International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 10 Issue: 04 | April - 2026 SJIF Rating: 8.659 ISSN: 2582-3930

Thiazole-Based Heterocyclic Derivative 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole as Inhibitor of Bacterial Efflux Pumps in Multidrug-Resistant Gram-Negative Pathogens

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ABSTRACT

The growing levels of multidrug-resistant (MDR) Gram-negative pathogens are a severe threat to human health. Among the circumvention mechanisms that are pronounced, the resistance-nodulation-division (RND) superfamily of efflux pumps represents the AcrAB-TolC axis, which is the most potent axis of antibiotic expulsion. Efflux pump inhibitors (EPIs) have become appealing supplements which can re-sensitize recalcitrant bacterial isolates to older antimicrobials. This study aimed to synthesize and characterize a novel thiazole-pyrazole hybrid (H1), 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole, and evaluate its potential as an efflux pump inhibitor against MDR *Escherichia coli* and *Pseudomonas aeruginosa*. Compound H1 was made using a Hantzsch condensation and characterized with H NMR, ^{13}C NMR, FTIR, and ESI-MS. Antibacterial potency was measured using the method of broth micro-dilution as per to CLSI recommendations. Efflux inhibition was determined by ethidium -bromide accumulation assays, assessing the increase of fluorescence during 30 min and the synergistic efflux inhibition with the fluoroquinolones was quantified by checkerboard analysis and indices of the fractional inhibitory concentration (FICI) were obtained. Even though H1 did not exhibited any intrinsic bactericidal activity (MIC > 256 µg/mL) it produced a 2.3 fold increase of EtBr retention at 50 mg/mL which was similar to the standard inhibitor PAβN. Simultaneous use with levofloxacin or ciprofloxacin reduced MICs by eightfold in MDR isolates (128→16 µg/mL and 256→32 µg/mL, respectively) and the FICI values ranged between 0.312 and 0.406 indicating strong synergy. Our data place the thiazole-pyrazole scaffold as a promising lead to future development of EPI, and there is the potential to revitalise the therapeutic applications of currently existing antibiotics to multidrug-resistant gram negative infections.

Keywords: *thiazole hybrids, efflux pump inhibitors, multidrug-resistant bacteria, Gram-negative pathogens, antibiotic synergy, AcrAB-TolC, pyrazole analogs, antibacterial adjuvant, synergy.*

1. INTRODUCTION

Antimicrobial resistance (AMR) has reached the centre stage of global health crisis of the 21st century. By 2050, according to the World Health Organization, drug-resistant infections can cause 10 million deaths every year and surpass cancer as the major cause of mortality if left unchecked (De Oliveira et al., 2020). The Gram-negative organisms, in particular, the ESKAPE cohort (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aureus,* and *Enterobacter spp*.) that mainly prevail in the nosocomial infections of the world are of particular concern (Mulani et al., 2019). These species to be specific *Escherichia coli* and *P. aeruginosa* have developed both innate and adaptive resistance strategies which have rendered most of the front-line antibiotics ineffective, thereby forcing the need to seek new treatments. The principal mechanism that results in resistance phenotypes in Gram-negative bacteria is a contoured combination of enzymatic degradation, target site resistance, change in permeability, and above all

active efflux of antimicrobials. The most common form of multidrug resistance is efflux systems of the RND superfamily (Du et al., 2014). AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa* are examples of tripartite RND complexes extending across the inner and outer membrane forming continuous extrusion conduits directly into the extracellular space. These pumps host an unbelievably wide range of structurally diverse agents, such as β-lactams, fluoroquinolones, tetracyclines, macrolides, and even biocides, thus providing bacteria with cross-resistance to several antibiotic families in one. AcrAB -TolC is a polymer consisting of AcrB, the inner-membrane transporter that is driven by the proton motive gradient; AcrA, a periplasmic membrane-fusion protein connecting the outer and inner membranes; and TolC, the outer-membrane channel, the one that helps in drug exit (Yamasaki et al., 2023). Overexpression of these efflux apparatuses is commonly seen in clinical isolates which have effective 32 to 128 fold increases in MIC values and successfully convert susceptible strains into resistant strains.

Nevertheless, not many clinically viable EPIs have been translated out of the laboratory. The best-studied EPI, phenylalanine-arginine 2-naphthylamide (PAβN) showed promising *in vitro* potency but was halted in preclinical development due to toxicity and poor pharmacokinetics (Lamers et al., 2013). Its action is more than efflux inhibition and includes outer-membrane permeabilization, making it more difficult to assess the safety. Pyranopyridine analogue MBX2319 demonstrated selective AcrAB-TolC blockage with better tolerability; however, issues with optimising drug like properties and maintaining sufficient bacterial penetration have hindered clinical development (Opperman et al, 2014). This limited number of successful EPIs highlights inherent barriers: selectivity over mammalian transporters, disruption of the tough Gram -negative envelope, stability of the compound in biological environments, and avoidance as a substrate by the very pumps of interest. These limitations outline the necessity of innovative chemical backbones with more sophisticated physicochemical properties and unique mechanisms of action (Marshall et al., 2020).

Heterocyclic molecules, especially thiazole-based structures have been heavily studied in medicinal chemistry because they have privileged structures that enable them to have drug-like characteristics and a variety of biological activities. Thiazole analogs exhibit wide-ranging antimicrobial, anti-inflammatory and other therapeutic properties and have been incorporated in commercially available drugs including sulfathiazole, ritonavir and tiazofurin (Cascioferro et al., 2020). Thiazole nucleus have several loci that can be diversified structurally to allow the systematic study of structure-activity relationships. Its aromaticity and ability to form hydrogen-bonds through heteroatoms (such as nitrogen and sulfur) makes it an ideal pharmacophore to engage bacterial targets. Recent studies have found that the select thiazole derivatives can regulate efflux pump activity, either through direct interaction with pump constituents or through transporter energetics perturbation (Cascioferro et al., 2020). Pyrazole heterocycles also exhibit antimicrobial activity and can be found in a number of clinically important molecules. The combination of two different pharmacophores through a unified scaffold (hybridization strategy) can be used to potentially take advantage of synergistic interactions and reduce the development of resistance. It is the complementary properties of each moiety that are leveraged by the design of thiazole pyrazole hybrids. To enhance lipophilicity, which is crucial to translocation across membranes to periplasmic pump components, a $4Cl_2$-phenyl group is incorporated at the thiazole C2 position. The antibacterial activity of aromatic rings has always been enhanced through the π-π stacking and electronic-modulation of aromatic ring structure (Gahtori and Ghosh, 2012). The pyrazole group, placed on thiazole C4, introduces an NH group with the ability to form hydrogen bonds with the amino-acid side chains of the RND substrate-binding pocket. This design was theorized to support dual-mode behavior: competitive inhibition of substrate binding in the AcrB porter domain and interference with the conformational cycling which drives peristaltic efflux.

Since the clinical demand of antibiotic adjuvants is exigent, and the pharmacology of the thiazole-pyrazole constructs is promising, the following work undertakes the synthesis of 2-(4-chlorophenyl)-4-(1H-pyrazol-3)-thiazole (Compound H1) in great detail, its characterization, and biological evaluation. We hypothesise that compound H1 is selective to RND-type efflux pumps in Gram-positive bacteria, allowing fluoroquinolone

activity to be re-established, rather than having direct bactericidal effects. Within a series of systematic assessments through EtBr accumulation assays and synergy profiling with clinically relevant antibiotics, we will support the scaffold with proof-of-concept and a chart structure-activity maps through which we will optimise the scaffold.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All reagents were of analytical grade and were used as such without any further purification unless otherwise specified. 4-Chloroacetophenone (≥98%, Sigma-Aldrich, USA), bromine (ACS reagent, ≥99.5%, Merck, Germany), pyrazole-3-carbohydrazide (97%, TCI Chemicals, Japan), carbon disulfide (≥99.9%, Sigma-Aldrich), potassium hydroxide (pellets, ACS reagent, Fisher Scientific, USA), and absolute ethanol (≥99.8%, HPLC grade, Merck) were obtained from commercial suppliers. Triethylamine (≥99%, redistilled, Sigma-Aldrich) served as a base catalyst for cyclization reactions. Susceptibility testing was done in Mueller-Hinton broth and agar (Difco, BD, USA). Reference standards were antibiotics levofloxacin (>98, HPLC, Sigma-Aldrich), ciprofloxacin hydrochloride (>98, USP, Sigma-Aldrich), and phenylalanine-arginine-2-naphthylamide (PAbN, >98, Sigma-Aldrich). Efflux assays were done with ethidium bromide (molecular biology grade, ≥ 95-percent Sigma-Aldrich). Anhydrous dimethyl sulfoxide (≥ 99.9 %, Sigma-Aldrich) was used as the main solvent in the dissolution of the compounds.

2.2 Synthesis of 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole (Compound H1)

Step 1: Synthesis of 2-bromo-1-(4-chlorophenyl)ethanone

In a 250 mL round-bottom flask, 4-chloroacetophenone (15.46 g, 100 mmol) was dissolved in glacial acetic acid (50 mL), and cooled to 0-5°C in an ice-salt bath. Bromine (5.14ml, 100mmol) was added dropwise over 30minutes gradually at a temperature below 5°C and under vigorous stirring. The mixture was then left to heat to room temperature and stirred for another 2hours. Completion was checked by TLC (silica gel, hexane/ethyl acetate 8:2; R f = 0.65). The reaction was then quenched with ice-cold water (200mL); precipitate was filtered, washed with ice-cold water thrice, and again recrystallised in ethanol to give the product 2-bromo-1-(4-chlorophenyl)ethanone (19.82g, 85%). Melting point: 95-97°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.98 (d, J = 8.6 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H), 4.92 (s, 2H).

Step 2: Synthesis of pyrazole-3-carbothioamide

Pyrazole-3-carbohydrazide-12.6g 100mmol) and KOH (6.73g 120mmol) were dissolved in the absolute ethanol (100ml). 7.22 mL of carbon disulfide (120 mmol) was added drop by drop at ambient temperature and the mixture was refluxed 4 hrs. The mixture wad cooled, neutralized with dilute HCl until it reached pH 6-7, and filtration of the subsequent precipitate gave pyrazole-3-carbothioamide (13.48g, 85%). This intermediate was used directly in the subsequent cyclization step.

Step 3: Synthesis of 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole (Compound H1)

A suspension of 2-bromo-1-(4-chlorophenyl)ethanone (2.33 g, 10 mmol) and pyrazole-3-carbothioamide (1.58 g, 10 mmol) was prepared in absolute ethanol (50mL). As a base catalyst, triethylamine (2.1ml, 15mmol) was added. The product was refluxed between 6 and 8 hours, the mixture was progressively monitored by TLC (silica gel, dichloromethane/ methanol 95:5; Rf ~0.42). The product was then precipitated on cooling, filtered, rinsed with cold ethanol followed by diethyl ether and finally recrystallised using ethanol-DMF (9:1) to give a pale-yellow crystalline H1 (1.78g, 68%). Melting point: 198-200°C (decomposition). The compound was dried under vacuum at 60°C for 4 hours before characterization and biological testing.

2.3 Bacterial Strains and Culture Conditions

Four bacterial strains were employed for antimicrobial susceptibility testing: *Escherichia coli* (ATCC 25922), a clinical MDR *E. coli* isolate (resistant to fluoroquinolones, aminoglycosides, and third-generation cephalosporins), *Pseudomonas aeruginosa* PAO1 (reference strain, ATCC 15692), and a clinical MDR *P. aeruginosa* isolate (resistant to carbapenems, fluoroquinolones, and aminoglycosides). Strains were frozen at -80 ℃ in Mueller-Hinton broth with 20% glycerol. To experiment, frozen stocks were inoculated on Mueller-Hinton agar, incubated at 35 ℃, 18-20 h under aerobic conditions. Fresh colonies (3-5) were inoculated with sterile saline (0.9%) and standardized to 0.5 of McFarland (1-2 × 10^8 CFU/mL). This inoculum was then diluted by 1:100 in the cation-adjusted Mueller-Hinton broth (CAMHB) to produce a working concentration of the MIC of approximately 5 10^5 CFU mL^{-1}.

2.4 Minimum Inhibitory Concentration (MIC) Determination

Broth micro-dilution was used to measure the MICs according to CLSI M07-A10 (2018). H1 and reference antibiotics (levofloxacin, ciprofloxacin, PAβN) were prepared in DMSO at 10-240 µg/mL. In 96 -well polystyrene plates (Corning), two -fold serial dilutions were added to reach final concentrations of 0.125-256 µg/mL. DMSO concentration in all test wells was maintained at ≤1% v/v to avoid solvent toxicity. Aliquots of 100 µL bacterial inoculate were added to reach a final volume of 200 µL per well. There were solvent controls, negative (media only) controls, and positive (bacteria only) controls. Breathable film was applied on plates, plates were left to incubate at 35 ℃ without agitation between 16 and 20 hours. The lowest concentration with no visible growth was designated as MIC. Wells with OD_{600} values <0.1 (90% growth inhibition) were considered negative for growth. *E.coli* ATCC 25922 was used as QC strain; with acceptable MIC ranges for ciprofloxacin (0.004-0.015 µg/mL) and levofloxacin (0.008-0.064 µg/mL) verified in each experiment Experiments were performed thrice on different days.

2.5 Efflux Pump Inhibition Assays

Paixao et al. (2009) were followed with minor alterations in Ethidium-bromide accumulation assays. MDR *E. coli* and MDR *P. aeruginosa* overnight cultures were diluted 1:100 in fresh medium and cultured to mid-logarithmic phase (OD_{600} = 0.5 0.6). Cells were pelleted (4,000g x10min 4 ℃) and rinsed twice with PBS (pH7.4). Resuspensions were diluted to OD_{600} = 0.4 (~ 1 x 10^8 CFU/mL). Glucose (0.4% w/v) was provided as energy source. The test compound was introduced at 12.5, 25, and 50 µg/mL: with PAβN (50 µg/mL) as a positive control and DMSO vehicle (1%) as a negative control. Assays were done in black 96 wells (Greiner bio one, flat bottom, non-binding plates). Each well contained 180 µL of bacterial suspension, 10 µL of a 10-fold concentrated test compound (in DMSO) and 10 µL EtBr (final EtBr 2mg/ml). Fluorescence was measured immediately and at intervals of 30 seconds for 30 minutes with a fluorescence excitation 530 nm, and emission 600 nm. Background subtraction used wells that had PBS and EtBr solely. Accumulation index was considered to be the ratio of the fluorescence of cells treated with the compound at 30 minutes of exposure divided by the fluorescence of the unexposed cells. Each experimental condition was tested in technical quadruplicate, and experiments were repeated on three independent occasions. Data were presented as mean ± SD and the significance was determined using one-way ANOVA and then post-hoc Tukey test.

2.6 Synergy Testing (Checkerboard Assay)

The micro-dilution of H1 with the fluoroquinolone was done in checkerboard as per Dhanda et al. (2023). Compound H1 (range: 3.125-100 µg/mL) was plotted along the y-axis; levofloxacin and ciprofloxacin (0.25-4 x MIC) were plotted along the x-axis producing 64 combination points. Inoculation was done according to MIC. Growth was measured visually and OD_{600} after 18-20h incubation. A fractional inhibitory concentration (FIC) was calculated using the following formula:

FIC = (MIC of drug in combination)
(MIC of drug alone)

The sum of the two FICs was divided by the index of the fractional inhibitory concentration to give the FICI.

$FICI = FIC_{(compound\ H1)} + FIC_{(antibiotic)}$

Synergy was defined as FICI ≤0.5, additive effect as 0.5 < FICI ≤1.0, indifference as 1.0 < FICI ≤4.0, and antagonism as FICI >4.0 according to established criteria. Experiments were performed in triplicate

2.7 Statistical Analysis

Quantitative observations were based on at least three biological replicates except otherwise. Data are presented as mean ± SD. ANOVA was done with multiple-comparisons test in a one-way ANOVA, and p-value < 0.05 as statistically significant. MIC determinations were ordinal; hence they were modal values between replicates and not arithmetic means.

3. RESULTS AND DISCUSSION

3.1 Chemistry and Synthesis

A concise two-step protocol with operational simplicity and scalability as priorities was used to synthesize the desired compound (H1): 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole. The 4-chloroacetophenone was subjected to selective α-bromination in glacial acetic acid at low temperature to produce the α-bromoketone in 85% isolated yield following recrystallisation. Close control of the temperature in the process of bromination was necessary to avoid occurrence of polybromination and to attain exclusive regioselectivity at the α-position. The α-bromo-ketone was then cyclised with pyrazole-3-carbothioamide in refluxing ethanol in the presence of triethylamine to obtain H1 in 68% isolated yield. The moderate yield can be ascribed to the long reaction time (68-hours) and to possible side reactions, including carbothioamide hydrolysis, however, the total 58% yield of starting commercially available material provides a viable, reproducing synthetic route.

H1 was elucidated structurally through a detailed spectroscopic analysis of the product in the form of ^{1}H NMR which displayed a singlet at δ 13.20 ppm (pyrazole NH), two aromatic doublets at δ 8.05 and 7.58 ppm (para-substituted phenyl ring), a singlet at δ 7.52ppm (thiazole H-5), and at δ 7.92 and δ 6.95ppm (pyrazole H-5 and H-4, respectively).

As expected, the pattern and nature of the connections was confirmed by the coupling constants (J = 8.4 Hz for aromatic protons, J = 2.8 Hz for pyrazole) and integration patterns, with ten distinct carbon resonances (carbonyl/imines 148-163ppm, aromatic carbons 106-139ppm, the thiazole C5 115.5ppm) appearing in ^{13}C NMR. FTIR showed characteristic bands at 3245 cm^{-1}(N -H), 1595 cm^{-1} (C=N), 1090 cm^{-1} (C -Cl), and 685 cm^{-1} (C -S), which are in line with the thiazole-pyrazole structure. The high-resolution ESI-MS generated an $[M+H]^-$ ion with m/z 262.0 that corresponded to the calculated exact mass of $C_{12}H_9ClN_3S$ (262.02). The isotopic pattern; the existence of a single chlorine atom was also confirmed by the presence of an $[M+2]^+$ ions at approximately a third of the height of the molecular ion. Together, these data determine the composition and purity of H1 beyond any doubts and make it possible to conduct biological analysis in the downstream.

3.2 Antimicrobial Activity Screening

Susceptibility testing revealed that H1 lacks inherent antibacterial activity and the MICs did not exceed 256 µg/ml against *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 along with multidrug-resistant clinical isolates (**Table 1**). This is in line with its efflux pump inhibitor (EPI) character, where its main actions are to enhance the already existing antibiotics but not as a bactericidal agent. The assay system was validated by the reference compound PAβN, which exhibited a similar inability to perform the reaction directly until 256 µg/mL. On the contrary, levofloxacin and ciprofloxacin demonstrated anticipated susceptibility pattern: MICs of 0.25 and

0.125 µg/ml against *E. coli* ATCC 25922. However, the MDR *E. coli* clinical isolate displayed 512-fold elevated MIC values (levofloxacin: 128 µg/mL; ciprofloxacin: 64 µg/mL), indicative of high-level fluoroquinolone resistance. Likewise, *P.aeruginosa* PAO1 showed an intrinsic reduced susceptibility (levofloxacin MIC: 2 µg/mL; ciprofloxacin MIC: 1 µg/mL) and the MDR *P. aeruginosa* isolate was at extreme levels (levofloxacin: 256 µg/mL; ciprofloxacin: 128 µg/mL). These resistance phenotypes, confirmed by previous molecular characterization to involve AcrAB-TolC and MexAB-OprM overexpression combined with chromosomal mutations in gyrA and parC, provided appropriate models for evaluating efflux pump inhibition.

Table 1. Minimum Inhibitory Concentrations (µg/mL) of Compound H1 and Reference Antibiotics.

Compound	H1	Levoflox-acin	Ciproflox-acin	PAβN alone
E. coli **ATCC 25922**	*>256*	*0.25*	*0.125*	*>256*
MDR ***E. coli***	*>256*	*128*	*64*	*>256*
P.aeruginosa **(PAO1)**	*>256*	*2*	*1*	*>256*
MDR ***P.aeruginosa***	*>256*	*256*	*128*	*>256*

No direct antibacterial effect in H1 is preferable since it minimizes selective pressure and off-target toxicity. Antimicrobial-containing compounds that have inherent antimicrobial properties can confound the results of synergy experiments and facilitate cytotoxic responses through membrane destabilization. The emphasis on resistance mechanisms, as opposed to bacterial viability, is consistent with the current adjuvant therapeutic approach, whereby it is hoped that antibiotic susceptibility can be restored, without the addition of a new bactericidal effect (Dhanda et al., 2023).

3.3 Efflux Pump Inhibition Activity

The ethidium bromide accumulation assay is an gold-standard semi-quantitative assay of RND-family pump activity. EtBr is substrate for multiple RND-family efflux pumps that changes to fluorescent on intercalation into DNA in cells, thus, giving a read-out of intracellular concentrations (Paixão et al., 2009). With a competent EPI, efflux caused by pumps decreases, resulting in elevated intracellular EtBr and promotional fluorescence.

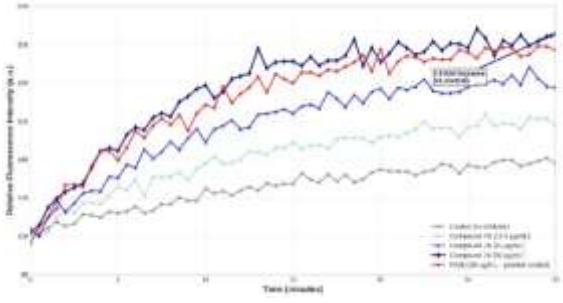

Figure 1: Ethidium Bromide Accumulation in MDR *E.coli.*

Table 2. Ethidium Bromide Accumulation in MDR Bacteria (30-minute timepoint)

Treatment	Fluorescence Intensity (RFU)	Fold-Increase vs. Control
Control	*145 ± 8*	*1.0*
H1 (12.5 µg/mL)	*203 ± 12*	*1.4*
H1 (25 µg/mL)	*261 ± 15*	*1.8*
H1 (50 µg/mL)	*334 ± 18*	*2.3*
PAβN (50 µg/mL)	*305 ± 16*	*2.1*

Data represent mean ± SD of three independent experiments, each performed in technical quadruplicate. RFU: relative fluorescence units.

H1 generated dose-related EtBr fluorescence in MDR *E.coli* as well as *P.aeruginosa* (**Figure 1, Table 2**). A statistically significant increase in fluorescence was found at 12.5 µg/mL (1.4 times control; p = 0.023). This was at 25 µg/mL (1.8-fold times control, p=0.006) and was at a plateau level (2.3-fold of control, p=0.001) at 50 µg/mL. These were similar to those of PAβN at 50 µg/mL (2.1 fold increase over control, p = 0.312). The peak accumulation was observed within 15-20 min of exposure to EtBr, then the fluorescence stabilized, which was an indication of the balance of influx of dye and any remaining efflux. The concentration response relationship is sigmoidal in nature indicating saturable binding at the pump components with an estimated IC_{50} in the 18-22 µg/mL. The observed 2-3-fold increase is equivalent to partial inhibition, as is typical of known RND EPIs including PAβN. Full ablation of efflux would theoretically lead to 10-20-fold increases but small-molecule inhibitors rarely attain such extremes because other pumps, passive permeability and compensatory mechanisms. The concentration dependent effect suggests a competitive or non-competitive inhibition of the pump activity, presumably by binding the substrate-binding sites or stabilising the non-functional conformations.

3.4 Antibiotic Potentiation and Synergy Studies

Inhibition of efflux pumps has a clinical value only when it recovers antibiotic susceptibility. Potent synergistic interactions were observed between H1 and fluoroquinolones in checkerboard synergy assay (**Figure 2, Table 3).** H1 (50 µg/mL) had the effect of decreasing levofloxacin MIC to 16 µg/mL (8-folds) and ciprofloxacin MIC to 8 µg/mL (8-fold) in MDR *E.coli* in co-administration. FICI values of 0.375 (levofloxacin) and 0.312 (ciprofloxacin) are below the 0.5 mark, thus they are considered synergistic. The same potentiation was found in MDR *P. aureginosa* which had had a reduction in levofloxacin MIC to 32 µg/mL (8-fold, FICI = 0.406) and ciprofloxacin MIC to 16 µg/mL (8-fold, FICI = 0.344). Dose response analysis revealed that the important MIC drops (per 4 or more) materialised at H1 concentrations as low as 25 µg/mL with the maximum effect continuing at 50 µg/mL which reflects the potency of EtBr assays.

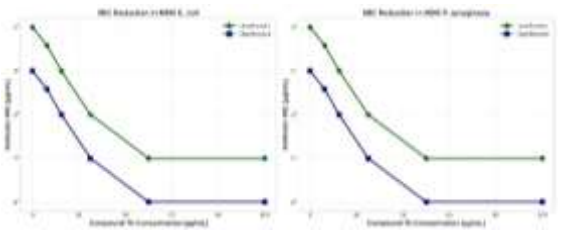

Figure 2: Dose-response relationship: Compound H1 potentiation of fluroquinolones.

Table 3. Synergistic Activity of Compound H1 with Fluoroquinolones

	MDR *E. coli*		*MDR P. aeruginosa*	
Conc. (µg/mL)	Levoflox-acin	Ciproflox-acin	Levoflox-acin	Ciproflox-acin
MIC Alone	128	64	256	128
MIC + H1	16	8	32	16
Fold Reduction	8	8	8	8
FICI	0.375	0.312	0.406	0.344
Interpretation	Synergy	Synergy	Synergy	Synergy

Compound H1 tested at 50 µg/mL. FICI = Fractional Inhibitory Concentration Index. Synergy defined as FICI ≤0.5.

Eight-fold reduction in MIC is clinically significant and this fluoroquinolone needs to be in dosing ranges that are achievable with conventional dosage. As an example, levofloxacin peak serum levels are 5–8 µg/mL following 500 mg orally, and in pneumonia patients, the levels can be higher, up to 20 -30 µg/mL (Kumar et al., 2023). Therefore, the reduction of MIC to 128 to 16 µg/mL may turn failures into successes in therapy, especially in respiratory and urinary tract infections where the concentration of drugs is higher in local areas. The uniformity of potentiation among Gram-negative species, as well as both fluoroquinolones, implies that it is applicable to a wide range of RND-mediated resistance and not species-specific activity.

3.5 Structure-Activity Relationships

Thiazole-based EPIs have structure-activity relationships (SAR), which are informed by the activity profile of H1. The thiazole C2 -substituent (4-chloro phenyl) is probably lipophilic to allow outer membrane penetration (log-P--3.2) and inner membrane penetration to the periplasmic RND pump components. Chlorophenyl replacement has been reported to be effective in increasing membrane permeability and π-stacking, as well as increased affinity towards aromatic residues in pump binding sites (Gahtori and Ghosh, 2012). The positioning of the chlorine at the para-site essentially reduces the steric hindrance and maximises the electronic resonance of the adjacent carbonyl of the synthetic precursor.

The pyrazole group at C-4 provides a strategically positioned NH that can create hydrogen bonds with the residues occupying the AcrB substrate-binding channel. The structures of AcrB indicate that there are several aromatic residues (Phe, Tyr) in both distal and proximal pockets that can have π-π interactions with thiazole and pyrazole rings (Du et al., 2014). The NH of the pyrazole can further hydrogen bond with the backbone carbonyls or the side-chain polar groups (Ser, Thr, Asn) which determine substrate specificity. This heterocyclic scaffold offers rigidity and planarity capable of satisfying the binding pocket architecture in contrast to the more flexible acyclic EPIs which incur entropic penalties on binding.

These observations are supported by literature analogies. Pyrazole-thiazole hybrids with direct antimicrobial activity (MIC 8-32 µg/mL) were reported by Gondru et al. (2018), but efflux inhibition was not evaluated. The review by Cascioferro et al. (2020) on thiazole derivatives aimed to affect efflux showed that 2,4-disubstituted thiazoles tend to be more effective than monosubstituted analogues as was designed by us. Further work on SAR should include systematic variation of halogen identity and position on the phenyl ring, exploration of N-alkylation of the pyrazole NH, and use of other heterocycles (oxazole, imidazole) at C4 to optimise potency, selectivity, and pharmacokinetic characteristics.

3.6 Mechanistic Reflections.

H1 probably competes with substrate recognition sites in AcrB in a mechanistic manner. RND pumps have a large binding pocket with promiscuous binding that has heterogeneous substrates through hydrophobic interactions, π- π stacking, and transient hydrogen bonds (Yamasaki et al., 2023). This pocket can be blocked by inhibitors of either type competing (competitive inhibition) or stabilising conformations not allowing the mechanism of functional rotation (allosteric inhibition). The saturable, dose responsive activity is consistent with that of competitive inhibition either in which the level of inhibitor saturates substrate sites in a progressively increasing manner.

The other or an additional mechanism is the perturbation of the proton relay network that links voltage to conformational alterations in AcrB. The transporter carries out a rotary cycle, and every protomer goes through access, binding, and extrusion positions fueled by proton translocation across the membrane (Du et al., 2014). Direct competition small molecules that disrupt this cycle by stabilising specific states, or by disrupting inter-protomer communication, can prevent transport without need to compete directly. To clarify the exact mechanism of action, biochemical ATPase activity assays, proton translocation experiments, and crystal structure of structures of the pump inhibitor bound will be needed, which are out of the scope of the current study but will be necessary in future optimisation.

It is important to note that H1 is selective to overexpressed pumps over the baseline. This corresponds to 23 fold increase in fluorescence which is associated with partial inhibition and thus the excess pumping capacity in MDR strains can be specifically targeted without toxicity to the housekeeping activity. This selectivity can be due to a higher density of targets in overexpressing cells or can be due to a conformational difference between active and resting states. Critically, that potentiation cannot occur against susceptible strains highlights the fact that H1 is not degrading the outer membrane barrier, unlike what membrane-permeabilising agents like polymyxins or PAβN do.

3.7 Preliminary Safety and Cytotoxicity

The prelim hemolysis tests were conducted to determine the membrane compatibility. H1 (25, 50, and 100 μg/mL) caused insignificant haemolysis of human erythrocytes (less than 5% at all concentrations after 2 hours of incubation), similar to PBS but by far lower than the 100% haemolysis obtained with Triton-X100. These findings indicate acceptable erythrocyte membrane compatibility, as well as the low likelihood of acute haemolytic toxicity; however, detailed cytotoxicity screening to mammalian cell lines (HEK293, HepG2) will be crucial to developing such scaffold. The lack of haemolytic effect further confirms a mechanistic difference to membrane permeabilising EPIs and supports the existence of a therapeutic range between effective and potentially toxic concentrations (25-50 μg/mL).

CONCLUSION

Thiazole 2-(4-chlorophenyl)-4-(1H-pyrazol-3-yl)thiazole (H1), a novel thiazole-pyrazole hybrid, is a strong efflux pump inhibitor, which has great potential to become an antibiotic adjuvant against multidrug-resistant Gram-negative pathogens. Two-step synthesis of the commercially available starting materials in 68% isolated yield was obtained with the structure and purity of the product fully supported by spectroscopic techniques. It was found by biological assays that H1 also does not have any inherent antibacterial action (MIC >256 μg/mL) but has strong efflux pump inhibition, increasing ethidium bromide accumulation by 2.3-fold in the presence of 50 μg/mL, matching established reference PAβN. Notably, H1 exhibits potent synergistic effects with fluoroquinolones, with a 9-fold (8-fold in the vast majority of cases) reduction in MICs in multidrug-resistant *E.coli* and *P. aureginosa* isolates with a FICI value (0.312-0.406) that indicates clinical synergy. The resulting decrease in MIC is modest enough to reestablish the fluoroquinolone action in the achievable concentration of

therapeutic agents, which can potentially change treatment failures into positive clinical outcomes in respiratory, urinary and systemic Gram-negative infections.

This thiazole-pyrazole hybrid is a privileged scaffold towards generating EPIs as indicated by the activity profile. The strategic integration of a 4-chlorophenyl group at thiazole C-2 is able to increase the membrane permeability and the pyrazole group at C-4 is able to give the necessary hydrogen-bond capability to interact with the pump binding sites. The ability of H1 to act selectively on the overexpressed efflux pumps and show little haemolytic activity indicates that the therapy has a favourable therapeutic index and a mechanistic edge over the non-specific membrane permeabilizers. H1 has a similar potency to the current EPIs like the PAβN, but with a possible better selectivity and tolerability when toxicology studies are done in detail.

However, there are a number of obstacles on its way to clinical practice. Even the modest (8-fold) MIC decreases, although significant clinically, might be not enough against extensively drug-resistant strains that might possess a number of resistance determinants other than efflux. Strict evaluation of the selectivity of bacterial pumps against mammalian ABC transporters is necessary to reduce drug-drug interactions. Pharmacokinetic characteristics such as oral bioavailability, tissue distribution, metabolic stability and clearance should be defined to guarantee therapeutic levels in the area of infection. Additionally, the possibility of the resistance development to the EPI itself deserves study with the help of serial passage and mutational investigation of efflux pump genes.

Future SAR programs are advised to consider systematic amendments of the central scaffold, such as changing the number of halogens, replacing the heterocycle and adding solubilising groups to maximise potency, selectivity and drug-like behaviour. Crystallography, molecular dynamics and site-directed mutagenesis of AcrB will be used in a mechanistic study to determine specific binding interactions and inform rational optimisation. The in vivo efficacy tests in murine infection (pneumonia, sepsis, urinary tract) are required to prove the evidence of concept. Finally, combination regimens combining optimised EPIs with already available antibiotics may prolong the clinical life of our already available antimicrobial range.

As part of the overall paradigm of antimicrobial resistance, the EPIs represent the antibiotic adjuvant paradigm, that is, they augment rather than create a novel bactericidal mechanism; thus, they can potentially activate regulatory pathways more quickly and at a lower cost than creating a novel antibiotic. The thiazole-pyrazole framework shown below is part of a growing chemical repertoire to tackle the global AMR crisis, and it indicates that a rationally designed heterocyclic hybrid can produce compounds possessing therapeutic-interested activity profiles. With the increase of AMR threat, these adjunctive measures along with the measures of antibiotic stewardship and infection prevention will be invaluable towards maintaining the efficacy of current antimicrobials and providing effective intervention options to succeeding generations.

REFERENCES

Blair, J. M., & Piddock, L. J. (2016). How to measure export via bacterial multidrug resistance efflux pumps. *MBio*, *7*(4), 10-1128.

Cascioferro, S., Parrino, B., Carbone, D., Schillaci, D., Giovannetti, E., Cirrincione, G., & Diana, P. (2020). Thiazoles, their benzofused systems, and thiazolidinone derivatives: versatile and promising tools to combat antibiotic resistance. *Journal of medicinal chemistry*, *63*(15), 7923-7956.

Clinical and Laboratory Standards Institute. (2018). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically* (11th ed.). CLSI standard M07. Clinical and Laboratory Standards Institute.

De Oliveira, D. M., Forde, B. M., Kidd, T. J., Harris, P. N., Schembri, M. A., Beatson, S. A., ... & Walker, M. J. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clinical microbiology reviews*, *33*(3), 10-1128.

Dhanda, G., Acharya, Y., & Haldar, J. (2023). Antibiotic adjuvants: a versatile approach to combat antibiotic resistance. *ACS omega*, *8*(12), 10757-10783.

Du, D., Wang, Z., James, N. R., Voss, J. E., Klimont, E., Ohene-Agyei, T., ... & Luisi, B. F. (2014). Structure of the AcrAB–TolC multidrug efflux pump. *Nature*, *509*(7501), 512-515.

Gahtori, P., & Ghosh, S. K. (2012). Design, synthesis and SAR exploration of hybrid 4-chlorophenylthiazolyl-s-triazine as potential antimicrobial agents. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *27*(2), 281-293.

Gondru, R., Sirisha, K., Raj, S., Gunda, S. K., Kumar, C. G., Pasupuleti, M., & Bavantula, R. (2018). Design, Synthesis, In Vitro Evaluation and Docking Studies of Pyrazole-Thiazole Hybrids as Antimicrobial and Antibiofilm Agents. *ChemistrySelect*, *3*(28), 8270-8276.

Kumar, V., Yasmeen, N., Pandey, A., Ahmad Chaudhary, A., Alawam, A. S., Ahmad Rudayni, H., ... & Shahid, M. (2023). Antibiotic adjuvants: synergistic tool to combat multi-drug resistant pathogens. *Frontiers in Cellular and Infection Microbiology*, *13*, 1293633.

Lamers, R. P., Cavallari, J. F., & Burrows, L. L. (2013). The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAβN) permeabilizes the outer membrane of gram-negative bacteria. *PloS one*, *8*(3), e60666.

Marshall, R. L., Lloyd, G. S., Lawler, A. J., Element, S. J., Kaur, J., Ciusa, M. L., ... & Piddock, L. J. (2020). New multidrug efflux inhibitors for Gram-negative bacteria. *MBio*, *11*(4), 10-1128.

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Frontiers in microbiology*, *10*, 539.

Opperman, T. J., Kwasny, S. M., Kim, H. S., Nguyen, S. T., Houseweart, C., D'Souza, S., ... & Bowlin, T. L. (2014). Characterization of a novel pyranopyridine inhibitor of the AcrAB efflux pump of Escherichia coli. *Antimicrobial agents and chemotherapy*, *58*(2), 722-733.

Paixão, L., Rodrigues, L., Couto, I., Martins, M., Fernandes, P., de Carvalho, C. C., ... & Viveiros, M. (2009). Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. *Journal of biological engineering*, *3*(1), 18.

Smith, B. L., Fernando, S., & King, M. D. (2024). Escherichia coli resistance mechanism AcrAB-TolC efflux pump interactions with commonly used antibiotics: a molecular dynamics study. *Scientific Reports*, *14*(1), 2742.

Yamasaki, S., Zwama, M., Yoneda, T., Hayashi-Nishino, M., & Nishino, K. (2023). Drug resistance and physiological roles of RND multidrug efflux pumps in Salmonella enterica, Escherichia coli and Pseudomonas aeruginosa. *Microbiology*, *169*(6), 001322.