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Murepavadin is a novel peptide antibiotic that exhibits potent antibiofilm activity and enhances the efficacy of conventional antibiotics against *Pseudomonas aeruginosa*

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ABSTRACT

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections are difficult to treat due to biofilm formation. This study evaluates the antimicrobial and antibiofilm efficacy of murepavadin (MUR), a novel peptidomimetic targeting the LptD protein, alone and combined with conventional antibiotics against clinical isolates. The minimum inhibitory concentrations (MICs) of 50 isolates against MUR and five antibiotics were determined. Synergy in planktonic cultures was assessed by checkerboard assays. For biofilm, the Bliss independence model was applied using MBIC (minimum biofilm inhibitory concentration) and the concentration yielding ≥90% biomass reduction, as measured by crystal violet (CV) staining. MUR showed potent activity against planktonic cells ($MIC_{50}/_{90}$: 0.25/0.5 mg l^{-1}). Synergy tests revealed consistent MUR-colistin synergy across all strains, with notable interactions against carbapenemase-producers when combined with meropenem. In biofilms, MUR demonstrated significantly lower MBIC and lower concentrations required for ≥90% biomass reduction (CV staining). Bliss analysis confirmed strong synergistic biofilm inhibition in all combinations, most pronounced with tobramycin. MUR exhibits high efficacy against both planktonic and biofilm-forming MDR *P. aeruginosa*. Its synergy with colistin and tobramycin highlights its potential as a strategic combination partner.

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Pseudomonas aeruginosa; murepavadin; antibiotic; synergy; biofilm

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, contributing significantly to morbidity and mortality in patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and ventilator-associated infections (Qin et al. 2022). Low membrane permeability, multiple drug elimination pumps and remarkable intrinsic and acquired resistance mechanisms, including chromosomally encoded β-lactamases, pose a critical challenge in clinical management (Horcajada et al. 2019). Consequently, the World Health Organization (WHO) has classified *P. aeruginosa* as a high-priority ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp.) pathogen, emphasizing the imperative need for novel antimicrobial strategies (Pang et al. 2019).

A major barrier to effective treatment is the organism's capacity to form robust biofilms (Thi et al. 2020). These structured bacterial communities are encased in a self-produced matrix of extracellular polymeric substances (EPS), composed of polysaccharides, proteins and extracellular DNA (eDNA), which facilitates significant tolerance to both host immune responses and conventional antibiotics. The EPS matrix acts as a physical shield, limiting drug penetration and rendering biofilm-associated infections notoriously difficult to eradicate (Díez-Aguilar et al. 2021a, 2021b). Furthermore, the Gram-negative outer membrane (OM) constitutes an additional permeability barrier that protects the cell from environmental stresses and contributes to the failure of current therapeutic regimens (Ramesh et al. 2025).

Current guidelines often recommend combination therapies, typically pairing a conventional β-lactam with either a β-lactamase inhibitor (eg avibactam, vaborbactam) or a second antibiotic from a different

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class, such as an aminoglycoside or a fluoroquinolone to enhance coverage against multidrug-resistant (MDR) organisms or to provide synergistic activity (Goodlet et al. 2017). While colistin (COL) remains a "last-resort" option for multidrug resistant (MDR) strains, its clinical utility is frequently constrained by significant nephrotoxicity and neurotoxicity risks (Korkmaz Ekren et al. 2017). This clinical gap has accelerated the search for novel agents that can act as membrane-permeabilizing adjuvants to sensitize recalcitrant bacteria to existing antibiotics (Lehman and Grabowicz 2019).

Among emerging therapeutic candidates, murepavadin (MUR), formerly POL7080, represents a strategic first-in-class peptidomimetic antibiotic (Martin-Loeches et al. 2018). Unlike traditional antibiotics, MUR specifically targets the LptD protein, a key component of the LptABCDEFG complex responsible for transporting lipopolysaccharide (LPS) to the OM (García-Gros et al. 2024). By disrupting OM biogenesis, MUR compromises bacterial cell integrity and demonstrates potent activity against MDR and extensively drug-resistant (XDR) strains, including carbapenemase-producers (Sader et al. 2018a, 2018b). Recent evidence reveals that MUR-mediated LptD inhibition leads to a lethal accumulation of LPS within the cytoplasmic membrane (CM), a mechanism that may overcome the conventional limitations of antibiotic failure in both planktonic and sessile communities (Robinson 2019). However, despite these promising preclinical and early-stage clinical attributes, the Phase III PRISM trials evaluating its intravenous formulation for the treatment of nosocomial pneumonia (NCT03409679) were terminated due to an unacceptably high frequency of acute kidney injury, raising significant safety concerns for its systemic use (Elmassry et al. 2023).

This study evaluated the *in vitro* activity of MUR against 50 clinical *P. aeruginosa* isolates, including phenotypically confirmed carbapenemase producers. It further investigated the synergistic potential of MUR in combination with five diverse antibiotics using checkerboard assays and evaluated its anti-biofilm efficacy through the Bliss independence model.

Materials and methods

Antibiotics and murepavadin

The active components of antibiotics used in this study were obtained from MedChemExpress (Monmouth Junction, NJ, USA). These include tobramycin (TOB; HY-B0441), ciprofloxacin (CIP; HY-B0356), colistin (COL; HY-A0089), meropenem (MRP; HY-13678), ceftazidime (CAZ; HY-B0593) and murepavadin (MUR; HY-P1674A).

Bacterial isolates

A total of 50 *P. aeruginosa* strains were isolated from clinical samples (blood, sputum, bronchial aspirates, wounds, urine and endotracheal tubes) received by the Medical Microbiology laboratory of Balıkesir University Health Practice and Research Hospital. These isolates were identified using the Phoenix-M50 automated bacterial identification system (BD, Sparks, MD, USA) and stored at −80 °C for later use. Reference strains *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PAO1 were used as controls.

Carbapenem inactivation method (CIM)

The carbapenem inactivation method (CIM) was used to determine carbapenemase production in clinical isolates (Gauthier et al. 2017). Briefly, 10 µl of bacterial culture was suspended in 400 µl of sterile distilled water. A 10 µg MRP disk was immersed in the suspension and incubated at 37 °C for 2 h. After incubation, the disk was transferred onto a Mueller-Hinton agar plate inoculated with *Escherichia coli* ATCC 29522 (adjusted to 0.5 McFarland turbidity) and incubated at 37 °C for 18–24 h. Carbapenemase production was indicated by the growth of *E. coli* around the MRP disk, demonstrating inactivation of the antibiotic. Non-carbapenemase-producing isolates exhibited a clear inhibition zone around the disk (Supplementary Figure S1).

Broth microdilution method

The MICs of MUR and other antibiotics were determined using the broth microdilution method in 96-well plates, following ISO 20776–1 guidelines recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Yilmaz et al. 2024). All tests were performed in cation adjusted Mueller–Hinton broth (CAMHB) (Oxoid Ltd., Hampshire, UK) for MUR and antibiotics. Fifty µl of CAMHB were pipetted into each well of the microplates. Serial dilutions of MUR and antibiotics were prepared to 64 mg l^{-1} and 0.03 mg l^{-1}, respectively. Bacterial suspensions of overnight cultures prepared to a McFarland density of 0.5 ($\sim 10^8$ CFU ml^{-1}) were diluted in CAMHB (1:100 v/v), and 50 µl of this

suspension was added to each well of the microplates (final concentration 5×10^5 CFU ml^{-1}). One well in the microplate was used for growth control (CAMHB + bacteria), solvent control for MUR and antibiotics (CAMHB + bacteria + distilled water), medium control (CAMHB) and sterility control (CAMHB + MUR/antibiotic). The microtiter plates were incubated at 37 °C for 18–24 h. Bacterial growth was indicated by turbidity in the well. The lowest concentration at which no bacterial growth was observed was determined as the MIC value. All experiments were three independent biological replicates, and average values were recorded.

Checkerboard method

The checkerboard method was used to assess the synergistic interaction between MUR and other antibiotics (TOB, CIP, COL, MRP and CAZ) against 10 *P. aeruginosa* strains selected to reflect the general resistance profiles of the study population. The 10 selected *P. aeruginosa* isolates were chosen based on their phenotypic diversity in terms of carbapenemase production, antibiotic resistance profiles and the variety of clinical samples from which they were isolated, thus representing different resistance mechanisms and sources of infection.

All tests were performed in CAMHB. CAMHB was dispensed into the initial microplate in a volume of 50 µl and the initial antibiotic was diluted from top to bottom in a vertical plane starting from 2–3 dilutions above and 5–6 dilutions below the MIC value. Fifty µl of CAMHB were dispensed into the second microplate and the MUR was diluted horizontally from right to left, from 2–3 dilutions above the MIC to 5–6 dilutions below. The dilutions from the second microplate were transferred to the first microplate with 50 µl in exactly the same well, obtaining different combinations of the two agents in each well (Eliopoulos and Moellering 1996; Yilmaz et al. 2024).

Bacterial suspensions were prepared as described in the microdilution method and added to each well of the microplates, except for the control wells, at a final concentration of 5×10^5 CFU ml^{-1}. The control wells used in the first microplate were growth control (CAMHB + bacteria), medium control (CAMHB), solvent control (CAMHB + bacteria + distilled water) and sterility control (CAMHB + MUR/antibiotic). Microplates were incubated at 37 °C for 18–24 h. All experiments were three independent biological replicates, and average values were recorded. The interaction between MUR and antibiotics was determined by calculating the fractional inhibitory concentration index (FICI) using the following formula (Odds 2003):

$$FICI = \left(\frac{MIC_{Murepavadin} \text{ in combination}}{MIC_{Murepavadin} \text{ alone}}\right) + \left(\frac{MIC_{Antibiotic} \text{ in combination}}{MIC_{Antibiotic} \text{ alone}}\right) \quad (1)$$

The interpretation of FICI values was based on previously determined breakpoints:

Synergy: $FICI \leq 0.5$

No interaction: $0.5 < FICI \leq 4.0$

Antagonism: $FICI > 4.0$

Evaluation of biofilm formation and anti-biofilm activity

Biofilm formation assay

The biofilm-forming ability of 50 clinical *P. aeruginosa* isolates was evaluated using a standard microtiter plate assay (Stepanović et al. 2007). Briefly, bacterial suspensions were prepared in brain heart infusion (BHI) broth from freshly grown colonies and incubated overnight at 37 °C. The overnight cultures were then diluted 1:100 in BHI broth supplemented with 0.25% (w/v) glucose. Aliquots of 200 µl of the diluted suspensions were dispensed into sterile, flat-bottom 96-well microtiter plates. P. aeruginosa PAO1 and uninoculated BHI broth served as positive and negative controls, respectively. The plates were incubated statically for 24 h at 37 °C. All experiments were performed in triplicate. After incubation, biofilm biomass was quantified using crystal violet (CV) staining. The planktonic cells were carefully removed, and each well was gently washed three times with phosphate-buffered saline (PBS) to eliminate non-adherent bacteria. The adherent biofilms were fixed at 60 °C for 30 min and subsequently stained with 1% (w/v) CV for 30 min at room temperature. Excess stain was removed by rinsing the plates with PBS, and the plates were air-dried. The bound dye was then solubilized using a destaining solution consisting of 80% ethanol and 20% acetone. The optical density (OD) of the solubilized CV was measured at 570 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Waltham, MA, USA). In the evaluation, samples were grouped as follows: those with $ODs \leq ODc$ did not produce biofilm; those with $ODc < ODs \leq 2 \times ODc$ produced weak biofilm; those with $ODc < ODs \leq 4 \times ODc$ produced moderate biofilm; and those with $4 \times ODc < ODs$ produced strong

biofilm. ODc represents the mean OD_{570} value of the negative control + (3 × SD of the negative control), while ODs represents the OD_{570} values of the samples.

Determination of minimum biofilm inhibitory and biomass reduction concentrations

Minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration preventing visible biofilm formation. Minimum Biofilm Eradication Concentration (MBEC) (operationally defined as the lowest concentration reducing pre-formed biofilm biomass by ≥90% compared to control, as measured by CV) was used as the reference endpoint. The samples tested were the same 50 clinical *P. aeruginosa* isolates listed in Supplementary Table S1 (Thieme et al. 2019; Cruz et al. 2018). The MBIC assay was conducted to assess the prophylactic potential of the agents in preventing initial bacterial colonization. Two-fold serial dilutions of each antimicrobial agent were prepared in BHI broth supplemented with 0.25% glucose, which serves as a metabolic trigger to enhance biofilm matrix production. Bacterial suspensions were standardized to a 0.5 McFarland turbidity (~10^8 CFU ml^{-1}) and subsequently diluted 1:100 in the glucose-supplemented BHI to achieve a final inoculation density of approximately 10^6 CFU ml^{-1} per well. The 96-well flat-bottomed polystyrene plates were incubated statically for 24 h at 37 °C. Biofilm formation was quantified using the CV staining method. The MBIC was defined as the lowest concentration resulting in a ≥ 90% reduction in biofilm biomass compared to the untreated growth control. The percentage of biofilm inhibition was calculated as follows:

$$\text{Biofilm Inhibition } (\%) = [1 - (OD_{treated}/OD_{control})] \times 100 \quad (2)$$

where $OD_{treated}$ and $OD_{control}$ represent the mean optical density values (measured at 570 nm) of the treated wells and untreated growth control wells, respectively.

The MBEC assay was performed to evaluate the agent's ability to penetrate and disrupt pre-established mature biofilms. Mature biofilms were first established by incubating bacterial suspensions in 96-well plates for 24 h at 37 °C without antimicrobial intervention. Following the maturation period, the supernatant containing planktonic cells was aspirated, and the wells were gently washed twice with PBS to remove non-adherent cells while preserving biofilm integrity. Two-fold serial dilutions of the antimicrobial agents in fresh BHI broth were added to the pre-formed biofilms, and the plates were incubated statically for another 24 h at 37 °C. After treatment, the solutions were removed, the wells were rinsed with PBS, and the remaining biomass was quantified by CV staining. The MBEC was defined as the lowest concentration that achieved a ≥ 90% reduction in the pre-formed biofilm biomass compared to the untreated biofilm control. The percentage of biofilm biomass reduction was calculated using the same formula as for the inhibition assay. To ensure methodological rigor, all MBIC and MBEC tests were three independent biological replicates.

Bliss-based evaluation of established biofilm biomass reduction induced by murepavadin–antibiotic combinations

The combined antibiofilm activity of MUR and conventional antibiotics was evaluated on four potent biofilm-producing isolates (PA10, PA34, PA43 and PA50) by quantitatively determining biofilm biomass reduction using the CV staining method. Biofilms were established in 96-well microtiter plates, treated with individual agents and their combinations, and subsequently stained with CV. Absorbance values were measured at 570 nm. Biofilm inhibition (%) was calculated relative to untreated biofilm controls after subtraction of background absorbance from cell-free wells. All interaction experiments were conducted at sub-biofilm biomass reduction concentrations (MBEC/2, MBEC/4 and MBEC/8) to specifically assess anti-biofilm interactions under non-eradicating conditions. These concentration ranges were deliberately selected because they overlap with the MBIC and, in several cases, approach the planktonic MIC values of the tested agents. This strategy allowed the evaluation of synergistic antibiofilm effects at clinically and biologically relevant sub-inhibitory concentrations, while minimizing the confounding impact of near-complete biofilm biomass loss or nonspecific biomass loss.

The Bliss independence model was applied, as it is particularly suitable for the analysis of non-viability-based biofilm biomass data derived from CV assays (Kovács et al. 2016; Vikelouda et al. 2021). For each concentration pair, the expected non-interactive effect (E_{Bliss}) was calculated from the fractional biofilm inhibition values of the individual agents (E_A for MUR and E_B for the antibiotic) using the equation:

$$E_{Bliss} = E_A + E_B - (E_A \times E_B) \quad (3)$$

The interaction effect (ΔE) was determined by subtracting the expected effect from the observed combination effect (E_{Obs}):

$$\Delta_E = E_{Obs} - E_{Bliss} \quad (4)$$

To ensure statistical robustness, ΔE values were calculated for all tested concentration pairs, and the mean ΔE for each drug combination was determined. Following the criteria described by Geladari et al. (2019), a combination was considered to exhibit statistically significant synergy when the mean ΔE was positive and its 95% confidence interval (CI) did not include zero. Conversely, significant antagonism required a negative mean ΔE with a 95% CI not crossing zero. Interactions with 95% CIs including zero were classified as indifferent.

Statistical analyses

Bliss Δ values were analyzed using non-parametric tests due to the small sample size ($n = 4$ clinical isolates). Differences between antibiotic combinations at each concentration were assessed using the Friedman test for repeated measures. For each antibiotic combination, the effect of all three concentrations was evaluated using the Friedman test. When a significant effect was detected overall ($p < 0.05$), post-hoc pairwise comparisons were performed using the Dunn test with Bonferroni correction. Statistical significance was defined as $\alpha = 0.05$ for the main tests and Bonferroni corrected $\alpha = 0.005$ for post-hoc comparisons (10 pairwise comparisons). All analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM, but *p*-values are based on rank tests.

Results

Antibacterial activity of murepavadin and conventional antibiotics

Antibiotic susceptibility testing revealed that all 50 *P. aeruginosa* isolates were susceptible to COL ($MIC_{50}/_{90}$: 0.5/1 mg l^{-1}) according to EUCAST v16.0 criteria. Resistance rates for other agents were documented as follows: CIP (42%), CAZ (16%), TOB (16%) and MRP (12%) (Supplementary Table S1). Phenotypic screening *via* the CIM assay identified carbapenemase activity in 10% of the isolates. MUR demonstrated potent *in vitro* activity against the tested strains ($MIC_{50}/_{90}$: 0.25/0.5 mg l^{-1}), with 80% of isolates exhibiting MIC values ≤0.25 mg l^{-1}.

Synergistic interactions of murepavadin and antibiotics against planktonic strains

Synergy studies were conducted on 10 selected *P. aeruginosa* strains, with detailed information on MIC values, clinical sample origins, and carbapenemase production status provided in Table 1.

Synergistic interactions were observed between MUR and several conventional antibiotics from different classes (eg COL, CIP, MRP) commonly used to treat *P. aeruginosa* infections. Notably, COL and MUR demonstrated consistent synergy (FICI range 0.240–0.500) across all tested strains (Table 2).

MBIC and biofilm biomass reduction values of murepavadin and antibiotics

Biofilm formation was assessed using the microtiter plate method. Among the 50 clinical *P. aeruginosa* isolates screened, 18 exhibited moderate and four exhibited strong biofilm formation (Figure 1 and Table 3). MUR showed significantly lower MBIC (2–8 mg l^{-1}) and lower MBEC values (8–32 mg l^{-1}; here MBEC refers to ≥90% biomass reduction by crystal violet, not viability-based eradication) compared to antibiotics, while antibiotics required higher MBIC (8–512 mg l^{-1}) and MBEC (32 to >512 mg l^{-1}) (Supplementary Figure S2).

Bliss-based evaluation of antibiofilm synergy in biofilm biomass reduction

The antibiofilm activity of MUR in combination with five clinically relevant antibiotics was evaluated against four *P. aeruginosa* clinical isolates using the Bliss independence model at sub-biofilm biomass reduction concentrations (MBEC/2, MBEC/4 and MBEC/8) (Supplementary Figure S3). At MBEC/2, all MUR–antibiotic combinations exhibited Bliss Δ values above the synergy threshold (+10) in all four isolates, indicating consistent synergistic biomass reduction. The Friedman test revealed a significant difference among the five combinations ($\chi^2 = 10.48$, $df = 4$, $p = 0.033$). Post-hoc analysis with Bonferroni correction ($\alpha = 0.005$) showed that MUR+TOB (mean $\Delta = 28.45 \pm 1.66$) was significantly more synergistic than MUR+CIP (22.48 ± 0.23, $p = 0.004$) and MUR+CAZ (19.83 ± 0.95, $p = 0.001$). No other pairwise differences reached significance ($p > 0.005$ for all). At MBEC/4 and MBEC/8, all Bliss Δ values were below +10, indicating non-synergistic interactions. For every antibiotic combination, the Friedman test confirmed a significant effect of concentration (all $p = 0.018$), with

Table 1. *In vitro* activity of murepavadin and antibiotics against clinical isolates selected for combination study (mg l^{-1}).

Strain	TOB	CIP	COL	MRP	CAZ	MUR	Carbapenemase activity	Clinical specimen
PA6	0.5	0.25	0.25	0.25	4	0.125	Negative	Sputum
PA10	8	8	1	16	16	0.5	Positive	Sputum
PA11	4	16	1	16	16	1	Positive	Wound
PA20	0.5	1	1	0.25	4	0.25	Negative	Wound
PA23	0.25	0.06	0.125	0.125	2	0.06	Negative	Urine
PA34	8	16	1	16	16	1	Positive	Tracheal aspirate
PA43	64	16	1	16	8	0.5	Positive	Urine
PA48	0.25	0.125	0.125	0.5	2	0.25	Negative	Urine
PA49	0.5	0.5	0.5	1	4	0.5	Negative	Sputum
PA50	8	4	1	16	16	0.25	Positive	Tracheal aspirate

Abbreviations: TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; MRP, meropenem; CAZ, ceftazidime; MUR, murepavadin.

Table 2. Interactions of MUR and antibiotics against *P. aeruginosa* strains.

	MUR				
	TOB	CIP	COL	MRP	CAZ
Strain	FICI values				
PA6	0.528	0.620	0.258	0.720	0.508
PA10	0.560	0.504	0.254	0.310	0.375
PA11	0.370	0.490	0.258	0.258	0.625
PA20	0.730	0.300	0.251	0.490	0.516
PA23	0.480	0.484	0.406	0.480	0.330
PA34	0.566	0.504	0.500	0.504	0.254
PA43	0.406	0.530	0.310	0.406	0.504
PA48	0.490	0.512	0.240	0.625	0.290
PA49	0.550	0.530	0.406	0.550	0.533
PA50	0.531	0.490	0.490	0.503	0.365
P. aeruginosa ATCC 27853	0.524	0.524	0.248	0.274	0.245

Abbreviations: TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; MRP, meropenem; CAZ, ceftazidime; MUR, murepavadin; FICI, fractional inhibitory concentration index. Green color indicates synergistic interactions (FICI $\leq$ 0.5). Red color indicates indifferent interactions (0.5 < FICI $\leq$ 4.0).

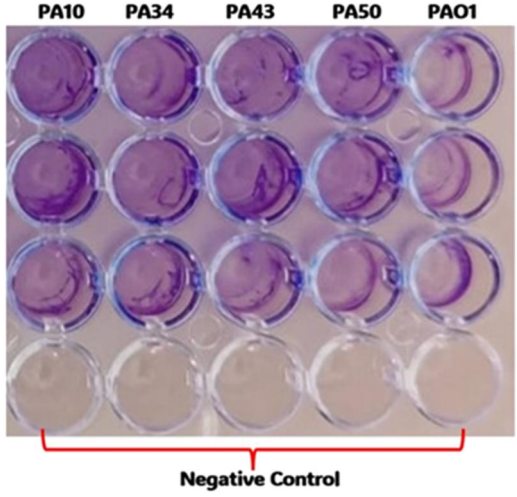

Figure 1. Strong biofilm formation of *P. aeruginosa* isolates as determined by the crystal violet microtiter plate test. Microtiter plate image showing biofilm production of four clinical isolates (PA10, PA34, PA43 and PA50) and the reference strain PAO1. The bottom row represents the negative control (medium only).

MBEC/2 values being markedly higher than those at MBEC/4 and MBEC/8. This concentration-dependent response was consistent across all four clinical isolates. Figure 2 depicts the heat map of Bliss Δ scores for MUR–antibiotic combinations across different MBEC concentrations in the tested *P. aeruginosa* clinical isolates.

Table 3. Minimum biofilm inhibitory concentrations (MBICs) of murepavadin and conventional antibiotics against strong biofilm-producing *P. aeruginosa* strains (mg l^{-1}).

	Strong biofilm-producing strains							
	PA10		PA34		PA43		PA50	
Antimicrobial	MBIC	MBEC	MBIC	MBEC	MBIC	MBEC	MBIC	MBEC
TOB	32	128	64	256	128	512	32	128
CIP	64	128	64	256	128	256	32	128
COL	8	32	16	64	16	128	8	32
MRP	128	256	64	256	128	256	128	256
CAZ	64	256	128	256	512	>512	256	512
MUR	2	8	4	16	8	32	2	8

Abbreviations: TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; MRP, meropenem; CAZ, ceftazidime; MUR, murepavadin; MBIC, minimum biofilm inhibitory concentration; MBEC, minimum biofilm biomass reduction concentration, operationally defined as the lowest concentration reducing pre-formed biofilm biomass by ≥90% compared to untreated control (crystal violet staining).

Percentage reduction in biofilm biomass corroborated the Bliss analysis, with combination treatments achieving a reduction of 67.7–84.2% at MBEC/2, followed by 21.9–32.5% at MBEC/4 and 4.8–7.2% at MBEC/8. The synergistic antibiofilm interactions assessed using the Bliss independence model are presented in Supplementary Figure S4, and the corresponding biofilm biomass reduction percentages for each clinical isolate are presented in Supplementary Figure S5.

Discussion

In this study, the *in vitro* activity of MUR, alone and in combination with conventional antibiotics, was evaluated against clinical *P. aeruginosa* isolates under both planktonic and biofilm conditions. The results demonstrated that MUR exhibited strong antibacterial activity against MDR isolates and showed synergistic interactions with several antibiotics, particularly COL, MRP and TOB. In addition, MUR-based combinations demonstrated variable antibiofilm activity

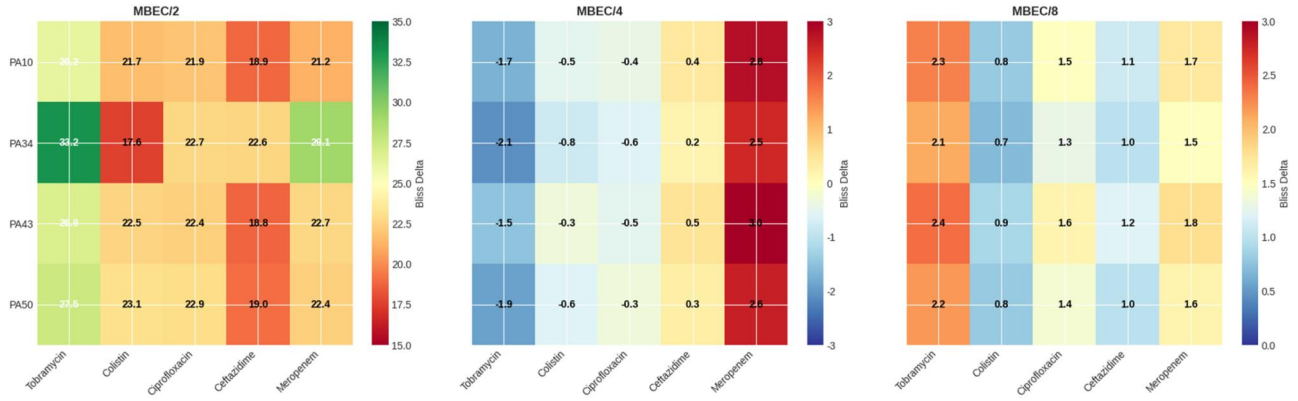

Figure 2. Heat map of Bliss delta scores (Δ) for MUR–antibiotic combinations against four *P. aeruginosa isolates* (PA10, PA34, PA43, PA50). Δ was calculated at MBEC/2, MBEC/4 and MBEC/8 (MBEC: ≥90% biomass reduction by crystal violet). Δ ≥ 10 indicates synergy in biomass reduction. Statistical comparisons (Friedman test with Dunn's post-hoc, Bonferroni corrected) revealed that at MBEC/2, MUR+TOB was significantly more synergistic than MUR+CIP and MUR+CAZ ($p < 0.005$). Data are from three independent biological replicates ($n = 4$ isolates). Data were coded with Python 3.14.2 (Python Software Foundation, Wilmington, DE, USA) and visualized *via* Google Colab (Google LLC; https://colab.research.google.com).

depending on the tested concentration and combination profile.

The potent *in vitro* activity of MUR against susceptible and resistant *P. aeruginosa* populations has been reported in a limited number of studies; MIC_{50} and MIC_{90} values generally range between 0.03–1.6 and 0.03–4 mg l^{-1}. As summarized in Table 4, the findings were generally consistent with the available literature and support the high antipseudomonal activity of MUR against MDR clinical isolates. Díez-Aguilar et al. (2021a) and Díez-Aguilar et al. (2021b) also reported low MIC values for MUR against clinical *P. aeruginosa* isolates, supporting the findings obtained in the present study. In addition, Sader et al. (2018a) emphasized the retained activity of MUR against contemporary MDR and XDR *P. aeruginosa* isolates collected worldwide.

Post-hoc analysis with Bonferroni correction revealed that MUR+TOB was significantly more synergistic than MUR+CIP and MUR+CAZ ($p < 0.005$), but not significantly different from MUR+COL or MUR+MRP. Synergistic interactions between MUR and COL have been detected, and similar synergistic profiles have been previously reported for MUR-COL combinations against resistant *P. aeruginosa* isolates (Sabnis et al. 2021). Earlier mechanistic studies suggested that inhibition of the LptD protein by MUR may alter OM organization and facilitate access of polymyxins to lipid A targets (Sabnis et al. 2021). However, no membrane permeability or molecular mechanistic analyses were performed in the present study. Therefore, the current findings should be interpreted as phenotypic evidence of synergy based on MIC, Minimum Bactericidal Concentration (MBC) and biofilm inhibition analyses. Romano et al. (2019) also reported that *pmrB*-associated lipid A modifications may contribute to reduced susceptibility to both MUR and COL, suggesting that possible cross-resistance mechanisms should be considered in future studies. In addition, Tsuji et al. (2019) highlighted the clinical importance and toxicity limitations of polymyxin-based therapies, supporting the potential value of combination approaches aimed at reducing effective polymyxin exposure.

The synergistic activity detected between MUR and MRP was also notable, particularly among carbapenem-resistant isolates. Similar observations have been reported in previous studies investigating MUR-based β-lactam combinations (Wei et al. 2023). Although the precise mechanism underlying this interaction was not investigated in the present study, previous reports proposed that MUR-associated OM alterations may facilitate β-lactam penetration into the periplasmic space and improve access to penicillin-binding proteins (Wei et al. 2023). Some non-carbapenemase-producing isolates demonstrated weaker or absent synergistic responses, suggesting that additional resistance mechanisms, such as efflux systems or target-associated alterations, may also influence combination efficacy.

MUR combinations with TOB and CIP also demonstrated synergistic or partially synergistic profiles in several isolates. Previous studies suggested that MUR exposure may increase aminoglycoside susceptibility through membrane-associated effects facilitating intracellular antibiotic uptake (Wei et al. 2024b). Similarly,

Table 4. Comparative study results of murepavadin and antibiotics against *P. aeruginosa* (mg l–1).

	MUR		TOB		CIP		COL		MRP		CAZ	
Reference	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
Sader et al. (2018b)	0.12	0.25	8	>8	-	-	1	2	16	>32	32	>32
Wei et al. (2023)	0.06	0.03	-	-	-	-	-	-	>64	>64	-	-
Wei et al. (2024a)	0.03	0.03	-	-	0.12	0.25	-	-	-	-	-	-
Sabnis et al. (2021)	1.6	0.8	-	-	-	-	1	0.8	-	-	-	-
Ekkelenkamp et al. (2020)	0.12	2	1	16	1	8	1	2	0.25	16	2	64
Ghassani et al. (2024)	0.12	4	2	32	1	8	1	2	1	32	4	64
Sader et al. (2018a)	0.12	0.12	0.5	>16	0.12	>8	1	1	0.5	16	2	>32
Díez-Aguilar et al. (2021b)	≤0.5	1	2	16	-	-	1	4	-	-	-	-
This study	0.25	0.5	0.5	8	0.25	16	1	0.5	0.5	16	4	8

Abbreviations: TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; MRP, meropenem; CAZ, ceftazidime; MIC, minimum inhibitory concentration.

earlier reports proposed that alterations in LPS organization following MUR exposure could indirectly affect multidrug efflux systems and intracellular fluoroquinolone accumulation (Wei et al. 2024a). Nevertheless, since no permeability, membrane potential, or efflux-related analyses were conducted in the present study, these mechanisms remain speculative in the context of the findings of this study. The observed synergistic interactions should therefore be interpreted primarily according to the phenotypic checkerboard and bactericidal assay results. The synergistic MUR concentrations observed in this study (MIC range 0.06–1 mg l^{-1} for the tested isolates) are substantially lower than the plasma Cmax achieved in healthy volunteers at a 4.5 mg kg^{-1} intravenous dose (approximately 9.5–12.8 µg ml^{-1}; Wach et al. 2018). This indicates that the effective concentrations are clinically achievable. However, the same phase I study reported dose-dependent adverse events including paresthesia, dizziness and infusion site reactions (all mild to moderate). No serious adverse events were attributed to MUR alone, but combination toxicity with COL, TOB, or other antibiotics has not been evaluated. Moreover, later phase III trials have reported pulmonary adverse events not observed in phase I. Therefore, the *in vitro* findings are hypothesis-generating; future animal efficacy and toxicity studies are required to determine the therapeutic window of MUR–antibiotic combinations. Clinical development of murepavadin was halted due to nephropathy. Amponnawarat et al. (2021) reported that MUR activates human mast cells *via* the MRGPRX2 receptor, leading to degranulation and pro-inflammatory cytokine release. This mechanism may contribute to acute renal injury and may be risky in terms of hypersensitivity reactions. However, the same study also suggests that this immunomodulatory effect may also promote extensive clearance and wound healing. Therefore, *in vivo* studies should carefully evaluate not only antibacterial efficacy but also renal function and mast cell-mediated immune responses.

Biofilm-associated infections caused by *P. aeruginosa* remain difficult to treat because of limited antimicrobial penetration into the EPS matrix and the reduced metabolic activity of biofilm-associated cells (Li et al. 2023). In addition, biofilm cells exhibit markedly higher antibiotic tolerance than planktonic counterparts, highlighting the need for combination-based therapeutic strategies (Touati et al. 2025). Recent studies have demonstrated that antibiotic combinations and adjuvant approaches may enhance activity against resistant *P. aeruginosa* and biofilms.

Several combination therapies have shown improved efficacy *in vitro* and in clinical settings. For instance, ceftazidime–avibactam combined with aztreonam has demonstrated promising activity against metallo-β-lactamase-producing strains (Liu et al. 2026), while gentamicin–cefepime combinations have shown strong antibiofilm effects *in vitro* (Usman et al. 2023). In addition, matrix-targeting strategies such as enzymatic degradation by alginate lyase or chelation-mediated disruption using EDTA have been reported to enhance antibiotic penetration and activity against biofilms (Charęza et al. 2023; Hale et al. 2024).

Beyond conventional combinations, modulation of biofilm regulatory pathways has also been explored. Antimicrobial peptides, for example, may enhance antibiotic activity through quorum sensing inhibition and efflux pump regulation (Shang et al. 2021). Collectively, these findings support the concept that combination-based or adjuvant-supported approaches may improve treatment outcomes in biofilm-associated infections. In the present study, Bliss independence analysis indicated that MUR+antibiotic combinations exhibited concentration-dependent synergistic or non-interactive profiles. Higher synergy scores observed at MBEC/2 concentrations became non-interactive at lower concentrations, suggesting a threshold effect for activity against mature biofilms.

Among all tested combinations, MUR+TOB showed the highest antibiofilm activity, achieving up to 84.2% biomass inhibition. Importantly, MBEC was defined based on CV staining; therefore, values reflect biomass reduction rather than complete eradication of viable cells.

Only limited studies have investigated the antibiofilm activity of MUR. Previous reports have shown $MBIC_{50}/_{90}$ and $MBEC_{50}/_{90}$ values ranging from 2/32 mg l^{-1} to 16/64 mg l^{-1}, highlighting its relatively strong activity against *P. aeruginosa* biofilms compared with other inhaled antibiotics (Tré-Hardy et al. 2008; Díez-Aguilar et al. 2021a). Overall, our findings are consistent with the growing evidence supporting combination strategies for chronic biofilm-associated infections, including CF-related *P. aeruginosa* infections (Ghassani et al. 2024).

Despite these findings, the study has several limitations. First, all experiments were conducted under *in vitro* conditions that may not fully reflect host–pathogen interactions observed *in vivo*. Second, mechanistic analyses evaluating membrane permeability, efflux pump activity, or molecular resistance pathways were beyond the scope of this study. Therefore, mechanistic interpretations discussed here are based primarily on previously published studies and should not be considered direct experimental findings of the present work. Furthermore, the lack of standardized clinical breakpoint criteria for MUR currently limits the direct interpretation of MIC findings in clinical settings. Future studies should focus on physiologically relevant models such as artificial sputum medium and investigate the molecular basis of MUR-associated synergistic interactions and potential resistance mechanisms.

Conclusion

In summary, this study reveals that MUR demonstrates potent *in vitro* activity against MDR and carbapenemase-producing *P. aeruginosa* isolates. Its findings substantiate MUR's role as a strategic membrane-permeabilizing adjuvant that facilitates the efficacy of diverse antibiotic classes through OM destabilization. Notably, the robust synergy documented with COL and MRP underscores a compelling rationale for dose-optimization strategies aimed at mitigating clinical toxicity. Furthermore, the concentration-dependent anti-biofilm activity of MUR-based combinations, most prominently with TOB, attests to its potential in managing recalcitrant biofilm-associated infections. While these results validate MUR as a promising therapeutic candidate, extensive *in vivo* investigations and the establishment of standardized clinical breakpoints are imperative to confirm its safety and long-term clinical success.

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Author contributions

CRediT: **Umut Yılmaz**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing; **Özlem Erkoç Güleryüz**: Conceptualization, Investigation, Writing – original draft, Writing – review & editing; **Mehmet Ünlü**: Supervision, Writing – original draft, Writing – review & editing; **Gülhan Vardar Ünlü**: Data curation, Supervision, Writing – original draft, Writing – review & editing.

Ethical approval

This study was approved by the Non-Interventional Research Ethics Committee of Balıkesir University Faculty of Health Sciences (Approval No. 2025/236).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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