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ORIGINAL ARTICLE

Exploring novel amides as efflux pump inhibitors for overcoming antibiotic resistance in multidrug-resistant *Pseudomonas aeruginosa*

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa (P. aeruginosa)*, a multidrug-resistant bacterium, represents a considerable risk in healthcare environments owing to its capacity to induce various infections. The resistance of *P. aeruginosa* is frequently linked to efflux pumps that actively remove antibiotics from the bacterial cell. This study investigates novel amide compounds as potential alternatives to address *P. aeruginosa* isolates exhibiting multidrug resistance mediated by efflux pumps.

Materials and Methods: Gram staining and biochemical assays revealed thirty-three multi-drug-resistant *P. aeruginosa* isolates from a tertiary care hospital Peshawar. After antibiotic susceptibility testing, efflux pumps were detected using Ethidium Bromide (EtBr) agar cartwheel technique and UV transilluminator. Novel amides were tested for efflux pump and anti-pseudomonal action against efflux pump-positive isolates utilizing agar well diffusion and micro broth dilution, including synergy with ciprofloxacin and gentamicin.

Results: Three high efflux pump activity *P. aeruginosa* isolates were chosen using ETBr agar cartwheel technique. Novel amides (ITC, ITD, ITE, DEP) block efflux pump, although TEM-cu is very antimicrobial. TEM-cu, DEP, ITC, and ITE have 0.19, 0.78, and 0.78 mg/ml MICs. Effectiveness against efflux pump-expressing *P. aeruginosa* is lowest with ITE (1.56 mg/

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ml). Together with ciprofloxacin and gentamicin, TEM-cu and DEP improved antimicrobial effectiveness.
Conclusion: TEM-cu is highly effective against efflux pump-positive *P. aeruginosa*, while amides like ITC, ITD, ITE, and DEP block these pumps. With significant reductions, DEP and TEM-cu improve ciprofloxacin and gentamicin efficacy. This method may help overcome *P. aeruginosa* efflux pump-mediated resistance.

Keywords: Anti-bacterial agents; Checkerboard assay; Drug resistance; Efflux pump; *Pseudomonas aeruginosa*

INTRODUCTION

P. aeruginosa commonly infects animals, plants, and humans (1). The severity of disease ranges from minor self-limiting to chronic serious infections with high rate of morbidity and mortality. *P. aeruginosa* is known to cause a variety infections in both healthy individuals and those with weak immune system and is the leading cause of death and disease in cystic fibrosis (CF) (2). It is frequently found in the environment and different sources like aqueous and vegetative environmental sources (3). *P. aeruginosa* can grow in almost any environment including at extreme temperatures, because of its nominal nutritional requirements. It is widely distributed in nature and hospital settings, such as bed rails, sinks and nurses' hands (4). When the host defense is compromised, it can lead to invasion and damage to the host. Bacterial resistance to antibiotics is on the rise, while the development of new antibiotics remains limited. If resistance continues to increase at its current rate, it will be difficult to combat superbugs and people will experience significant morbidity and death, as earlier (5).

Efflux Pump is a mechanism involved in antibiotic resistance (6). The efflux pump system is responsible for transporting a variety of substrates from inside to outside of the cell like dyes, antibiotics, detergents, and other molecules (7). For bacteria to cause infection and grow biofilm, efflux pumps are critical (8, 9). The efflux pump plays a crucial role in antibiotic resistance and contributes to multidrug resistance phenotype (10). *P. aeruginosa* efflux mechanism uses three proteins to remove antibiotics and other substances from the cell: cytoplasmic transporter proteins that use energy as a proton motive force, outer membrane porins, also known as OprM, and a periplasmic connective protein (11). In *P. aeruginosa* the most expressed efflux pump isolates are MexAB-OprM, MexXY, MexEF-OprN, and MexCD-OprJ and play an important role in multidrug resistance phenotype in clinical isolates (12). Efflux pump inhibitors have been shown in studies to reduce the minimum inhibitory concentration (MIC) of several antibiotics against *P. aeruginosa* (12). Efflux pump inhibitors (EPIs) are increasingly vital in combating multidrug-resistant (MDR) *P. aeruginosa*, a major nosocomial pathogen (13, 14). Efflux pumps are crucial in *P. aeruginosa*, as they actively expel a range of antimicrobial drugs, reducing their effectiveness and increasing the risk of treatment failure (10, 15). Several studies have shown that when efflux pump inhibitors are used in conjunction with antibiotics, the antibiotics work better against resistant pathogens (16).

Amides are functional groups where a carbonyl carbon atom is bonded to a nitrogen atom, as well as a hydrogen or a carbon atom, by a single bond. Amides can also be called carboxamide or organic amides (17, 18). Amide derivatives are associated with a broad spectrum of biological activities including anti-tuberculosis (19), anti-convulsant (20), anti-inflammatory (21), insecticidal (22), anti-fungal (23), and anti-tumor properties (24). Morphine derivatives are found to have a wide spectrum of antimicrobial, anti-helminthic, bactericidal and insecticidal activity (25).

Considering the resistance mechanisms in *P. aeruginosa*, this research project utilized amides (ITC, ITD, ITE, TEM-cu, and DEP) to investigate their potential in inhibiting the efflux pumps in *P. aeruginosa* isolates.

MATERIALS AND METHODS

Sample collection and processing. The study was conducted at the Institute of Basic Medical Sciences (IBMS), Department of Microbiology, Khyber Medical University (KMU) in Peshawar. The study involved collecting multidrug-resistant *P. aeruginosa* samples from three tertiary hospital laboratories. Samples were obtained from pus, urine, and burn patients, and were transported to the Microbiology laboratory at KMU in Eppendorf tubes under strict aseptic conditions and protocols. Confirmation of *P. aeruginosa* involved assessing colony morphology, gram staining, and biochemical tests (catalase, oxidase, triple sugar iron).

Antibiotic susceptibility test and agar well diffusion method. Following CLSI 2019 guidelines, antibiotic susceptibility testing was conducted to identify multidrug-resistant *P. aeruginosa* isolates. Initial confirmation of MDR isolates utilized the Kirby-Bauer disk diffusion method with antibiotic discs recommended by CLSI 2019 (26): ceftazidime 30 µg, aztreonam 30 µg, meropenem 10 µg, amikacin 30 µg, ciprofloxacin 5 µg, and gentamicin. *P. aeruginosa* ATCC 25619 served as a control, and Muller Hinton Agar was the medium for susceptibility testing. Moreover, agar well diffusion technique was performed to find out the antibacterial activity of novel amides (ITC, ITD, ITE, DEP and TEM-cu). This method is similar to antibiotic susceptibility test but instead of antibiotic disc well were made in the media. This method is mostly done for liquid compounds.

Identification of efflux pump in *P. aeruginosa* using ETBr agar cartwheel approach. All isolates were tested for their efflux pump activity after conformation of multidrug resistant *P. aeruginosa*, and the activity was verified by a useful, simple, and cost-effective method called ETBr Agar Cartwheel Method (27). The ETBr agar cartwheel approach is a simple technique used to detect the efflux pump activity in isolates. Tryptic soya agar plates containing 0.5µl of ETBr were prepared in the test. In this process, the purpose of adding ETBr is that it acts as an efflux pump substrate and the bacteria push EtBr from within the cell to outside, as the bacteria also extrude the antibiotic from the cell.

Determination of minimum inhibitory concentration (MIC) using broth microdilution methods. The MIC of ITC, ITD, ITE, TEM-cu, DEP, ciprofloxacin, and gentamicin were determined using the microbroth dilution method as described by CLSI guidelines (28). Serial two-fold dilutions of the amides (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 mg/mL) were prepared and added to 96-well microtiter plates. Similarly, ciprofloxacin and gentamicin were diluted (2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 µg/mL) and added to separate wells. Following the addition of bacterial suspensions from efflux pump-positive *P. aeruginosa* isolates, plates were incubated to allow bacterial growth. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible bacterial growth in the wells after incubation, thereby indicating antimicrobial activity.

Determination of minimum bactericidal concentration (MBC) for amides. The MBC is defined as the lowest concentration of an antibiotic, natural compound, or synthetic compound that results in bacterial cell death (28). This assessment is typically conducted following the MIC determination to establish whether the tested compound is bactericidal rather than merely bacteriostatic. While bacteriostatic agents inhibit bacterial growth, bactericidal agents actively kill the microorganisms. Determining whether bacterial growth is simply inhibited in the MIC assay can be challenging. Therefore, the MBC assay is essential to confirm whether the compound truly eliminates viable bacteria.

Synergistic checkerboard assay. The checkerboard assay is a technique used to evaluate how two antimicrobial agents interact whether their combined effect is synergistic, additive, or antagonistic. In this method, the two antimicrobials are serially diluted in microtiter plates: one along the rows and the other along the columns, creating a grid of various concentration combinations. Each well is then inoculated with a standardized bacterial suspension and incubated to allow growth. After incubation, microbial growth is typically assessed by measuring turbidity, revealing how the two agents work together against the microorganisms (29). To assess the synergistic effect of amides (ITC, ITD, ITE, TEM-cu, and DEP) with ciprofloxacin and gentamicin against active efflux pump *P. aeruginosa*, a high MIC value isolate was selected. In the 1st row of 96-well plates, 100 µl of culture was combined with 100 µl of ciprofloxacin, while in the 2nd row, 100 µl of culture was mixed with 100 µl of gentamicin. Subsequent rows involved combining 100 µl of culture with 50 µl of each amide and 50 µl of ciprofloxacin or gentamicin. The MIC plate was incubated at 37°C for 18-24 hours, and results were observed visually, following the checkerboard assay methodology (30).

RESULTS

Identification of *P. aeruginosa.* Thirty-three samples collected from hospital were cultured MacConkey agar, followed by 37°C incubation for 24 hours. *P. aeruginosa* is a non-lactose fermenter and its colonies on MacConkey agar, a selective medium for gram-negative bacteria, (31) are shown in Fig. 1. Fur-

thermore, these isolates were oxidase positive and no reaction on triple sugar iron (TSI) agar indicates that the organism did not ferment any of the sugars (glucose, lactose, or sucrose) present in the medium (32).

Antibiotic susceptibility test for *P. aeruginosa*. The assessment of antibiotic susceptibility (Fig. 2) shows that amikacin (39%), gentamicin (34%), and meropenem (29%), had high efficacy. On the other hand, ceftazidime (19%), ciprofloxacin (21%), and aztreonam (18%) showed low efficacy.

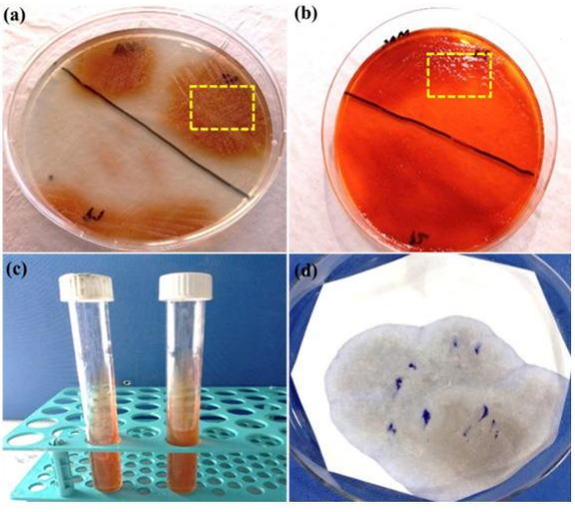

Fig. 1. (a) brown red color pigment on nutrient agar (b) swarming colonies on MacConkey agar (c) no color change at the bottom and on the slant, no gas and H2S production and (d) change of color to blue indicates positive oxidase test for *P. aeruginosa*.

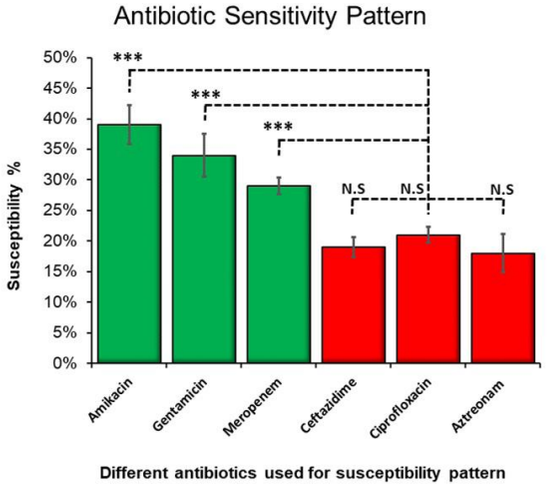

Fig. 2. Antibiotics susceptibility pattern of *P. aeruginosa*

Antibacterial activity of amides against *P. aeruginosa*. All the experiments were performed in triplicates, mean and standard deviation were calculated. After 24 hours of incubation, the antibacterial activity of amides against *P. aeruginosa* was assessed as shown in Fig. 3, revealing varying zones of inhibition. Notably, TEM-cu 2-((1-methoxy-4-(methylthio)-1-oxobutan-2-yl) carbamoyl) benzoic acid) and DEP 2-((1-methoxy-3-methyl-1-oxopentan-2-yl) carbamoyl) benzoic acid) demonstrated high sensitivity, with respective zones of 18 mm and 19 mm, while ITC N-(2,4-Dichlorophenyl) dodecanamide), ITD N-(2-Trifuolromethylphenyl) dodecanamide), and ITE showed intermediate activity with zones of 10 mm ± 1, 9 mm ± 1.3, and 13 mm ± 1.7 respectively.

Prevalence of efflux pump positive *P. aeruginosa*. In a sample set of 33 multidrug-resistant (MDR) *P. aeruginosa*, 8.82% exhibited efflux pump activity as shown in Fig. 3.

Inhibition of efflux pump by amides. After a 24-hour incubation, plates with ETBr alone exhibited no fluorescence, indicating active efflux pumps. Plates containing ETBr with amides (ITC, ITD, ITE, and DEP) displayed fluorescence, signifying good efflux pump inhibitory activity (Fig. 4). However, the plate with ETBr and TEM-cu showed no fluorescence, suggesting a lack of efflux pump inhibitory activity for TEM-cu.

Minimum inhibitory and bactericidal concentration. The bacteriostatic or bactericidal nature of the tested amides against an efflux pump-expressing isloates of *P. aeruginosa* was evaluated through a minimum inhibitory concentration (MIC) assay. The results in Table 1 revealed potent bactericidal effects for

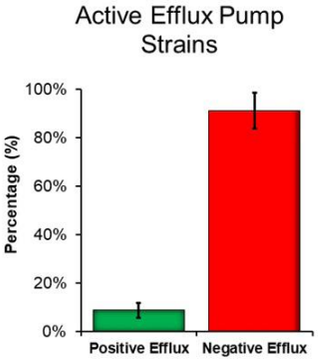

Fig. 3. Prevalence of efflux pump producing *P. aeruginosa*

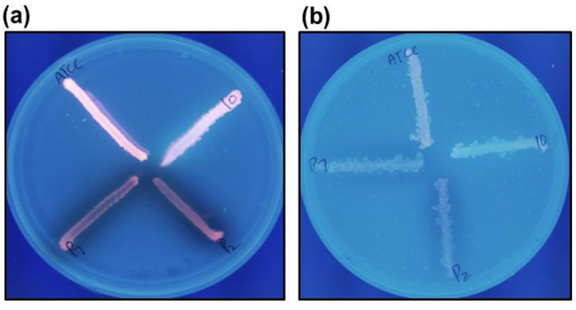

Fig. 4. (a) Negative control plate containing only EtBr. Fluorescence is absent, indicating active efflux pump function in *P. aeruginosa* isolates (P1, P2) and reference strains (ATCC, ID), which extrude EtBr and prevent its intracellular accumulation. (b) Test plate containing EtBr and amide compounds. Fluorescence is observed, suggesting that the amide compounds inhibit efflux pump activity in the isolates and strains, leading to EtBr retention and visible fluorescence.

Table 1. Bactericidal effect of amides against efflux pump expressing isolates of *P. aeruginosa*

Amides	MIC value	MBC
ITC	0.78 mg/ml ± 0.01	0.78 mg/ml
ITD	1.56 mg/ml ± 0.03	1.56 mg/ml
ITE	0.78 mg/ml ± 0.05	0.78 mg/ml
DEP	0.19 mg/ml ± 0.01	0.19 mg/ml
TEM-cu	0.19 mg/ml ± 0.04	0.19 mg/ml

all examined amides, including ITC (MIC: 0.78 mg/ml ± 0.01), ITD (MIC: 1.56 mg/ml ± 0.03), ITE (MIC: 0.78 mg/ml ± 0.05), DEP (MIC: 0.19 mg/ml ± 0.01), and TEM-cu (MIC: 0.19 mg/ml ± 0.04). Subsequently, the minimum bactericidal concentration (MBC) values mirrored the MIC values for each amide, reaffirming their robust bactericidal activity.

Minimum inhibitory concentration of ciprofloxacin and gentamicin. Ciprofloxacin displayed activity at 16 µg/ml, while gentamicin showed activity at 512 µg/ml. The minimum inhibition concentration (MIC) values confirmed ciprofloxacin's effectiveness at 16 µg/ml and gentamicin's activity at 128 µg/ml.

Synergistic effect of ciprofloxacin and gentamicin along with amides. Ciprofloxacin and gentamicin with amides (ITC, ITD, ITE, TEM-cu, and DEP), enhanced activity was noted for ciprofloxacin with TEM-cu and DEP, as well as for gentamicin with TEM-cu and DEP. Synergistic effects were evident, with consistent MIC values for amides ITC (0.78 mg/ml), ITD (1.56 mg/ml), ITE (0.78 mg/ml), DEP (0.19 mg/ml), and TEM-cu (0.19 mg/ml). Ciprofloxacin displayed reduced MIC (16 µg/ml) in combination with amides, while gentamicin showed decreased MIC (32 µg/ml) with TEM-cu and DEP, indicating potential synergy. All the data is summarized in Table 2.

DISCUSSION

P. aeruginosa, a prominent nosocomial pathogen affecting immunocompromised and burn patients, heightens morbidity and mortality rates (33). With increasing antibiotic resistance, there is a pressing global health concern. Research, focusing on precise susceptibility patterns and antibiotic adjuvants, has become crucial for addressing the growing resistance issue.

This research determined a high antibiotic resistance profile in *P. aeruginosa*, with piperacillin, piperacillin-tazobactam, and ciprofloxacin displaying notable resistance. Meropenem, ceftazidime, and aztreonam exhibited comparatively lower resistance. These results highlight the importance of selecting antibiotics carefully in clinical settings, offering important new information for improved treatment strategies. Notably, A. Rehman et al. reported heightened resistance to piperacillin, ceftazidime, and ciprofloxacin in *P. aeruginosa* (34), whereas our study found sensitivity to amikacin and resistance to gentamicin, ceftazidime, meropenem, and aztreonam. Variations in resistance levels may be attributed to differences in sample size, antibiotics used, and the specific environment yielding multi-drug resistant *P. aeruginosa* (35, 36).

In this study, inhibitors (ITC, ITD, ITE, TEM-cu, and DEP) were applied to TSA plates with EtBr, and fluorescence, indicating efflux pump activity which was observed using a UV transilluminator. Increased fluorescence was noted in samples with inhibitors, while minimal or no fluorescence was observed in EtBr-only samples. Recently a study utilized EtBr in TSA media found that the presence of the inhibitor PABN led to higher fluorescence, demonstrating inhibited efflux pump activity (37). Similarly, our study, employing synthetic amides as inhibitors, showed inhibitory activity against *P. aeruginosa*'s efflux pump (37, 38). Previous studies which had

Table 2. Synergistic effect of ciprofloxacin and gentamicin with amides (ITC, ITD, ITE, TEM-cu and DEP)

Amides	MIC of Amides	MIC of Ciprofloxacin in combination with amides	MIC of Gentamicin in combination with amides
ITC	0.78 mg/ml	16 µg/ml	128 µg/ml
ITD	1.56 mg/ml	16 µg/ml	128 µg/ml
ITE	0.78 mg/ml	16 µg/ml	128 µg/ml
DEP	0.19 mg/ml	8 µg/ml	32 µg/ml
TEM-cu	0.19 mg/ml	4 µg/ml	32 µg/ml

used UV transilluminators observed increased fluorescence with efflux pump inhibition, supporting our findings. These inhibitors not only block efflux pump action but also exhibit antibacterial activity, crucial for understanding their dual role (39, 40).

In our investigation, synthetic amides (DEP and TEM-cu) with ciprofloxacin and gentamicin displayed a 3-fold reduction, highlighting synergy. Matsumoto et al. (41) found a two-fold reduction with phenyl arginine naphthylamide and ciprofloxacin; however, our synthetic amides (TEM-cu and DEP) showed a 3-fold reduction. Talebi et al. (42) used CCCP as an efflux inhibitor with ciprofloxacin, resulting in a two-fold reduction, while our study found a 3-fold reduction with synthetic amides (TEM-cu and DEP) in combination with ciprofloxacin. This finding underscores the significance of efflux-mediated resistance in a subset of MDR isolates, necessitating further exploration for targeted therapeutic strategies. The MBC values for each amide matched the MIC values, confirming their strong bactericidal activity. The consistency of MIC and MBC values suggests that these amides may be effective antimicrobials, which could lead to new treatments for bacterial infections, particularly those involving efflux pump mechanisms in *P. aeruginosa*.

Chiu-Fai Kuok identified luteolin as an inhibitor against MRSA and MSSA, displaying partial synergy when combined with aminoglycosides and quinolones. Gentamicin, an aminoglycoside, is impacted slightly by the extract of *Daphne genkwa*, binding irreversibly to the bacterial ribosome's 30S subunit to disrupt protein synthesis (43). MRSA's aminoglycoside resistance mechanisms align with Khameneh B et al.'s 2014 study, where gentamicin combined with piperine showed a 16-fold reduction (44, 45). In our study, novel amides (TEM-cu and DEP) exhibited a 3-fold reduction, while amides (ITC, ITD, and ITE) showed no MIC reduction.

CONCLUSION

The novel amide derivatives ITC, ITD, ITE, and DEP exhibited the ability to inhibit efflux pumps in Pseudomonas aeruginosa isolates, thereby enhancing the retention of antimicrobial agents. Among these, TEM-cu displayed strong intrinsic antibacterial activity. When used in combination with ciprofloxacin and gentamicin, both TEM-cu and DEP significantly improved the efficacy of these antibiotics, resulting in a two-fold reduction in their minimum inhibitory concentrations (MICs). Notably, DEP combined with ciprofloxacin achieved a three-fold MIC reduction, while TEM-cu with ciprofloxacin produced a two-fold reduction. These results suggest that DEP and TEM-cu act synergistically with conventional antibiotics, likely through efflux pump inhibition and complementary antibacterial mechanisms.

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