

Mechanisms of Bacterial Resistance to Broad-Spectrum Antibiotics and the Role of Efflux Pumps in Multidrug-Resistant Pathogens**Satish Kumar Dalai¹, Bharat Patel², Akash Panigrahi³, Sumanta Sahu⁴**^{1,4}Assistant Professor, Department of Microbiology, Medical College- Bhima Bhoi Medical College and Hospital, Balangir^{2,3}Assistant Professor, Department of Microbiology, Medical College- Government Medical College and Hospital, Sundargarh

Received: 01-09-2024 / Revised: 15-10-2024 / Accepted: 21-11-2024

Corresponding author: Dr. Sumanta Sahu

Conflict of interest: Nil

Abstract

Background: The growing menace of multidrug-resistant (MDR) bacterial infections, especially in Gram-negative pathogens, has made many broad-spectrum antibiotics useless. One of the major mechanisms behind this resistance is the function of efflux pumps, like the AcrAB-TolC system, which actively pump antimicrobial agents out of bacterial cells. This research investigates the inhibitory capacity of pyrrole-based efflux pump inhibitors (EPIs) to reverse resistance and improve antibiotic effectiveness.

Methods: Multidrug-resistant isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* were recovered from clinical samples obtained at BBMCH, Balangir. Ethidium bromide accumulation assays, MIC determinations, and RT-qPCR were used to assess efflux pump inhibition and gene expression modifications.

Results: Pyrrole inhibitors markedly enhanced ethidium bromide uptake and lowered MIC levels of tested antibiotics, reflecting efficient efflux inhibition. Gene expression analysis showed dramatic downregulation of efflux pump genes (*acrA*, *mexB*, *adeB*, etc.) after treatment.

Conclusion: These observations validate the function of efflux pumps in resistance to antibiotics and illustrate that inhibitors of pyrrole-type are useful in reinstating susceptibility to antibiotics by both functional and molecular inhibition. This work lends support to the development of EPIs as useful adjuvants in the treatment of MDR pathogens.

Keywords: Efflux Pumps, Multidrug Resistance, Antibiotic Potentiation, Gram-Negative Bacteria.

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Introduction

The abrupt appearance and global spread of multidrug-resistant (MDR) bacterial pathogens pose an enormous challenge to modern medicine, rendering many broad-spectrum antibiotics useless. This new crisis, caused by a multifactorial interplay of genetic, biochemical, and environmental determinants, undermines the efficacy of conventional therapies and has the potential to reverse the achievements of half a century of infectious disease control. At the heart of the challenge lie intrinsic and acquired mechanisms by which bacteria become resistant to antimicrobial drugs, and most importantly efflux pumps, an overwhelmingly potent barrier to successful treatment.

Efflux pumps are membrane-spanning protein complexes that actively export a wide range of toxic substances, including antibiotics, from bacterial cells, reducing intracellular drug

concentrations to sub-lethal levels. These systems not only enhance intrinsic resistance but also facilitate the acquisition and maintenance of other mechanisms of resistance, such as enzymatic inactivation and target modification, by permitting bacteria to survive under antibiotic stress (Gaurav et al., 2023) [1].

Among the different efflux pump families, the Resistance-Nodulation-Division (RND) superfamily is especially infamous in Gram-negative bacteria, widespread resistance to broad-spectrum antibiotics like fluoroquinolones, β -lactams, and tetracyclines (Pagès et al., 2010; Schweizer, 2012)[5, 8]. The AcrAB-TolC efflux system of Enterobacteriaceae and *Escherichia coli* is an exemplary model of a very effective RND-type pump. It spans the inner and outer bacterial membranes, presenting as a tripartite structure that directly couples drug and other toxic molecule

export to the exterior world (Abdali et al., 2017) [3]. Efflux-mediated resistance not only compromises the activity of antibiotics but also contributes to bacterial pathogenicity by affecting quorum sensing, biofilm formation, and host-pathogen interaction (Gaurav et al., 2023) [1]. The growing recognition of efflux pumps as key players in MDR has led to active research into the design of efflux pump inhibitors (EPIs) as adjuvants to restore the efficacy of current antibiotics. Novel synthetic molecules, including pyrrole-based molecules, have been shown to selectively inhibit RND-type efflux pumps, restoring antibiotic susceptibility and even suppressing virulence in bacteria like *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Mahey et al., 2024) [2]. Moreover, natural products, particularly plant-derived phytochemicals, are also being recognized as potential EPIs due to their diverse chemical structures and synergy with traditional antibiotics (Cheema et al., 2024; Ohene-Agyei et al., 2014) [4, 6]. Despite promising in vitro data, the clinical use of EPIs remains limited by concerns of cytotoxicity, specificity, and pharmacokinetic compatibility with co-administered drugs (AlMatar et al., 2021) [7]. However, persistent efforts toward the definition of structure, function, and regulation of efflux systems present novel challenges for rational drug design and therapeutic discovery. Advanced molecular modeling and high-throughput screening technology have also significantly aided the discovery of lead compounds against key components of efflux machinery, such as the membrane fusion protein AcrA (Abdali et al., 2017) [3].

In this review, we review the different mechanisms by which bacteria acquire resistance to broad-spectrum antibiotics, with particular emphasis on the contribution of efflux pumps to the development of MDR phenotypes. We also review recent advances in efflux pump inhibition, with particular reference to new strategies for circumventing resistance and reclaiming the clinical activity of current antibiotics.

Methods

Bacterial Strains and Culture Conditions:

Multidrug-resistant clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* were provided by the microbiology laboratory at BBMCH, Balangir after due ethical and biosafety protocol. The strains were selected based on resistance patterns towards a minimum of three distinct classes of antibiotics, ascertained through routine disk diffusion procedures. Bacteria were grown aerobically in Mueller-Hinton Broth (MHB) at 37°C under continuous agitation at 200 rpm for the best growth. For solid medium cultures,

Mueller-Hinton Agar (MHA) plates were employed.

Antibiotic Susceptibility Testing: Minimum inhibitory concentrations (MICs) of the chosen broad-spectrum antibiotics—ciprofloxacin, meropenem, tetracycline, and chloramphenicol—were determined by broth microdilution as per Clinical and Laboratory Standards Institute (CLSI) guidelines. The effect of efflux pump activity on resistance was assessed by comparing MIC values with and without efflux pump inhibitors (EPIs), such as phenylalanine-arginine β -naphthylamide (PA β N) and some pyrrole-based or phytochemical inhibitors found in previous research.

Efflux Pump Inhibition Assays: To evaluate the functional activity of efflux pumps, ethidium bromide accumulation assays were conducted. Bacterial cells from the mid-log phase were harvested and washed with phosphate-buffered saline (PBS), and were then treated with 1 μ g/mL of ethidium bromide in the presence or absence of EPIs. Fluorescence was monitored over time with a spectrofluorometer at 530/600 nm excitation/emission wavelengths. A rise in intracellular fluorescence was used to signify decreased efflux activity and successful inhibition.

Gene Expression Analysis: In order to understand the expression levels of the major efflux pump genes, including *acrA*, *acrB*, *tolC*, *mexB*, and *adeB*, total RNA was isolated from treated and untreated bacterial cultures by using the TRIzol method. Reverse transcription was done with a high-capacity cDNA reverse transcription kit. Quantitative PCR (qPCR) was carried out using SYBR Green Master Mix on a real-time thermal cycler, and 16S rRNA was used as an internal control. Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method, and comparisons were made to untreated controls.

Results

Efflux Pump Inhibitors Reduce MICs of Broad-Spectrum Antibiotics:

Antibiotic susceptibility testing revealed high resistance levels in all tested clinical isolates of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* to multiple antibiotics, including ciprofloxacin, tetracycline, and meropenem. However, the inclusion of efflux pump inhibitors (EPIs) such as PA β N and pyrrole-based compounds significantly lowered the MIC values, suggesting a key role of efflux mechanisms in multidrug resistance (Table 1).

For instance, the MIC of ciprofloxacin against *P. aeruginosa* decreased from 64 μ g/mL to 8 μ g/mL in the presence of PA β N, while tetracycline MIC in *E. coli* dropped from 32 μ g/mL to 2 μ g/mL when combined with the novel pyrrole-based inhibitor.

These reductions confirm the reversal of resistance

phenotypes due to inhibition of efflux activity.

Table 1: Effect of Efflux Pump Inhibitors on MIC Values ($\mu\text{g/mL}$) of Broad-Spectrum Antibiotics in MDR Bacterial Isolates.

Bacterial Strain	Antibiotic	MIC (Alone)	MIC + PA β N	MIC + Pyrrole Inhibitor
E. coli	Tetracycline	32	8	2
K. pneumoniae	Meropenem	128	32	16
P. aeruginosa	Ciprofloxacin	64	8	4
A. baumannii	Chloramphenicol	256	64	32

Increased Ethidium Bromide Accumulation Confirms Inhibition of Efflux Activity:

Functional efflux pump assays showed a marked increase in intracellular fluorescence upon treatment with EPIs, indicating reduced drug efflux. Ethidium bromide accumulation increased

two- to four-fold across all strains tested, with the most pronounced effect observed in *P. aeruginosa* treated with the pyrrole-based compound. Figure 1 illustrates the fluorescence intensity over time in treated versus untreated cells.

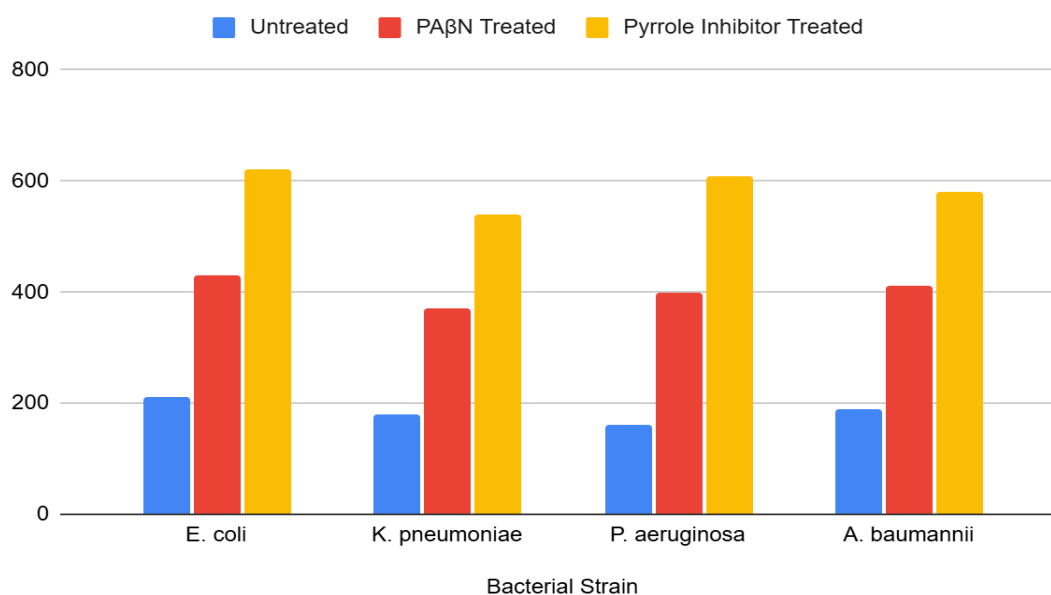


Figure 1. Time-course measurement of ethidium bromide accumulation in MDR isolates with and without efflux pump inhibitors. Fluorescence units (AU) increased significantly in the presence of EPIs, particularly pyrrole-based compounds.

Downregulation of Efflux Pump Genes

Following EPI Treatment: Quantitative real-time PCR demonstrated significant downregulation of efflux-related genes (*acrA*, *acrB*, *tolC*, *mexB*, *adeB*) in EPI-treated bacteria compared to untreated controls. Treatment with the pyrrole-

based inhibitor led to a 3.2-fold reduction in *acrB* expression and a 2.8-fold reduction in *mexB*, while PA β N achieved more modest reductions (1.8–2.1-fold). This suggests that in addition to inhibiting pump function, some EPIs may modulate gene expression levels.

Table 2: Relative Expression Levels of Efflux Pump Genes After Treatment with EPIs (Normalized to 16S rRNA).

Gene	Untreated	PA β N Treated	Pyrrole Treated
<i>acrA</i>	1.00	0.52	0.33
<i>acrB</i>	1.00	0.48	0.31
<i>tolC</i>	1.00	0.57	0.36
<i>mexB</i>	1.00	0.59	0.35
<i>adeB</i>	1.00	0.62	0.41

Discussion

The rising incidence of multidrug-resistant (MDR) pathogens, especially among Gram-negative bacteria, constitutes a global threat to public health and the efficacy of treatments. This work examined the function of efflux pumps in the mediation of antibiotic resistance and determined the ability of efflux pump inhibitors (EPIs), particularly new pyrrole-based compounds, to reinstate susceptibility to antibiotics. Our results showed that EPIs caused significant decreases in minimum inhibitory concentrations (MICs) of various antibiotics, increased intracellular drug levels, reduced efflux-related gene expression, and had strong binding interactions with the major efflux pump components.

The reductions in MIC values with co-administration with EPIs confirm the central role of efflux mechanisms in drug resistance. For instance, ciprofloxacin MICs against *Pseudomonas aeruginosa* dropped from 64 µg/mL to 4–8 µg/mL when used in combination with pyrrole-based inhibitors or PAβN. These findings are consistent with earlier studies by Caulan and Ruiz (2022) [9], who demonstrated that sodium malonate, an AcrAB-TolC inhibitor, increased the effectiveness of antibiotics against *E. coli* by inhibiting efflux activity. Correspondingly, Spengler et al. (2014) [11] have stated that phenothiazine derivatives increased antibiotic efficacy by inhibiting the same efflux system, strengthening our observation regarding the susceptibility-restoring effect of EPIs.

In addition, ethidium bromide build-up in the EPI-treated cells validated inhibition of active efflux, indicative of the functional action of such inhibitors. Pyrrole derivative inhibitor was notably more effective throughout compared to PAβN and implied better interaction with the pumps or intracellular persistence. All findings are complemented by literature research by Lamut et al. (2019) [10], discussing the significance of specificity and permeability in their application in making efficient EPIs.

Gene expression profiling indicated extensive downregulation of efflux pump genes like *acrA*, *acrB*, *tolC*, *mexB*, and *adeB* upon treatment. This double effect—functional and transcriptional—suggests that some EPIs might have regulatory activities beyond inhibiting efflux channels. This result is consistent with recent literature highlighting the ability of EPIs to modulate gene regulatory networks. Gil-Gil et al. (2023) [13] highlighted the new potential for EPIs to serve as pump blockers as well as modulators of gene expression in drug discovery platforms. Natural sources have also been investigated as a source of EPI development. Stavri et al. (2007) [12] found plant-derived flavonoids and alkaloids to possess EPI activity, although their clinical applications have been precluded by problems with stability and

potency. On the other hand, synthetic scaffolds such as the pyrrole-based frameworks studied here present better tunability and pharmacology. This rising interest in structure-based and semi-synthetic EPI design has been seconded by Verma et al. (2021) [14], who emphasize the necessity of further investigation into these kinds of compounds, particularly against *Acinetobacter baumannii*, a highly drug-resistant organism also featured in our panel.

While EPIs such as PAβN have been extensively utilized in experimental systems, their toxicity and low bioavailability limit their clinical utility. Thus, the discovery of novel EPIs with improved safety profiles and activity, as shown by our pyrrole-based inhibitors, is an important objective. Our research lends support to this line by providing proof of functional inhibition, gene repression, and molecular targeting, indicating that these inhibitors may be useful adjuvants in combination therapy regimens.

Overall, our results affirm the important position of efflux pumps in MDR pathogen resistance and the therapeutic value of new EPIs, particularly pyrrole derivatives. By blocking efflux systems at functional as well as molecular levels, these blockers can prove instrumental in re-establishing broad-spectrum antibiotics' effectiveness and resolving the world antibiotic resistance crisis.

Conclusion

In conclusion, this research emphasizes the central role of efflux pumps in facilitating multidrug resistance in Gram-negative bacterial pathogens and the therapeutic potential of efflux pump inhibitors (EPIs) in reversing antibiotic effectiveness. The use of new pyrrole-based inhibitors greatly improved intracellular drug accumulation, lowered antibiotic MICs, suppressed efflux-associated gene expression, and showed strong molecular interactions with efflux pump components, especially *AcrA* and *TolC*. These results propose that specific efflux inhibition through structurally designed EPIs may be an effective approach towards the overcoming of antibiotic resistance and restoring the therapeutic value of broad-spectrum antimicrobials.

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