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Synergistic combinations of novel polymyxins and rifampicin with improved eradication of colistin-resistant *Pseudomonas aeruginosa* biofilms

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ABSTRACT

Background: Increased prevalence of antimicrobial resistance coupled with a lack of new antibiotics against Gram-negative bacteria emphasize the imperative for novel therapeutic strategies. Colistin-resistant *Pseudomonas aeruginosa* constitutes a challenge, where conventional treatment options lack efficacy, in particular for biofilm-associated infections. Previously, synergy of colistin with other antibiotics was explored as an avenue for the treatment of colistin-resistant infections, and recently we reported our efforts towards colistin analogs capable of combating planktonic colistin-resistant strains.

Aims: The aim of the present study was to investigate whether analogs of polymyxin B with improved potency in wild-type and moderate resistant Gram-negative pathogens would retain similarly increased activity in highly colistin-resistant clinical *P. aeruginosa* isolates (in planktonic and biofilm growth) when applied alone and in combination with rifampicin.

Materials and methods: In this *in vitro* study, we tested three analogs of polymyxin B prepared by solid-phase peptide synthesis. Antimicrobial susceptibility testing was performed by measurement of minimum inhibitory concentrations via the broth microdilution method. Interactions between two antimicrobials was quantified in a checkerboard broth microdilution assay by calculating the fractional inhibitory concentration index for each combination. For testing of antibiofilm activity a previously described model with alginate beads encapsulating a biofilm culture was applied. The minimum biofilm eradication concentrations (MBECs) were evaluated, and the fractional biofilm eradication concentration indices were calculated. Three recently identified colistin analogs (CEP932, CEP936 and CEP938) were tested against three isogenic pairs of colistin-susceptible and colistin-resistant *P. aeruginosa* clinical isolates as well as the reference strain PAO1.

Results: For bacteria in planktonic growth CEP938 retained almost full potency in all three resistant isolates, while exhibiting similar activity as colistin in susceptible isolates. Against biofilms CEP938 was slightly more potent against PAO1 as compared to colistin, while also retaining activity against a biofilm of the colistin-resistant strain 41,782/98. Next, synergy between CEP938 and the antibiotic rifampicin was explored. Interestingly, CEP938 did not exhibit synergy with rifampicin in planktonic cultures. Importantly, for colistin-

Abbreviations: All, allyl; Bip, biphenyl; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; CFU, colony-forming unit; CstR, colistin-resistant (strain); 2-CTC, 2-chlorotrityl chloride; Dab, 2,4-diaminobutanoic acid; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, N,N′-dimethylformamide; EPS, extracellular polymeric substances; FICI, fractional inhibitory concentration index; Fmoc, fluorenylmethyloxycarbonyl; HBTU, hexafluorophosphate benzotriazole tetramethyluronium; HOAt, 1-hydroxy-7-azabenzotriazole; HPLC, high-performance liquid chromatography; ivDde, 4,4-dimethyl-2,6-dioxocyclohexane-1-carboxamide-3-methylbutyl; LPS, lipopolysaccharide; MBEC, minimum biofilm eradication concentration; *mcr*, mobile colistin resistance; MDR, multidrug-resistant; MeCN, acetonitrile; MHB, Müller-Hinton Broth; MIC, minimum inhibitory concentration; MoA, mode of action; PyAOP, (7-azabenzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate; *t*Bu, *tert*-butyl; TCS, two-component regulatory system; TFA, trifluoroacetic acid.

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resistant biofilms the CEP938-rifampicin combination demonstrated activity superior to that found for the colistin-rifampicin combination.
Conclusions: The present study showed *in vitro* efficacy of CEP938 against both colistin-susceptible and colistin-resistant *P. aeruginosa* biofilms as well as an ability of CEP938 to synergize with rifampicin in biofilm eradication.

1. Introduction

A decline in development of new antibiotics and treatment strategies together with continuous emergence of antimicrobial resistance have led to a situation with multidrug-resistant (MDR) bacteria, for which no or few treatment options are available. Colistin is a highly cationic lipopeptide antibiotic in clinical use for infections caused by Gram-negative bacteria [1]. Colistin is often the last-resort antibiotic when no alternative treatments are available for infections caused by Gram-negative MDR bacteria [2]. However, its usage is restricted due to concerns regarding nephro- and neurotoxicity [3]. Nevertheless, these issues can usually be managed by adopting a cautious dosing regimen with careful monitoring of indicators of kidney function as well as use of the colistin prodrug colistinmethate sodium [3]. Development of colistin resistance is of particular concern, since it often develops in bacteria that already are resistant to many other classes of antibiotics, such as carbapenems, which in some cases renders the bacteria pan-resistant [4–9].

MDR pathogens belonging to the ESKAPE group (***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*** and ***Enterobacter*** spp.) are of particular concern due to an increasing prevalence of cases in which few or no antibiotics remain efficacious [10]. Generally, infections caused by MDR *P. aeruginosa* are difficult to treat, and colistin may be the only effective treatment option [11]. *P. aeruginosa* infections can be particularly severe in patients who are severely immuno-compromised or suffering from advanced chronic obstructive pulmonary disease (COPD) with bronchiectasis, cystic fibrosis (CF) or cilia dyskinesia syndrome [12]. The chronic respiratory infections seen in COPD and CF patients are often caused by pathogens with a strong tendency for biofilm formation, which in turn can lead to rapid development of MDR strains [12].

The main mode of action (MoA) for colistin involves initial interaction with the outer membrane of Gram-negative bacteria, specifically the lipid A moiety of lipopolysaccharide (LPS) [13]. Colistin penetrates the outer membrane through displacement of divalent cations bound to lipid A, thereby permitting colistin molecules to reach the inner membrane by a self-promoted uptake. As in the outer membrane, LPS in the inner membrane is the target for lysis by displacement of the divalent cations, and thus killing of the bacteria [14–17].

Colistin resistance typically arises from: (I) Plasmid-borne mobilized colistin resistance (*mcr)* genes, encoding a phosphoethanolamine transferase that via modification of lipid A renders the bacteria resistant to colistin [14]; (II) Mutations in chromosomal genes, or (III) Induction of reversible tolerance by addition of L-4-aminoarabinose to phosphate groups of lipid A, which commonly is regulated by a two-component regulatory system (TCS) [15]. Thus, colistin resistance usually results from membrane modifications that confer a reduced negative surface charge [16,17], which diminishes the electrostatic interaction between colistin and LPS [15].

In *P. aeruginosa*, colistin resistance typically arises either via acquisition of *mcr* plasmids or mutations in chromosomal genes involved in regulation of lipid A modification [15,18]. These comprise among others PhoPQ and PmrAB, which constitute the most common two-component regulatory systems (TCSs) that upon mutation may upregulate the *arn* operon. This ultimately leads to introduction of L-4-aminoarabinose onto lipid A, and thus colistin binding becomes too weak to induce membrane destabilization [18]. Interestingly, even when colistin has lost its direct antibacterial activity towards colistin-resistant bacteria, it may retain an ability to permeabilize the outer membrane of Gram-negative bacteria to a degree that promotes uptake of hydrophobic antibiotics such as rifampicin [19].

Biofilms are aggregates of bacteria enclosed within an extracellular polymeric matrix (EPS), when adhered to a foreign material surface or tissue surface [20]. Non-adhering biofilm aggregates can also be found within secretions or tissues, e.g., in the lungs of CF patients [21,22]. Bacteria growing as biofilms exhibit distinct characteristics, e.g., variations in metabolism as well as both responses and susceptibility to antibiotics. Biofilms tend to develop increased tolerance to antibiotics due to restricted penetration of antibiotics through the EPS matrix and reduced metabolic activity within the inner biofilm regions [21,23,24]. For colistin, slow penetration into the biofilm creates a decreasing concentration gradient of colistin across the EPS, which enables a rapid development of colistin tolerance (through activation of TCSs as mentioned above) in metabolically active cells within the outer layer of the biofilm, since these become exposed to sub-killing levels of colistin, which promotes inducible tolerance and thereby resistance to colistin [25].

One strategy of combating colistin resistance focuses on novel colistin analogs that retain activity against colistin-resistant species, and recent examples comprise CEP932, CEP936 and CEP938, which display a more hydrophobic unnatural amino acid than present in polymyxin B (Figs. 1 and 2) [26]. The resulting slight increase in hydrophobicity conferred improved activity against both colistin-susceptible and -resistant *Escherichia coli, A. baumannii* and *P. aeruginosa* [26]. Structurally, CEP932, CEP936 and CEP938 are similar to polymyxin B except for replacement of the N-terminal fatty acid (i.e., octanoic acid instead of 6-methyloctanoic acid), and modifications in the hydrophobic segment of the cyclic core. Another example is the recently discovered polymyxin macolacin and its analog Bip-macolacin [27], which proved to be particularly active against colistin-resistant *K. pneumoniae* strains [26,27].

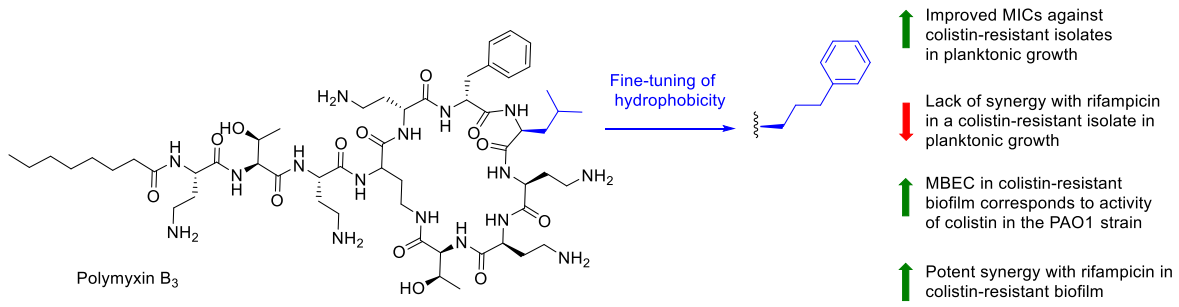

Fig. 1. Overall aim and results obtained with the most promising polymyxin tested.

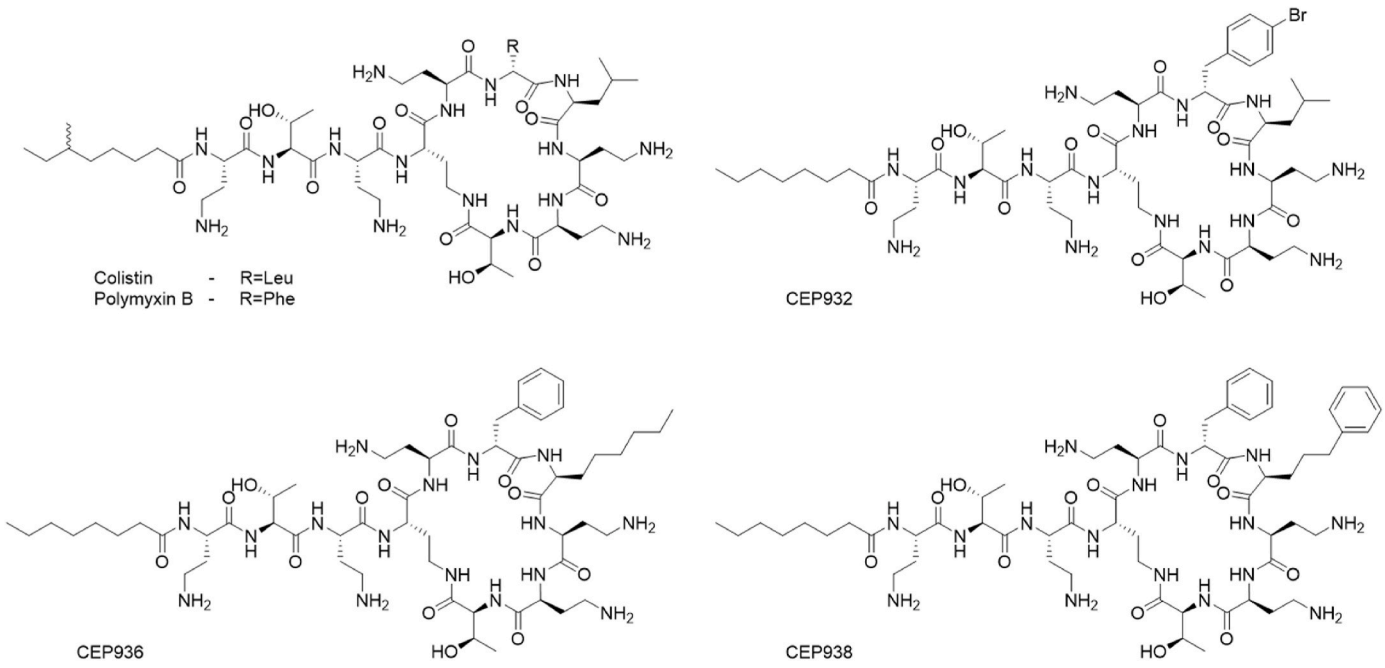

Fig. 2. Chemical structures of colistin and polymyxin B analogs CEP932, CEP936 and CEP938.

Combination therapy is particularly beneficial in reducing the likelihood of resistance development, since the bacteria need to develop resistance towards several antibiotics simultaneously to survive. Notably, colistin exhibits synergy with several anti-Gram-positive antibiotics (e.g., rifampicin) in colistin-resistant Gram-negative bacteria [28]. Rifampicin is a hydrophobic antibiotic that inhibits the DNA-dependent RNA synthesis in bacteria [29], but it is devoid of activity in most Gram-negative bacteria, since it is unable to cross their outer membranes [30]. However, when rifampicin is used in combination with compounds (e.g., colistin) that permeabilize or disrupt the outer membrane of Gram-negative bacteria, it may exert enhanced activity in Gram-negative bacteria [30].

In the present study we aimed to investigate whether the increased direct antibacterial activity of CEP932, CEP936 and CEP938 against planktonic Gram-negative pathogens could be translated into retained activity towards highly colistin-resistant clinical isolates of *P. aeruginosa*. But most importantly, it remained to be resolved whether CEP932, CEP936 and CEP938 exerted a similarly improved antibiofilm activity either alone or when applied in combination with rifampicin.

2. Materials and methods

2.1. General

Materials and solvents were purchased from commercial suppliers (Iris Biotech, Markredwitz, Germany; Fluorochem, Hadfield, United Kingdom; and Merck, Darmstadt, Germany) and used without further purification. Rifampicin and colistin sulfate were purchased from Sigma-Aldrich. Water used for analytical and preparative HPLC was filtered through a 0.22-μm membrane filter (LaboStar® Pro TWF), while water used for biological assays was sterile-filtered and autoclaved.

For this study, several *P aeruginosa* strains were used. PAO1 was used as a reference strain, and several isolates from CF patients were provided by Copenhagen Cystic Fibrosis Center (i.e., isolates 9A, 4137 07, 19,848/98, 41,782/98, 20,164/85 and 40,049/96); of these several were colistin-resistant (i.e., 4137 07, 41,782/98 and 40,049/96). Isogenic sequential pairs of susceptible and resistant isolates comprise 9A and 4137 07, 19,848/85 and 41,782/85 as well as 20,164/85 and 40,049/96. Whole-genome sequencing of the isolates revealed the colistin-resistant phenotype to arise from different mutations as can be seen in Supplementary Table S1. Antimicrobial susceptibility tests and antibiotic synergy assays were performed in Müller-Hinton Broth (MHB) with Mg^{2+} and Ca^{2+} added to a final concentration of 4 mg/L each.

2.2. Ethics statement

The clinical isolates were obtained from Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark. All bacterial strains were revived from freeze storage. According to the Danish Science Ethics Committee, the study did not need approval from the Committee (Protocol nr H-2-2013-FSP45).

2.3. Peptide synthesis

Peptides were synthesized as previously described [26]. Briefly, Fmoc-Dab-OAll (0.8 mmol, 4 eq.) was dissolved in dry DCM (6 mL) and added to a 2-CTC resin (1.0 g, loading: 0.2 mmol/g). After 2 h, the resin was drained and washed with dry DCM, and then capped with a mixture of DCM–DIPEA–MeOH (17:2:1). The linear intermediate peptide was then assembled through manual solid-phase peptide synthesis. Fmoc deprotection was performed by treatment with 20 % piperidine in DMF (2 × 10 min), followed by washing with DMF (× 5). Couplings were performed with HBTU (4 eq.), the appropriate Fmoc-protected amino acid building block (4 eq.) and DIPEA (8 eq.), which all were dissolved in DMF and shaken for 10 min before being added to the freshly Fmoc-deprotected resin, and then coupling was continued for 1 h at room temperature. Before branching of the linear peptide, the ivDde group was removed by treatment with 4 % hydrazine hydrate in DMF (3 × 5 min). Then Fmoc-L-Thr(*t*Bu)-OH was coupled to the free side-chain amine in the same manner as used in previous peptide couplings. The allyl group was removed with $Pd(PPh_3)_4$ (0.2 eq.) and $PhSiH_3$ (10 eq.) in dry DCM (2 mL; 2 × 15 min). After allyl removal, the resin was washed sequentially with DMF (× 5), then with 0.5 % sodium diethyldithiocarbamate in DMF (× 3), DCM (× 3), and finally with DMF (× 3). On-resin cyclization was then performed by addition of PyAOP (4 eq.), HOAt (4 eq.) and DIPEA (8 eq.) dissolved in DMF. The resin-bound cyclic peptide was washed with DMF (× 3) and dry DCM (× 5), and was then simultaneously cleaved from the linker and globally deprotected

with a solution of TFA–TIS–H_2O (95:2.5:2.5) for 1 h, followed by elution of the resin with TFA (2 × 2 mL) and DCM (2 × 5 mL). The crude peptide was then obtained upon rotary evaporation of the combined eluates, and subsequent lyophilization from MeCN–H_2O. The final compounds were obtained upon purification by preparative HPLC on a Phenomenex Luna Omega Polar C18 column (250 × 21.2 mm; particle size: 5 μm; pore size: 100 Å) on a Shimadzu Prominence system using a gradient of 10–40 % buffer B over 20 min. Eluents used for analytical and preparative HPLC were: Eluent A (MeCN–H_2O–TFA 4.95:95.95:0.1) and eluent B (MeCN–H_2O–TFA 94.95:4.95:0.1). All peptides had a purity >95 %, as measured by analytical HPLC on a Phenomenex Luna C18 HST column (100 mm × 3 mm; particle size: 2.5 μm; pore size: 100 Å) on a Shimadzu Prominence and Shimadzu Nexera system. Total yields were approx. 15–20 % based on the initial 2-CTC resin loading.

2.4. Antimicrobial susceptibility testing

The minimum inhibitory concentrations of the polymyxins were evaluated by the broth microdilution method in non-binding polystyrene microtitre plates (Nunc #260860; Thermo Fisher, Waltham, USA). Bacteria were used in a final concentration of 1×10^6 CFU/mL in MHB broth (with Mg^{2+} and Ca^{2+} added to a final concentration of 4 mg/L each). Test compounds were dissolved in sterile 0.9 % saline and aliquots of the stock solution of each compound was then transferred to the microtitre plate, whereafter the solution was serially diluted in the wells to reach a concentration range of 256-0.25 μg/mL. Finally, the bacterial culture was added to each well. For all handling of compounds and transfer of solutions containing test compounds low-binding sterile tubes and tips were used. Microtitre plates were incubated for 20 h at 37 °C. The antibacterial activity was then determined as the lowest concentration of each compound that inhibited bacterial growth as determined by the absorbance measured in the well, being equal to a negative control.

Similarly, interaction between two antimicrobials was quantitatively determined through a checkerboard broth microdilution assay following a similar methodology, though here one antimicrobial (colistin or one of the colistin analogs CEP932, CEP936 or CEP938) was serially diluted down one axis of the microtitre plate, while rifampicin was serially diluted down the other axis. The fractional inhibitory concentration index for each combination was calculated by using the following equation:

$$FICI = \frac{MIC_{A_{comb}}}{MIC_A} + \frac{MIC_{B_{comb}}}{MIC_B}$$

where MIC_A and MIC_B are the MICs of test compounds A and B when used alone, while MIC_{Acomb} and MIC_{Bcomb} are the concentrations of test compounds A and B when used in combination. All experiments were performed in duplicates.

Strain description: The non-mucoid *P. aeruginosa* strain PAO1 [31] was used as control strain in this study. Three pairs of isogenic sequential isolates (colistin-susceptible and -resistant, respectively) from the sputum of patients affected by cystic fibrosis (CF) (i.e., 9A and 4137 07; 19,848/85 and 41,782/98 as well as 20,164/85 and 40,049/96) were obtained from from the Copenhagen CF Center. Whole-genome sequencing results are shown in Suppl. Mat. All selected clinical strains with *phoQ* and/or *pmrB* mutations showed high resistance to colistin (see Suppl. Mat. Table S1).

2.5. Biofilm bead formation

For every experiment involving biofilm alginate beads, the beads were prepared the day prior to testing. The alginate beads were prepared by first mixing a sterile-filtered solution of 1 % seaweed alginate (Protanal LF 10/60, FMC BioPolymer N-3002 Drammen, Norway) in 0.9 % saline with a washed and diluted overnight culture of either PAO1 or 41,782/98 in LB broth. The 1-day old alginate biofilm beads were matured at 37 °C for one day following bead formation, while 6-day old biofilms were matured for six days, changing the medium every day [32]. This mixture was then sprayed as a fine mist through a nozzle at 20 ml/h (using a Graseby 3100 Syringe Pump; Ardus Medical Inc., Watford, UK) into a bath containing Tris buffer with added calcium chloride under magnetic stirring at 300 rpm. After all the alginate solution had been added to the solidifying bath, the suspension was filtered for any large non-uniform beads, centrifuged, and finally the alginate beads were washed several times in a sterile solution consisting of 0.9 % saline with added calcium chloride. Beads were plated, and the CFUs were counted to determine the initial CFU loading to be used the following day.

2.6. Biofilm susceptibility testing

The minimum biofilm eradication concentrations (MBECs) of the compounds were evaluated through a modified protocol similar to the previously described broth microdilution protocol. Alginate biofilm beads were used in a final concentration corresponding to 1×10^6 CFU/mL in sterile 0.9 % saline [32]. All handling of test compounds and transfer of solutions containing test compounds was performed with low-binding sterile tubes and tips, and experiments were performed in non-binding polystyrene microtitre plates. Compounds were dissolved in sterile 0.9 % saline, and aliquots of the stock solution of each compound were then transferred to the microtitre plate, whereafter the solution was serially diluted into the wells to reach a concentration range of 1024-2 μg/mL. Finally, the alginate beads (containing the bacteria) were added to each well. The microtitre plates were then incubated for 20 h at 37 °C. The MBEC was determined as the lowest concentration of each compound for which no bacteria could be regrown on lactose agar plates after recovery from the alginate beads. To recover bacteria from the biofilm, the alginate beads were washed, dissolved in an autoclaved citrate buffer (pH of 6.8), and then plated onto lactose agar plates for CFU counting. All experiments were performed in duplicate.

Interaction between two antimicrobials was quantitatively determined through a protocol following a similar methodology (as for planktonic bacteria), where one antimicrobial (colistin or CEP938) was serially diluted down one axis of the microtitre plate, while rifampicin was serially diluted down the other axis. The fractional biofilm eradication concentration index for each combination was calculated by the following equation:

$$FBECI = \frac{MBEC_{A_{comb}}}{MBEC_A} + \frac{MBEC_{B_{comb}}}{MBEC_B}$$

Where $MBEC_A$ and $MBEC_B$ are the MBECs of test compounds A and B when used alone, while $MBEC_{Acomb}$ and $MBEC_{Bcomb}$ are the concentrations of test compounds A and B when used in combination.

3. Results and discussion

Three polymyxin B analogs (CEP932, CEP936 and CEP938) were tested for their ability to inhibit the growth of a panel of colistin-susceptible and -resistant *P. aeruginosa* clinical isolates. Colistin was included as a control to determine whether compounds CEP932, CEP936 and CEP938 had potential as leads with improved activity. Previously, these analogs were found to exhibit improved antimicrobial activity (with MICs below the EUCAST clinical breakpoint for colistin-resistant strains, i.e., 2 μg/mL) against several colistin-resistant Gram-negative isolates tested [26]. In the present work, these three polymyxins and colistin were tested in another panel of seven *P. aeruginosa* isolates, consisting of three isogenic pairs of a colistin-resistant strain with an MIC of 64 μg/mL or higher and a colistin-susceptible strain as well as the reference PAO1 strain (see Table 1).

Table 1
Minimum inhibitory concentrations (MIC values in μg/mL) of colistin, CEP932, CEP936 and CEP938 against a panel of colistin-susceptible and -resistant *P. aeruginosa* strains.

P. aeruginosa strain[a]	MIC in μg/mL			
	Colistin	CEP932	CEP936	CEP938
PAO1	1–2	1–2	1	1–2
9A	<0.125	0.25	0.25–0.5	0.25–0.5
4137 07 (CstR)	>64	>64-64	8–32	1–4
19,848/85	1	0.25–1	0.5–1	1
41,782/98 (CstR)	>64	16–32	0.5–2	1–2
20,164/85	0.25	1–2	0.5	0.5–1
40,049/96 (CstR)	64	16	2–8	2–8

[a] Colistin-susceptible strains comprise: PAO1, 9A, 19,848/85 and 20,164/85, while colistin-resistant (CstR) strains include 4137 07, 41,782/98 and 40,049/96.

Generally, polymyxins CEP932, CEP936 and CEP938 exhibited improved antimicrobial activity against all colistin-resistant *P. aeruginosa* strains, except for CEP932, which was devoid of activity against colistin-resistant isolate 4137 07. Although isolate 4137 07 was still found to be resistant towards CEP936, the MIC was reduced as compared to that of colistin (i.e., MIC of 8–32 μg/mL for CEP936 and MIC >64 μg/mL for colistin). All three compounds were found to inhibit growth of the colistin-susceptible strains with a similar potency as colistin. Overall CEP938 was found to exhibit excellent activity against all isolates, with MIC values below or near the EUCAST clinical breakpoint for colistin [33]. The EUCAST clinical breakpoint is a useful metric for determining whether a bacterium exhibits tolerance or resistance towards colistin, but it is not representative of the concentration of colistin administered, as much higher concentrations are reached both in serum by i. v. injection and in sputum through inhalation treatment [34,35].

3.1. Synergistic combinations of polymyxins and rifampicin

The outer membrane in Gram-negative bacteria constitutes the main barrier for sufficient spontaneous internalization of hydrophobic antibiotics (e.g., rifampicin) that act on an intracellular target. In order to enable repurposing of such antibiotics for use in Gram-negative pathogens like *P. aeruginosa* it is necessary to apply these in combination with compounds (e.g., colistin) that partially disrupt the integrity of the LPS layer that is the main component in the outer leaflet of the outer membrane of Gram-negative bacteria.

To further characterize the potential utility of CEP932, CEP936 and CEP938, their synergy with rifampicin was examined through an antimicrobial checkerboard assay with colistin included as reference (see Table 2). Overall, synergy with rifampicin was absent for all tested compounds against two of the colistin-susceptible strains (PAO1 and 19,848/85). This is in accordance with recently reported results for colistin-rifampicin combinations [28]. However, for the colistin-resistant strain 41,782/98 synergy with rifampicin was seen for combinations with colistin, CEP32 and CEP936, which in themselves had high MICs (i.e., >2 μg/mL).

Thus, colistin was unable to inhibit growth of the tested colistin-resistant strain (41,782/98) by itself due to an MIC of 256 μg/mL, i.e., much higher than the EUCAST clinical breakpoint (>2 μg/mL) for colistin-resistant bacteria [33]. However, colistin was capable of potentiating rifampicin (to achieve synergy) even at 4 μg/mL, thus reducing the MIC of rifampicin from >256 μg/mL to 2 μg/mL.

Similarly, CEP932 (having an MIC of 16 μg/mL against 41,782/98) at 2 μg/mL potentiated rifampicin at a concentration of 4 μg/mL, which thus constituted a synergistic combination. Compound CEP936 was more active against 41,782/98 with an MIC of 4 μg/mL, while 1 μg/ml of CEP936 was capable of potentiating rifampicin (at 8 μg/ml), which thus constituted a synergistic combination. For CEP938 a synergistic effect could not be observed for combinations with rifampicin, since the concentration required to improve entry of rifampicin was similar to the MIC of CEP938 when applied alone. Hence, polymyxins that are substantially more potent than colistin (e.g., CEP938) appear to exert direct killing already at a concentration equivalent to the membrane-permeabilizing concentration.

Furthermore, the bactericidal concentrations (MBCs) for colistin and CEP938 were determined against two *P. aeruginosa* strains, namely PAO1 and 41,782/98 to assess whether treatment with CEP938 only resulted in improved inhibition of bacterial growth or whether it prevented regrowth after end of exposure. Colistin and CEP938 exhibited similar MBCs against *P. aeruginosa* PAO1 at 1 μg/mL and 1–2 μg/mL, respectively. However, against the colistin-resistant *P. aeruginosa* 41,782/98 strain, the MBC for colistin proved to be > 128 μg/mL, while the MBC for CEP938 was 8 μg/ml, which is 4–8 fold higher than the MIC against this strain.

The MoA for colistin potentiation of other antibiotics has previously been shown to involve a permeabilizing effect on the outer membrane of Gram-negative bacteria, enabling hydrophobic antibiotics (e.g., rifampicin; normally repelled by the negatively charged outer membrane) to enter the cytosol where they interact with their target(s) [36].

Here, colistin and CEP932 were found to exhibit synergy with rifampicin against the resistant 41,782/98 isolate at concentrations of 2–4 μg/mL, while CEP936 appeared to permeabilize the membrane to a sufficient degree already at 1 μg/mL, indicating a slightly improved interaction with LPS. In contrast, CEP938 alone exhibited a direct killing effect at 1–2 μg/mL. These findings suggest that synergy of polymyxins with rifampicin requires a threshold concentration for the polymyxin of approx. 1–2 μg/mL to permeabilize the outer membrane. These concentrations are similar, albeit slightly higher than the concentrations at which these compounds were previously found to permeabilize the membrane of *E. coli* MG1655, in a bacterial envelope disruption assay [26].

3.2. CEP938 efficiently eradicates colistin-resistant biofilms

Based on the results of the antibacterial inhibition assays on planktonic bacteria, we decided to investigate the potential effects of CEP938

Table 2
Fractional inhibitory concentration indices (FICI) of colistin, CEP932, CEP936 and CEP938 when applied in combination with rifampicin against *P. aeruginosa* strains PAO1, 41,782/98 (CstR) and 19,848/85 (susceptible). MIC of rifampicin was found to be > 256 μg/mL for all strains. FICI are calculated as the sum of the ratios between the MICs in combination and the MICs alone. Concentrations are stated as μg/mL.

Strain	Colistin	Colistin/Rifampicin combination	FICI	Strain	CEP932	CEP932/Rifampicin combination	FICI
PAO1	1	0.5/2	~0.51	PAO1	1	2/1	~2.00
19,848/85	1	0.5/1	~0.50	19,848/85	0.5	0.5/1	~1.00
41,782/98 (CstR)	256	4/2	<0.02	41,782/98 (CstR)	16	2/4	<0.13
Strain	CEP936	CEP936/Rifampicin combination	FICI	Strain	CEP938	CEP938/Rifampicin combination	FICI
PAO1	1	2/1	~2.01	PAO1	1	2/1	~2.00
19,848/85	1	1/1	~1.00	19,848/85	1	1/1	~1.00
41,782/98 (CstR)	4	1/8	<0.28	41,782/98 (CstR)	1	2/4	~2.02

Table 3
Minimum biofilm eradication concentration (MBEC) of colistin and CEP938 against *P. aeruginosa* in an alginate bead biofilm model. PAO1 is colistin-susceptible, while 41,782/98 is a colistin-resistant strain. MBEC was determined for both young (1 day old) and mature (6 day old) biofilms. The young biofilm was matured for one day at 37 °C following bead formation, while the old biofilm was matured for six days prior to determination of MBEC.

Minimum Biofilm Eradication Concentration:			
PAO1		41,782/98	
1 day old biofilms			
Colistin	CEP938	Colistin	CEP938
64 μg/mL	16 μg/mL	>1024 μg/mL	64 μg/mL
6 day old biofilms			
Colistin	CEP938	Colistin	CEP938
64 μg/ml	32 μg/mL	>1024 μg/mL	64 μg/mL

Table 4
Fractional biofilm eradication concentration indices (FBECI) of colistin or CEP938 in combination with rifampicin against alginate-bead encapsulated biofilms of *P. aeruginosa* PAO1 and 41,782/98. FBECI is calculated as the sum of the ratios between the MBEC in combination and the MBEC alone. Concentrations are in μg/mL.

Biofilm	Colistin	Rif	Colistin/Rif	FBECI
PAO1	64	>512	32/16	~0.53
41,782/98	>1024	>512	64/32	<0.125
Biofilm	**CEP938**	**Rif**	**CEP938/Rif**	**FBECI**
PAO1	16	>512	16/16	~1.03
41,782/98	64	>512	8/8	<0.14

on both a colistin-resistant and a colistin-susceptible *P. aeruginosa* biofilm, since this compound exhibited the highest antimicrobial activity against the entire panel of tested *P. aeruginosa* strains in planktonic growth. We also wished to determine whether synergy between CEP938 and rifampicin might occur in a biofilm model. In a previous study, synergy between colistin and rifampicin was seen in both planktonic *P. aeruginosa* and in a *P. aeruginosa* biofilm model of both PAO1 and 41,782/98 [28].

Both colistin and CEP938 were tested in an alginate bead biofilm model for their ability to eradicate the biofilm and prevent regrowth (Table 3). For colistin a minimum biofilm eradication concentration (MBEC) of 64 μg/mL was found in both a young (1 day post bead formation) and an old (6 days post bead formation) biofilm of PAO1, which corroborates previously reported MBECs [28]. In contrast, colistin was unable to eradicate the colistin-resistant 41,782/98 biofilm even at the highest tested concentration (i.e., MBEC >1024 μg/mL). For CEP938 lower MBECs were found for both young and old PAO1 biofilms (i.e., 16 μg/mL and 32 μg/mL, respectively), corresponding to 4-fold and 2-fold lowered MBECs as compared to those of colistin. Moreover, CEP938 was able to eradicate the 41,782/98 biofilm regardless of the age with an MBEC of 64 μg/ml, which is similar to the MBEC for colistin in the PAO1 biofilm. These results suggest that CEP938 may provide improved efficacy against *P. aeruginosa* biofilms as compared to colistin, regardless of colistin resistance (see Table 4).

3.3. CEP938 and colistin exhibit synergy with rifampicin in eradication of biofilms of P. aeruginosa 41,782/98

Although CEP938 and rifampicin did not exhibit synergy in planktonic colistin-resistant *P. aeruginosa*, we investigated whether this also would be the case in biofilms, since a previous study had established that synergy between colistin and rifampicin may occur in biofilms [28]. Thus, *P. aeruginosa* biofilm alginate beads (containing PAO1 or 41,782/98) were treated with varying concentrations of either colistin or CEP938 and rifampicin. For biofilms of *P. aeruginosa* PAO1, a slightly lower concentration of CEP938 (i.e., 16 μg/mL) than of colistin (32 μg/ml) was able to potentiate rifampicin (at 16 μg/ml). Thus, an additive interaction was observed between colistin and rifampicin for eradication of the PAO1 biofilm (FBECI ~0.53). The concentration of CEP938 needed to achieve complete PAO1 biofilm eradication remained unchanged at 16 μg/mL when combined with rifampicin, and thus no synergy was observed. For biofilms of *P. aeruginosa* 41,782/98 colistin (at 64 μg/mL) was found to interact synergistically (with a FBECI below 0.125) with rifampicin (at 32 μg/mL) equaling a previous study, where a biofilm peg model was used, thus inferring cross-validity of the different biofilm models when studying the interactions of colistin with other antibiotics [28]. Also, CEP938 proved to exhibit synergy with rifampicin in eradication of a colistin-resistant biofilm, with CEP938 (at 8 μg/mL) potentiating rifampicin (at 8 μg/mL), resulting in an FBECI below 0.14. In fact, this corresponds to eradication of the biofilm at the same concentration as the minimum bactericidal concentration observed for CEP938 against planktonic 41,782/98 (see Supplementary Table S2).

Expression of colistin resistance is often accompanied by a significant cost of fitness leading to an increased susceptibility to other types of antibiotics [37], which may explain why the combination of CEP938 and rifampicin exert efficient eradication of 41,782/98 biofilm, while no synergistic effects were observed against the PAO1 biofilm. Currently, the MoA of CEP938 against planktonic bacteria is not completely elucidated, albeit it proved capable of disrupting the bacterial membrane in a similar, but more rapid and pronounced manner as compared to colistin [26].

Also, it remains to be elucidated why CEP938 exert synergy with rifampicin towards a colistin-resistant biofilm, whereas synergy was absent against planktonic cells. Assuming that CEP938 acts similarly to colistin on the Gram-negative outer membrane, an explanation could be that the concentration at which CEP938 disrupts the outer membrane to allow for uptake of other antibiotics is close to its bactericidal concentration (Table S2), whereas higher concentrations are needed to eradicate the biofilm, thus allowing CEP938 to exert a membrane-permeabilizing effect well below its MBEC. Another explanation could be, that the metabolic active planktonic cells, in contrast to the inactive center of biofilm-growing bacteria, are able to use their efflux pumps (e.g., MexAB-OprM) to prevent efficient uptake of rifampicin through the plasma membrane [38].

The results reported in the present work involved a limited number of *P. aeruginosa* strains, in particular the study of antibiofilm activity is confined to the study of the effect on a single clinical isolate. The risk of development of resistance to rifampicin due to occurrence of target mutations in *P. aeruginosa* during prolonged and repeated treatment with the combination has not been assessed, and this is especially relevant in the context of CF, where there is a high prevalence of hypermutable isolates that have the potential to acquire fast resistance to rifampicin.

4. Conclusion

Our results show that polymyxins developed to overcome colistin resistance may also possess an improved ability to exhibit synergy with other antibiotics against colistin-resistant biofilms. We found that analog CEP938 effectively inhibited growth of three colistin-resistant *P. aeruginosa* clinical isolates. In addition, CEP938 showed improved efficacy in biofilm eradication of colistin-resistant and colistin-susceptible *P. aeruginosa* biofilms. Furthermore, we found that CEP938 in combination with rifampicin was able to eradicate colistin-resistant *P. aeruginosa* biofilms at a concentration comparable to the bactericidal concentration of CEP938 alone against the same strain in planktonic growth. These findings show promise for further development of new strategies for overcoming colistin resistance, both in terms of novel polymyxins as antibacterial agents alone and as part of synergistic combinations with other antibiotics.

CRediT authorship contribution statement

Johan Storm Jørgensen: Writing – original draft, Investigation. **Anne Sofie Laulund Siebert:** Investigation. **Oana Ciofu:** Writing – review & editing, Resources. **Niels Høiby:** Writing – review & editing, Resources. **Claus Moser:** Writing – review & editing, Supervision, Resources. **Henrik Franzyk:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they do not have any competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100224.

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