigues Alves L, de Araújo Jacomé PRL, Bezerra Neto JP, Maciel MAV, Camargo de Morais MM. Biofilm production by clinical isolates of Pseudomonas aeruginosa and structural changes in LasR protein of isolates non biofilm-producing. Braz J Infect Dis 2018;22(2):129–36. doi:10.1016/j.bjid.2018.03.003.
[18] The European Committee on Antimicrobial Susceptibility Testing – EUCAST. Clinical breakpoints - breakpoints and guidance, https://www.eucast.org/clinical_breakpoints; 2023 [accessed].
[19] Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic activity of fosfomycin, ciprofloxacin, and gentamicin against Escherichia coli and Pseudomonas aeruginosa biofilms. Front Microbiol 2019;10:2522. doi:10.3389/fmicb.2019.02522.
[20] Di Luca M, Navari E, Esin S, Menichini M, Barnini S, Trampuz A, et al. Detection of biofilms in biopsies from chronic rhinosinusitis patients: in vitro biofilm forming ability and antimicrobial susceptibility testing in biofilm mode of growth of isolated bacteria. Adv Exp Med Biol 2018;1057:1–27. doi:10.1007/5584_2017_34.
[21] Gajdács M, Baráth Z, Kárpáti K, Szabó D, Usai D, Zanetti S, et al. No correlation between biofilm formation, virulence factors, and antibiotic resistance in Pseudomonas aeruginosa: results from a laboratory-based in vitro study. Antibiotics 2021;10(9):1134. doi:10.3390/antibiotics10091134.
[22] Carcione D, Leccese G, Conte G, Rossi E, Intra J, Bonomi A, et al. Lack of direct correlation between biofilm formation and antimicrobial resistance in clinical Staphylococcus epidermidis isolates from an Italian hospital. Microorganisms 2022;10(6):1163. doi:10.3390/microorganisms10061163.
[23] Duarte A, Ferreira S, Almeida S, Domingues FC. Clinical isolates of Acinetobacter baumannii from a Portuguese hospital: PFGE characterization, antibiotic susceptibility and biofilm-forming ability. Comp Immunol Microbiol Infect Dis 2016;45:29–33.
[24] de Campos PA, Royer S, da Fonseca Batistão DW, Araújo BF, Queiroz LL, de Brito CS, et al. Multidrug resistance related to biofilm formation in Acinetobacter baumannii and Klebsiella pneumoniae clinical strains from different pulsotypes. Curr Microbiol 2016;72(5):617–27. doi:10.1007/s00284-016-0996-x.
[25] Stracquadanio S, Torti E, Longshaw C, Santerre Henriksen A, Stefani S. In vitro activity of cefiderocol and comparators against isolates of Gram-negative pathogens from a range of infection sources: SIDERO-WT-2014–2018 studies in Italy. J Glob Antimicrob Resist 2021;25:390–8. doi:10.1016/j.jgar.2021.04.019.
[26] Taheri Y, Joković N, Vitorović J, Grundmann O, Maroyi A, Calina D. The burden of the serious and difficult-to-treat infections and a new antibiotic available: cefiderocol. Front Pharmacol 2021;11:578823. doi:10.3389/fphar.2020.578823.
[27] Delgado-Valverde M, Conejo MDC, Serrano L, Fernández-Cuenca F, Pascual Á. Activity of cefiderocol against high-risk clones of multidrug-resistant *Enterobacterales*, Acinetobacter baumannii, Pseudomonas aeruginosa and Stenotrophomonas maltophilia. J Antimicrob Chemother 2020;75:1840–9. doi:10.1093/jac/dkaa117.
[28] Simner PJ, Patel R. Cefiderocol antimicrobial susceptibility testing considerations: the Achilles' Heel of the Trojan Horse? J Clin Microbiol 2020;59(1):e00951 e00920. doi:10.1128/JCM.00951-20.
[29] Tascini C, Antonelli A, Pini M, de Vivo S, Aiezza N, Bernardo M, et al. Infective endocarditis associated with implantable cardiac device by metallo-β-lactamase-producing Pseudomonas aeruginosa, successfully treated with source control and cefiderocol plus imipenem. Antimicrob Agents Chemother 2023;67(3):e0131322. doi:10.1128/aac.01313-22.
[30] Basavegowda N, Baek K-H. Combination strategies of different antimicrobials: an efficient and alternative tool for pathogen inactivation. Biomedicines 2022;10(9):2219. doi:10.3390/biomedicines10092219.
[31] Bao J, Xie L, Ma Y, An R, Gu B, Wang C. Proteomic and transcriptomic analyses indicate reduced biofilm-forming abilities in cefiderocol-resistant Klebsiella pneumoniae. Front Microbiol 2022;12:778190. doi:10.3389/fmicb.2021.778190.
[32] Bulman ZP, Chen L, Walsh TJ, Satlin MJ, Qian Y, Bulitta JB, et al. Polymyxin combinations combat Escherichia coli harboring mcr-1 and bla_{NDM-5}: preparation for a postantibiotic era. mBio 2017;8(4):e00540 e00517. doi:10.1128/mBio.00540-17.
[33] Chen W, Zhang Y-M, Davies C. Penicillin-binding protein 3 is essential for growth of Pseudomonas aeruginosa. Antimicrob Agents Chemother 2017;61(1):e01651 e01616. doi:10.1128/AAC.01651-16.
[34] Cushnie TPT, O'Driscoll NH, Lamb AJ. Morphological and ultrastructural changes in bacterial cells as an indicator of antibacterial mechanism of action. Cell Mol Life Sci 2016;73(23):4471–92. doi:10.1007/s00018-016-2302-2.
[35] Thi MTT, Wibowo D, Rehm BHA. Pseudomonas aeruginosa biofilms. Int J Mol Sci 2020;21(22):8671. doi:10.3390/ijms21228671.
[36] 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. doi:10.1128/AAC.48.4.1175-1187.2004.
[37] Rahme C, Butterfield JM, Nicasio AM, Lodise TP. Dual beta-lactam therapy for serious Gram-negative infections: is it time to revisit? Diagn Microbiol Infect Dis 2014;80(3):239–59.
[38] Sader HS, Huynh HK, Jones RN. Contemporary in vitro synergy rates for aztreonam combined with newer fluoroquinolones and β-lactams tested against Gram-negative bacilli. Diagn Microbiol Infect Dis 2003;47(3):547–50. doi:10.1016/S0732-8893(03)00158-5.